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

Molecular insights in bovine endometritis and the intrauterine administration of *Lactobacillus buchneri* as a potential treatment of subclinical endometritis

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der Freien Universität Berlin

vorgelegt von
Sarah Peter
Tierärztin aus Köln

Berlin 2019
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Für meine Familie und für Roman

In der Wissenschaft gleichen wir alle nur den Kindern, die am Rande des Wissens hie und da einen Kiesel aufheben, während sich der weite Ozean des Unbekannten vor unseren Augen erstreckt.

- Sir Isaac Newton -

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List of abbreviations

16S rRNA	16S ribosomal ribonucleic acid
°C	degree Celsius
%	percent
AI	artificial insemination
AMP	antimicrobial peptide
CD45	cluster of differentiation 45; Leukocyte-common antigen
CE	clinical endometritis
CXCL1-5	chemokine (C-X-C motif) ligand 1-5
CXCR2	chemokine (C-X-C motif) receptor 2
DAMP	damage-associated molecule pattern
DEFB	beta-defensin
DIM	days in milk
DSM	German library for microorganisms (Deutsche Sammlung für Mikroorganismen)
E. coli	*Escherichia coli*
e.g.	for example
ELISA	enzyme-linked immunosorbent assay
F. necrophorum	*Fusobacterium necrophorum*
Fig.	figure
GnRH	gonadotropin-releasing hormone
h	hour
H	healthy
H_2O_2	hydrogen peroxide
H. somni	*Histophilus somni*
IL	interleukin
IL1A	interleukin 1 alpha
IL1B	interleukin 1 beta

IL1RN	interleukin 1 receptor antagonist
IL6	interleukin 6
IL8	interleukin 8
IL10	interleukin 10
kg	kilogram
L.	*Lactobacillus*
LAB	lactic acid bacteria
LH	luteinizing hormone
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
MMP1	matrix metallopeptidase 1
MOI	multiplicity of infection
MRL	maximum residue limit
mRNA	messenger ribonucleic acid
MT	metric ton
n	sample size
NEFA	non-esterified fatty acids
P. acidilactici	*Pediococcus acidilactici*
PAMP	pathogen-associated molecular pattern
PAF	platelet-activating factor
PG	prostaglandin
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin F_{2alpha}
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
p.i.	post inseminationem
PLA_2	phospholipase A_2

PMN	polymorphonuclear neutrophils
pp	postpartum
PTAFR	platelet-activating factor receptor
PTGDS	prostaglandin D_2 synthase
PTGES	prostaglandin E synthase
PTGES2	prostaglandin E synthase 2
PTGES3	prostaglandin E synthase 3
PTGFS	prostaglandin F synthase
PTGIS	prostacyclin synthase
PTGS1	prostaglandin-endoperoxide synthase 1
PTGS2	prostaglandin-endoperoxide synthase 2
PTP	protein tyrosine phosphatase
PTPRC	protein tyrosine phosphatase, receptor type C
RFM	retained fetal membranes
SAA	serum amyloid A
SCE	subclinical endometritis
sp.	single species (not identified)
spp.	several species (not identified)
T. pyogenes	*Trueperella pyogenes*
TLR	Toll-like receptor
TLR1-6	Toll-like receptor 1-6
TNF	tumor necrosis factor
vs.	versus

1. Introduction

Milk and dairy products are extremely valuable agricultural commodities that play a key role for global human nutrition (Muehlhoff et al., 2013). They are consumed daily by billions of people worldwide, while the world population is growing continuously. In less than three decades, the world milk production increased from around 540 million metric tons (MT) in 1990 to about 810 million MT in 2016 (FAOSTAT, 2019). Besides the milk of buffaloes (11 %), goats (2 %), sheep (1 %) and camel (0.4 %), cow milk accounted for around 85 % of the world milk production in 2013 (FAO, 2015).

Along with the growing global demand for milk, certain cattle breeds were selected for milk production and the milk yield per cow per year was highly increased by breeding programs (Van Vleck et al., 1986; Wiggans, 1991; Ashwell et al., 2001). For example in Germany, the average milk yield increased from 3395 kg/cow/year in 1960 to 4710 kg/cow/year in 1990 and to 7746 kg/cow/year in 2016 (BRS, 2018). But this systematic rise of the milk yield, accompanied by increasing metabolic stress for the cow, caused an increasing risk for dairy cows to develop a disease. It is suggested that a high milk yield and the associated metabolic stress impairs the innate immunity of the uterus, representing one aspect why high-producing dairy cows are more likely to have puerperal inflammatory uterine diseases (Duffield et al., 2009; Sheldon et al., 2018).

In fact, bovine endometritis is one of the most prevalent uterine diseases in the puerperium of high producing dairy cows (Gilbert et al., 2005; Sheldon et al., 2008). It is associated with subfertility (Sheldon et al., 2009a) and the delay of conception (Gobikrushanth et al., 2016), which results in unwanted reduced annual milk production (Auldist et al., 2007) or even the premature and cost-intensive replacement of the cow (Studer and Morrow, 1978).

Besides clinical endometritis, subclinical endometritis got into the focus of science especially in the past decade. It is defined by an increased proportion of immune cells present in the endometrium with the concurrent lack of clinical signs (Sheldon et al., 2006). Considering the need of special diagnostic techniques including the preparation of a smear, staining and microscopy, the detection of subclinical endometritis is laborious and yet not possible as a cow-side test. Therefore, subclinical endometritis virtually always remains unnoticed in dairy cows causing subfertility and substantial financial losses for the dairy industry (Gilbert et al., 2005; Sheldon et al., 2009a).

In this context, the two most frequently used therapeutic strategies for the treatment of endometritis - the use of antibiotics or the hormone prostaglandin $F_{2\alpha}$ - are questioned worldwide, e.g. due to the fear of residues in milk and meat and the potential spread of bacterial resistances or a questionable effectiveness, respectively. Especially if subclinical

endometritis comes to the focus, considering that there is no practicable diagnostic method, an interest grows to find substances that cure subclinical endometritis and can be used even preventive or on speculation without negative aspects for the cow and the consumer.

This brings *Lactobacillus* spp. into consideration. Lactobacilli and other lactic acid bacteria are already known to have probiotic properties (Ljungh and Wadstrom, 2006), which means that they are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). The assumption that lactic acid bacteria could also be potential probiotics in bovine reproductive medicine was confirmed when positive effects were shown after the intravaginal administration to cows around calving (Deng et al., 2015a; Genis et al., 2018). Hence, it can be suspected that certain *Lactobacillus* spp., if administered intrauterine and in adequate amounts, could have positive effects on the uterine health and the reproductive performance of cows.

To summarize, the occurrence of bovine subclinical endometritis is a relevant problem in dairy farming and there is huge interest in the establishment of new successful strategies for its treatment and prevention. Therefore, the present study contributes to the molecular understanding of endometrial defense mechanisms in cases of clinical and subclinical endometritis. One of the main objectives was the evaluation of the endometrial messenger ribonucleic acid (mRNA) expression of pro-inflammatory factors in the puerperium of healthy cows and of cows with subclinical or clinical endometritis. Furthermore, *Lactobacillus buchneri* DSM 32407 was chosen for the intrauterine administration to dairy cows on days 24-30 postpartum (pp) and the investigation of its influence on the endometrial mRNA expression of selected pro-inflammatory factors of cows with subclinical endometritis and on the subsequent reproductive performance.

2. Overview of literature

2.1. The bovine puerperium

The early pp period is called puerperium, which is generally defined as the period of time after parturition (which includes the delivery of the placenta) until the remission of the pregnancy-related changes in the mother's reproductive organs (Pschyrembel, 2014). This time span can vary individually between cows. The puerperium includes uterine involution and the re-establishment of the estrous cycle.

2.1.1. Uterine involution during the bovine puerperium

After parturition, the uterus decreases enormously in size and weight especially in the first 10 days pp and undergoes histological changes such as regeneration of endometrial epithelium over the caruncles (Gier and Marion, 1968; Wagner and Hansel, 1969; Archbald et al., 1972).

In a physiological puerperium, the decrease in size was completed around day 22 to 28 pp when diagnosed only by transrectal palpation and the regeneration of endometrial epithelium over the caruncles was completed by day 30 pp in normal cows (Wagner and Hansel, 1969; Bekana et al., 1996; Krueger et al., 2009). However, evaluating the changes within the uterine wall by using an ultrasonic linear scanner, completion of the uterine involution was confirmed to last approximately 40 days (Okano and Tomizuka, 1987). A more recent study examined the size of the uterine horns, the uterine body diameter and the uterine fluid volume by ultrasound and found that the completion of the uterine involution took about 49 days (Scully et al., 2013). Investigations of the uterine blood flow found a decrease of 86 % in the ipsilateral horn during the first 45 days pp and a subsequent 2 % reduction (Heppelmann et al., 2013).

This suggests that the puerperium of high-producing dairy cows takes about 6 to 7 weeks, a period of time when conception rate is considerably lower and physiological uterine involution appears to be a barrier to fertility even though the ovulation of an oocyte capable of fertilization can occur (Kiracofe, 1980; Breuel et al., 1993).

2.1.2. Re-establishment of the estrous cycle during the bovine puerperium

For dairy cows, the time span required to regain ovarian cyclicity after parturition has changed over the past decades. Studies completed before 1970 found that first ovulation occurred between 14 and 21 days after calving (Morrow et al., 1966; Marion and Gier, 1968) but with increasing productivity of dairy cows, the average interval from calving to first

ovulation extended. Even though a more recent study found that all cows (sample size [n] = 19) had resumed ovarian cyclicity also by day 21 (Scully et al., 2013), there are more studies that report higher time spans for the resumption of ovarian cyclicity in dairy cows with the means ranging from about 25 to 43 days (McDougall et al., 1995; Zain et al., 1995; Lucy, 2001; Sakaguchi et al., 2004). Usually, the first ovulation is not accompanied by visible signs of estrus (Sakaguchi et al., 2004; Crowe, 2008).

2.1.3. The influence of parturition and uterine diseases on the bovine puerperium

The time span of the bovine puerperium is influenced especially by the course of parturition and the occurrence of retained fetal membranes (RFM). Obstructed labour, also known as dystocia, describes a difficult parturition resulting in a prolonged expulsive phase of calving (Mee, 2008). RFM are inconsistently described as the expulsion of fetal membranes more than 8 h (Vannucchi et al., 2017), more than 12 h (Pohl et al., 2015) or more than 24 h (Kimura et al., 2002) after calving.

The occurrence of dystocia is a predisposing factor for RFM (Laven and Peters, 1996) and cows with RFM are more likely to develop inflammatory diseases of the bovine uterus (Bruun et al., 2002; Giuliodori et al., 2013). If inflammatory uterine diseases such as clinical metritis or endometritis occur during the bovine puerperium it is considered pathological and can be prolonged (Fonseca et al., 1983; Mateus et al., 2002; Heppelmann et al., 2013; Heppelmann et al., 2015), which is also linked to prolonged intervals from calving to conception (McDougall et al., 2007).

2.2. Inflammatory diseases of the bovine uterus

Inflammatory diseases of the bovine uterus are frequently diagnosed in dairy cows in the time span from calving to conception (Bondurant, 1999). Postpartum uterine inflammation can be resolved spontaneously during the puerperium and the prevalence declines with time pp (McDougall et al., 2007; Chapwanya et al., 2012).

2.2.1. Terms depending on histology

The terms for inflammatory diseases of the bovine uterus are derived from the histological subdivision of the bovine uterine wall into the perimetrium, the myometrium and the endometrium (Liebich, 2003). If inflammation affects the endometrium it is called endometritis, if it affects the perimetrium it is called perimetritis and if it affects all layers of the uterine wall it is called metritis.

The perimetrium (tunica serosa) is a single-layer epithelium on the outside of the myometrium. The myometrium (tunica muscularis) contains an outer longitudinal and an inner circular layer of smooth muscles divided by a vascular layer (stratum vasculosum). Towards the uterine lumen, the myometrium is covered by the endometrium (tunica mucosa), which forms numerous tubular glands and contains different immune cells (e.g. macrophages and lymphocytes) (Liebich, 2003). The endometrium is subdivided into the lamina propria mucosae (multi-layered stromal cells as connective tissue) and luminal and glandular epithelial cells (mostly single-layered; in places multi-layered). In the cow, the endometrium also forms caruncles, knob-like aglandular protrusions for the connection with the fetal placenta (Liebich, 2003). Throughout the estrous cycle, the cells of the endometrium proliferate and degenerate dependent on hormonal changes (Arai et al., 2013).

2.2.2. Definitions depending on clinical signs

Depending on the clinical signs, metritis is subdivided into clinical metritis and puerperal metritis and endometritis is subdivided into clinical endometritis (CE) and subclinical endometritis (SCE). Furthermore, there a cases of pyometra. The characterization of these postpartum reproductive tract diseases has been revised by Sheldon et al. (2006) to determine precise and consistent definitions.

2.2.2.1. Clinical and puerperal metritis

According to Sheldon et al. (2006), an abnormally enlarged uterus and purulent uterine discharge detectable in the vagina within 21 days after parturition define clinical metritis. The case of an abnormally enlarged uterus, fetid watery red-brown vaginal discharge, signs of systemic illness (e.g. toxemia, reduced milk yield, dullness) and fever (> 39.5 °C) within 21 days pp is defined as puerperal metritis. Cows with metritis have been shown to suffer from severe pain (Stojkov et al., 2015). The incidence of metritis depends on the definition and diagnostic method and ranges from about 13 up to 69 % (Urton et al., 2005; Mahnani et al., 2015). Metritis represents a risk factor for subsequent occurrence of endometritis, as metritis persist in about 20 % of the animals as CE (Sheldon et al., 2008).

2.2.2.2. Clinical endometritis

Purulent (> 50 % pus) vaginal discharge detectable after 21 days pp or mucopurulent (approximately 50 % pus, 50 % mucus) vaginal discharge after 26 days pp characterizes CE. CE is not accompanied by systemic signs of illness (Sheldon et al., 2006). In case of CE, the involution of the uterus may be delayed, but overall the identification of the size of the uterus by palpation lacks diagnostic accuracy (LeBlanc et al., 2002a). The prevalence of CE varies considerably among studies depending on the housing and general health condition of the herd, the diagnostic methods and the time point of the examination pp. Reports about CE

state its prevalence between 15 and 60 % (McDougall et al., 2007; LeBlanc, 2008; Plöntzke et al., 2011).

2.2.2.3. Subclinical endometritis

SCE defines an endometrial inflammation with the absence of purulent material in the vagina, a physiological size of the uterus and no systemic signs of illness. It is manifested through the infiltration of the endometrium with polymorphonuclear neutrophils (PMN) (Sheldon et al., 2006). SCE is diagnosed by cytological assessment (Kasimanickam et al., 2004; Melcher et al., 2014). Samples of the composition of cells in the endometrium are collected by flushing the uterine lumen or by using the cytobrush technique (Gilbert et al., 2005; Kasimanickam et al., 2005a).

There are different determinations about the percentage of PMN that define SCE in the literature. Thresholds range from 4 to 18 % PMN (Kasimanickam et al., 2004; Barlund et al., 2008; Galvão et al., 2009). In scientific studies, the prevalence of SCE varies from 16 % up to 90 % (Lincke et al., 2007; LeBlanc, 2008; Madoz et al., 2013). Barański et al. (2012) showed that the prevalence of SCE can range from approximately 19 % to 75 % even within one study depending on the use of two herds, two time points (fourth and sixth week pp) and four different cytological thresholds (18, 10, 8 and 5 % PMN).

2.2.2.4. Pyometra

An infrequent case of bovine uterine disease is pyometra. Pyometra is defined as a distended uterus with an accumulation of purulent or mucopurulent material in the uterine lumen (ultrasonographically detectable) and a persistent corpus luteum. There is a functional closure of the cervix, but some pus may discharge into the vaginal lumen (Sheldon et al., 2006). Of all cases of uterine diseases, less than 5 % are defined as pyometra (Sheldon et al., 2008).

2.2.3. Impact on bovine reproductive performance

Many studies showed the negative impact of metritis and endometritis on the reproductive performance of dairy cows.

Cows suffering from metritis had lower first breeding (artificial insemination [AI] or embryo transfer) pregnancy rates and an increased incidence of pregnancy loss after day 45 post inseminationem (p.i.) (Ribeiro et al., 2016). The chance of pregnancy was lower for cows with puerperal metritis on days 3-14 pp compared to healthy cows (Piccardi et al., 2016). The reproductive performance was also impaired when cows were diagnosed with (muco)purulent vaginal discharge on days 7-28 pp compared to cows with an absent or clear

vaginal discharge (Runciman et al., 2009). This study did not separate metritis from CE cows.

Cows with CE on days 24-26 pp were four times less likely to conceive at first service and had a decreased likelihood of pregnancy at 150 days pp (Gobikrushanth et al., 2016). The negative influence of CE on reproductive performance was also shown when the diagnosis of CE on days 21-41 pp was linked to a decreased first service conception rate (Tison et al., 2017), prolonged intervals from calving to conception and higher culling rates (Giuliodori et al., 2013).

Cytologically diagnosed endometritis (independent of the occurrence of vaginal discharge) on days 40-60 pp was associated with a lower first service and also overall pregnancy rate (Gilbert et al., 2005). Similarly, cows that were categorized to have high proportions of endometrial PMN (highest quartile; independent of vaginal discharge) on day 28 and 42 pp had an increased number of days from calving to conception and lower pregnancy rates (McDougall et al., 2011).

The influence of SCE (no purulent vaginal discharge) on reproductive performance is not consistent in scientific studies. SCE diagnosed on days 20-47 pp was associated with a reduced pregnancy rate (Kasimanickam et al., 2004; Ribeiro et al., 2013). However, a different study suggested a negative impact of SCE on reproductive performance only for multiparous but not for primiparous cows (Cheong et al., 2011). Two studies from Argentina also showed a contrary influence of SCE on reproductive performance. First service conception rate was not affected by SCE on days 18-38 pp (Plöntzke et al., 2010), whereas it was reduced by SCE on days 21-62 pp (Madoz et al., 2013). Another study suggests that the influence of SCE on reproductive performance depends on the time point pp. Cows with SCE on day 35 pp and on day 49 pp had an increased time to pregnancy, whereas that was not the case for cows with SCE on day 21 pp (Galvão et al., 2009). A study of Lima et al. (2013) showed similar results. SCE diagnosed on 25±3 days in milk (DIM), 32±3 DIM or 46±3 DIM depressed the number of pregnancies for timed AI on day 75±3 DIM and increased pregnancy loss (between 32 and 60 days p.i.) only when it persisted until day 46±3 DIM (Lima et al., 2013).

2.2.4. Economic consequences

Beyond the optimal time for first insemination (considering a voluntary waiting period), each additional day that a cow is not pregnant is costly (Groenendaal et al., 2004; Meadows et al., 2005). A herd conceiving at an early stage of the lactation (60 to 100 DIM) had a higher value than a herd conceiving at a later stage (150 to 200 DIM) (Kalantari and Cabrera, 2012). A different study calculated the net economic value of reproductive efficiency in dairy cattle

for three different (good, average and poor) reproductive performance scenarios considering the rates for ovulation, conception and embryonic death (Inchaisri et al., 2010). This study showed a mean net economic loss of €34/cow/year for an average reproductive performance and of €231/cow/year for a poor reproductive performance in comparison to the good scenario. Important factors were increased calving intervals, involuntary culling costs and the decline of milk production. Consequently, this leads to great economic losses for the dairy industry. The costs are also increased by the number of AI and veterinary attendance. The expenses for the veterinary treatment included in the calculation, endometritis reduces the profitability of dairy farming enormously. In 2009, the estimated annual cost for uterine diseases was 1.4 billion € for the European Union and 650 million $ for the United States (Sheldon et al., 2009a).

2.3. The role of bacteria in the bovine uterus

Besides impaired immunity, one of the causes for the development of uterine inflammation in the postpartum period is the invasion of bacteria into the uterine lumen (Griffin et al., 1974; Singh et al., 2008). During pregnancy, the cervix is closed and prevents the endometrium from bacterial contamination. During and after parturition, this anatomical barrier is breached for several days and allows bacteria to ascend to the uterus. The majority of bacterial invasion occurs periparturient and in the first days of the puerperium when there is a uterine environment of disrupted epithelial surfaces, debris and fluids which accelerates bacterial growth (Bondurant, 1999; Sheldon et al., 2002; Földi et al., 2006). Especially abnormal calving and RFM are accompanied with the invasion of bacteria to the uterus and they are among the strongest risk factors for metritis and endometritis (Salasel et al., 2010; Giuliodori et al., 2013; Prunner et al., 2014b; Bicalho et al., 2017c).

With culture-independent methods, bacteria can always be found in uterine samples from both healthy and metritic cows in the pp period. The composition of these bacterial communities varies considerably in between different uterine health groups (Santos et al., 2011; Santos and Bicalho, 2012; Wagener et al., 2015). This suggests that certain bacterial species take part in the pathogenesis of uterine inflammatory diseases and others do not.

Among several others, bacteria most frequently associated with uterine disease in dairy cows are *Escherichia* (*E.*) *coli* and *Trueperella* (*T.*) *pyogenes* (Williams et al., 2005; Wagener et al., 2015; Pohl et al., 2018). Within the first 3 weeks pp, dairy cows had a uterine contamination with potentially pathogenic *E. coli* and *T. pyogenes* from up to 60 % and 85 %, respectively (Wagener et al., 2014). Research showed that uterine infection was predominated by *E. coli*

in the first week pp whilst the presence of *T. pyogenes* increased to a peak around day 14 pp (Williams et al., 2007; Wagener et al., 2014; Gilbert and Santos, 2016).

Although there are several other bacteria that are potentially pathogenic for the bovine uterus, only *E. coli*, *T. pyogenes* and their virulence factors are introduced here. It should be noticed that the development of inflammatory diseases of the bovine uterus is dependent on the composition, interaction and characteristics of the whole bovine uterine microbiome (Wagener et al., 2015; Bicalho et al., 2017a; Bicalho et al., 2017b). However, the interplay of the endometrial microbiome during the bovine puerperium and its role in uterine diseases are not yet fully understood, part of other intensive research (Jeon et al., 2015; Knudsen et al., 2016; Wang et al., 2018) and will not be presented at this point.

The presence of *E. coli* in the bovine uterus is significantly associated with fetid vaginal discharge (Jaureguiberry et al., 2016) and with high levels of lipopolysaccharide (LPS), an endotoxin released from the bacterium that appears to be its major virulence factor (Dohmen et al., 2000; Sheldon et al., 2010). Endotoxin concentrations were significantly higher in the uterine fluid of cows with severe endometritis than in cows with mild endometritis (Mateus et al., 2003). Furthermore, a link can be drawn to ovarian dysfunction and therefore impaired reproductive performance. Cows with a high contamination of the uterus with bacterial pathogens on day 7 pp, including *E. coli*, had smaller dominant follicles, lower peripheral estradiol plasma concentrations and smaller corpora lutea that produced less progesterone (Williams et al., 2007). The systemic application of LPS to cows in diestrous suppressed both the structure and function of the corpus luteum (Herzog et al., 2012). Intravenous or intrauterine infusion with LPS delayed ovulation by interrupting the preovulatory estradiol rise, decreasing the luteinizing hormone (LH) pulse frequency and blocking or lowering the preovulatory LH surge in cows and heifers (Peter et al., 1989; Suzuki et al., 2001; Lavon et al., 2008).

These effects might be linked to changes in the function of the hypothalamus and pituitary. In sheep, the species most intensively studied for the effects of LPS in the ruminant brain, LPS suppressed both the frequency and amplitude of gonadotropin-releasing hormone (GnRH) and LH pulses and the sensitivity of the pituitary to exogenous GnRH (Karsch et al., 2002). However, follicle-stimulating hormone concentrations in the peripheral plasma are unaffected in cows with uterine disease (Sheldon et al., 2002).

Similar, a *T. pyogenes* infection of the bovine uterus is correlated with severe uterine lesions and purulent vaginal discharge, representing a significant risk factor for the development of CE and has long been in the focus of intensive research (Studer and Morrow, 1978; Bicalho et al., 2016). Cows that were positive for *T. pyogenes* at 35±3 DIM had remarkably 19.8

higher odds of being diagnosed with CE (Machado et al., 2012a). An *in vivo* study investigated the effects of an intrauterine infusion of *T. pyogenes* on the histological presentation of uterine tissue. All cows infused with *T. pyogenes* had moderate to severe signs of inflammation with transmigration of neutrophils and disruption of the epithelium (Lima et al., 2015). The same study showed that the mRNA expression of pro-inflammatory interleukins (ILs) was increased in cows after the intrauterine infusion of *T. pyogenes*. A different study demonstrated that after the intrauterine infusion of *T. pyogenes* to cows with a clinically healthy uterus, the development of persisting mucopurulent discharge was associated with the isolation of *T. pyogenes* four and six days after the challenge (Amos et al., 2014). This categorizes *T. pyogenes* as a primary uterine pathogen. An explanation for its virulence is the secretion of the pore-forming cholesterol-dependent pyolysin, which can cause endometrial cell lysis, especially of endometrial stromal cells (Jost and Billington, 2005; Amos et al., 2014).

There are studies suggesting that the contamination of the bovine uterus with *E. coli* early after parturition favors the development of a *T. pyogenes* infection (Dohmen et al., 2000; Gilbert and Santos, 2016). In contrast, it was also shown that *E. coli* negative cows were more likely to have a subsequent *T. pyogenes* infection than *E. coli* positive cows (Wagener et al., 2014).

However, it has to be taken into account that both *E. coli* and *T. pyogenes* can be isolated from the uterus of clinically affected and unaffected cows, which suggests a different pathogenicity of the isolates (Bicalho et al., 2010; Sheldon et al., 2010; Jeon et al., 2015). In this context, a *T. pyogenes* strain that was isolated from the puerperal uterus of a cow developing CE expressed more virulence factors, had a higher growth rate and induced a higher endometrial mRNA expression of pro-inflammatory factors *in vitro* than a *T. pyogenes* strain that was isolated from the puerperal uterus of a cow that remained healthy (Ibrahim et al., 2017).

There are also new insights about the localization of bacteria if present in the bovine endometrium. With fluorescence *in situ* hybridization, *T. pyogenes* and *E. coli* were detected only on the endometrial surface or debris (sometimes intracellularly in leukocytes), while e.g. *Fusobacterium (F.) necrophorum* and *Porphyromonas levii* were often also localized intraepithelially or in the lamina propria (Karstrup et al., 2017).

2.4. Defense mechanisms of the bovine uterus

Generally, microbial infections and tissue damage result in defense mechanisms mediated by the affected tissue (Chovatiya and Medzhitov, 2014). Anatomically, endometrial epithelial

cells are forming the line of initial defense in case of the invasion of the uterine lumen with pathogenic bacteria being more resilient than the underlying stromal cells (Amos et al., 2014; Healy et al., 2015).

When pathogenic bacteria ascend to the uterine lumen, the immune system of the bovine endometrium will be activated (Bondurant, 1999; Sheldon et al., 2014) and encompasses nonspecific but immediate defense mechanisms of the host against foreign substances (e.g. invading pathogens) and trauma by the recruitment of immune cells to the site of infection (Murphy et al., 2008). There is indication that the immune system reacts dependent on the bacterial load there is. In endometrial samples, the expression of genes of the innate immune system was correlated to the bacteriology score on day 29 pp (Walker et al., 2015) and the overall proportion of PMN increased by 0.03 % for every increase in counted bacteriologic colony within the first 4 weeks after calving (Prunner et al., 2014a).

2.4.1. Toll-like receptors for the recognition of bacterial infection

One mechanism of the innate immune system to respond to bacterial infection is the recognition of pathogen-associated molecular patterns (PAMPs) of the invading bacteria by Toll-like receptors (TLRs), likewise it can respond to trauma by the recognition of damage-associated molecular patterns (DAMPs) of damaged cells (Beutler et al., 2003; O'Neill et al., 2013; Sheldon et al., 2014).

It was shown that TLR1 to 10 are expressed in bovine endometrial tissue (Davies et al., 2008; Gärtner et al., 2016). For example, TLR4 appears to play a central role for the inflammatory cascade in the bovine uterus by recognizing the endotoxin LPS from Gram-negative bacteria (Sheldon et al., 2009a). The mRNA expression of TLR4 was up-regulated when bovine endometrial epithelial cells were stimulated with LPS from *E. coli* (Fu et al., 2013).

Other important TLRs in the bovine uterus for the recognition of Gram-positive bacteria are TLR1, TLR2 and TLR6 by binding bacterial lipopeptides and TLR5 by recognizing flagellin from both Gram-positive and Gram-negative bacteria (Hayashi et al., 2001; Sheldon et al., 2014). The activation of TLRs in the bovine endometrium by PAMPs subsequently initiates signalling pathways within the endometrial cell resulting in an enhanced synthesis of pro-inflammatory prostaglandins and cytokines, which can chemoattract immune cells such as PMN (Fu et al., 2013; Sheldon et al., 2014; Turner et al., 2014).

2.4.2. Polymorphonuclear neutrophils for the control of bacterial infection

PMN are leucocytes that are formed in the bone marrow and migrate into the tissue at the site of an infection as part of the innate immune response. They can eliminate pathogens by

chemotaxis towards the targeted cell, phagocytosis and the production of antimicrobials such as enzymes and reactive oxygen species (Hussain, 1989; Cooray and Bjorck, 1995; Mayadas et al., 2014).

There is indication that not only the amount but also the functionality of the PMN is decisive for the control of pathogens and this functionality can be reduced when pp metabolic stress occurs in cows (Hoeben et al., 2000; Zerbe et al., 2000; Ster et al., 2012). Furthermore, a periparturient depression of circulating PMN in terms of a lower production of super oxide, myeloperoxidase and hydrogen peroxide was associated with the development of bovine postpartum reproductive diseases such as metritis and endometritis (Islam et al., 2017). It was also observed that the periparturient reduction in phagocytic activity of neutrophils is more marked in older rather than younger cows (Gilbert et al., 1993), which might be a reason why uterine disorders in the pp period are more common in older cows than in heifers (Grohn et al., 1990).

2.4.3. Pro-inflammatory mediators, enzymes and receptors

Not only immune cells, such as PMN, are needed to conduct a regulated response. Also a large number of mediators, enzymes and receptors are important to allow the communication and mediation between cells for defense and regeneration.

Among many others, important members of this group are specific cytokines, specific prostaglandin synthases, matrix metallopeptidase 1 (MMP1), protein tyrosine phosphatase, receptor type C (PTPRC) and platelet-activating factor receptor (PTAFR). As these mediators, enzymes and receptors were chosen to be analyzed in the present study, they will be presented in detail below.

2.4.3.1. Cytokines

The group of cytokines includes ILs, tumor necrosis factor (TNF), chemokines and interferons. Cytokines are small proteins that are important for the regulation of cell communication, especially during the immune response of the host to infection and trauma, but also for reproductive events such as ovulation (Sheldon et al., 2014; Stassi et al., 2017). Cytokines can prevent propagation of pathogens by the activation of immune cells resulting in an ultimately elimination of the pathogens. Nearly all nucleated cells are capable of synthesizing and also responding to them via specific receptors, which in case of bovine endometritis involves endometrial and immune cells (Tecchio et al., 2014). There are pro-inflammatory cytokines which support the development of inflammation whereas anti-inflammatory cytokines reduce inflammation, e.g. by the suppression of the activity of pro-inflammatory cytokines (Dinarello, 2000).

2.4.3.1.1. Interleukin 1 alpha, beta and receptor antagonist

IL1 alpha (IL1A) and IL1 beta (IL1B) are pro-inflammatory cytokines that can affect virtually all cell types and organs binding to the same receptor (Garlanda et al., 2013). IL1A primarily is a mediator of local inflammation, rarely detected in blood circulation, whereas IL1B also has systemic hormone-like effects, e.g. also produced by blood monocytes (Dinarello, 1996; 2011). The endometrial mRNA expression of *IL1A* and *IL1B* was elevated in cows with SCE and CE (Gabler et al., 2009; Fischer et al., 2010; Kasimanickam et al., 2014; Johnson et al., 2015). Furthermore, the concentration of IL1B was higher in cervico-vaginal mucus from endometritic cows compared to healthy cows measured by an enzyme-linked immunosorbent assay (ELISA) (Adnane et al., 2017).

Generally, IL1A plays an important role in early inflammation inducing the release of IL1B and other cytokines and mediating neutrophil recruitment to damaged tissue (Rider et al., 2013). IL1B is able to influence the synthesis of prostaglandins by the induction of the synthesis of phospholipase A_2 (PLA_2) and prostaglandin-endoperoxide synthase 2 (PTGS2) (Dinarello, 2009). Furthermore, IL1B promotes the infiltration of immune cells into the extravascular space and tissue by its ability to increase the expression of adhesion molecules on mesenchymal and endothelial cells (Dinarello, 2009).

Although IL1A normally is an intracellular cytokine and classified as DAMP, it was released from bovine endometrial cells after cell damage, but only in combination with a prior pathogen (LPS) challenge *in vitro* (Healy et al., 2014). The same study showed that a treatment with heat killed bacteria (no cell damage) or cycles of freezing in liquid nitrogen and thawing (sterile cell damage without bacteria) did not increase the release of IL1A. The authors concluded that rather than a typical DAMP, IL1A is released from bovine endometrial cells especially in recognition that there is a combination of pathogen challenge followed by cell damage.

Furthermore, two *in vitro* studies showed that the endometrial mRNA expression of *IL1A* seems to be dependent on the bacterial species. A significantly higher mRNA expression of *IL1A* was observed in endometrial cells co-cultured with *Bacillus pumilus* with a multiplicity of infection (MOI) of 1 already after 2 h (Gärtner et al., 2016) and with *T. pyogenes* (MOI = 1) only after 8 h (Ibrahim et al., 2017). Interestingly, *T. pyogenes* (MOI = 0.1) also induced a significantly higher mRNA expression of *IL1A* in peripheral blood mononuclear cells (PBMC) already after 6 h of co-culturing (Ibrahim et al., 2017). Furthermore, the expression of *IL6* mRNA in endometrial cells co-cultured with *T. pyogenes* for 8 h was higher when PBMC were present (Ibrahim et al., 2017). The authors assumed that for the up-regulation of bovine

uterine inflammation, IL1A is derived from bacteria-activated immune cells rather than from damaged endometrial cells, which also contradicts the classification of a typical DAMP.

The naturally occurring IL1 receptor antagonist (IL1RN) competitively binds to the same receptors as IL1A and IL1B, which results in a very effective prevention of IL1 signal transduction (Dinarello, 1996). A significantly increased mRNA expression of *IL1RN* in bovine endometrial epithelial cells was shown in case of CE (Gabler et al., 2009).

2.4.3.1.2. Interleukin 6 and 8

IL6 is a pro-inflammatory cytokine that is promptly synthesized in case of tissue damage or inflammation due to infection or injury, stimulating acute-phase immune reactions and hematopoiesis (Tanaka and Kishimoto, 2014), whereas IL8 mainly promotes the chemoattraction and diapedesis of neutrophils to the site of infection (Hoch et al., 1996; Zerbe et al., 2003; Struyf et al., 2005). This leads to an increased phagocytosis and bacterial killing. Concentrations of IL6 and IL8 were higher in cervico-vaginal mucus from endometritic cows compared to healthy cows measured by an ELISA (Adnane et al., 2017). The same applied for IL6 and IL8 in uterine flush samples derived from cows with CE (Kim et al., 2014). In addition, CE and/or SCE led to a significantly increased mRNA expression in bovine endometrial epithelial cells of *IL6* and *IL8* (Fischer et al., 2010; Ghasemi et al., 2012; Kasimanickam et al., 2014; Johnson et al., 2015).

2.4.3.1.3. Interleukin 10

An anti-inflammatory effect is awarded to IL10 (Moore et al., 1993). It inhibits the capacity of monocytes and macrophages to present antigen and downregulates the expression of IL1, IL6, IL8 and TNF (Trifunovic et al., 2015). IL10 concentrations were higher in uterine flush samples derived from cows with CE (Kim et al., 2014). In contrast, endometrial *IL10* mRNA expression could not be associated with bovine uterine disease in two other studies (Herath et al., 2009a; Galvão et al., 2011).

2.4.3.1.4. Tumor necrosis factor

Pro-inflammatory TNF is mainly produced by monocytes and/or macrophages infiltrating to the site of an inflammation (Vilcek and Lee, 1991). It induces the release of IL1 (Dinarello et al., 1986) and stimulates, together with IL1, the production of the pro-inflammatory IL8 (Sica et al., 1990; Roach et al., 2002). This interplay of TNF, interleukins and neutrophils was also exemplified when the infusion of TNF into bovine uteri led to histological signs of moderate to severe inflammation with transmigration of neutrophils into the glands (Lima et al., 2015). Surprisingly, the mRNA expression of *TNF* was lower in uterine biopsies taken from cows with histologically classified severe endometrial inflammation compared to mild inflammation (Chapwanya et al., 2009). The authors of this study suggested that the downregulation of

TNF could be in favor of the resolution of an existing inflammation. In contrast, TNF concentrations (measured by ELISA) were higher in uterine flush samples derived from cows with CE compared to healthy cows (Kim et al., 2014) and in case of CE and/or SCE, also a significantly increased endometrial mRNA expression was shown for *TNF* (Fischer et al., 2010; Ghasemi et al., 2012; Kasimanickam et al., 2014; Johnson et al., 2015).

2.4.3.1.5. Chemokine ligands and chemokine receptor 2

Besides IL8, other strong chemoattractants that stimulate the migration of leukocytes to the site of infection are the chemokine (C-X-C motif) ligands 1 (CXCL1), CXCL2, CXCL3 and CXCL5 (Olson and Ley, 2002; De Filippo et al., 2013). The effects of these chemokine ligands and IL8 are promoted by the chemokine receptor 2 (CXCR2), which is expressed on granulocytes, macrophages and endothelial cells (Murphy, 1994; Addison et al., 2000). In former studies, it was shown that an increased endometrial mRNA expression of all of the mentioned chemokine ligands and their receptor can be associated with SCE and/or CE (Fischer et al., 2010; Gabler et al., 2010; Gärtner et al., 2016).

2.4.3.2. Prostaglandins and their synthesis

Prostaglandins (PGs) represent a group of lipid mediators with hormone-like autocrine and paracrine effects that are produced by nucleated cells in almost all tissues (Miller, 2006). Together with the leukotrienes they are referred to as eicosanoids. They regulate numerous (patho)physiological processes including platelet aggregation, mitogenesis and inflammation by the modulation of immunity (Goodwin and Webb, 1980; Goetzl et al., 1995; Harris et al., 2002). Furthermore, prostaglandins have long been known to be involved in important reproductive events such as luteolysis and ovulation (Armstrong, 1981; McCracken et al., 1981).

2.4.3.2.1. Prostaglandin H_2

For the synthesis of PGs, arachidonic acid is released from membrane phospholipids by the action of PLA_2 and enzymatically converted to PGH_2 (Miller, 2006). The conversion from arachidonic acid to PGH_2 is catalyzed by two key enzymes of the PG biosynthesis, PTGS1 and PTGS2, also known as cyclooxygenase 1 and 2, respectively.

PTGS1 and PTGS2 differ in their tissue distribution and expression pattern. PTGS1 is known to be a constitutive synthase for PGH_2 in all tissues and cells that synthesize PGs, whereas PTGS2 is inducible by inflammatory stimuli (Botting, 2006).

PGH_2, which is chemically unstable, is then metabolized into different bioactive products such as further PGs and thromboxanes. PGs that are emerged from PGH_2 are $PGF_{2\alpha}$, PGE_2, PGI_2 and PGD_2 (Harris et al., 2002).

2.4.3.2.2. Prostaglandin $F_{2\alpha}$

$PGF_{2\alpha}$ is produced from PGH_2 by prostaglandin F synthase (PTGFS) and known to take part in reproductive events especially by its luteolytic effect (McCracken et al., 1999).

2.4.3.2.3. Prostaglandin E_2

The enzyme for the catalysis of PGH_2 to PGE_2, which takes part in the estrous cycle, occurs in three forms; the membrane-bound prostaglandin E synthase (PTGES) and PTGES2, while PTGES3 is located in the cytosol. PTGES2 and PTGES3 are rather constitutively expressed in various cells and tissues and mostly unaffected by inflammation or tissue damage, whereas the expression of PTGES is induced by pro-inflammatory stimuli and tissue repair (Kudo and Murakami, 2005).

PTGES might be one of the best examples how endometrial inflammation can influence the cycle of reproductive tract events. In samples from healthy uteri, the mRNA expression of *PTGES* was found to be estrous cycle dependent with a higher expression in the post-ovulatory compared to the pre-ovulatory phase (Gabler et al., 2009). This pattern fits to the knowledge that PGE_2 influences the corpus luteum by its luteotrophic effect (Magness et al., 1981; Reynolds et al., 1983) and that increased concentrations of PGE_2 caused luteal persistence in cows (Gimenez and Henricks, 1983; Thibodeaux et al., 1992). The mRNA expression of *PTGES* was not influenced in repeat breeder cows (RBC = cows that were unsuccessfully inseminated for at least 3 times in a row within the same lactation) with SCE compared to RBC without SCE (Janowski et al., 2017). However, it was higher in endometrial samples of cows with SCE and CE compared to healthy cows on days 29-36 pp (Johnson et al., 2015) and higher amounts of PGE_2 were found with an enzyme immunoassay in cows with SCE compared to healthy cows on days 21-28 pp (Barański et al., 2013). Cows with severe metritis compared to mild metritis had higher concentrations of PGE_2 in uterine fluids within the first week pp (Mateus et al., 2003) and even peripheral blood concentrations of PGE_2 where higher in cows with a uterine *E. coli* infection one week after calving (Herath et al., 2009a). Bacterial superantigens induced the production of PGE_2 in bovine mononuclear cells (Hendricks et al., 2000) and when bacterial LPS was added to endometrial cells *in vitro* an endocrine switch from $PGF_{2\alpha}$ to PGE_2 was noted (Herath et al., 2009a).

Therefore, it is not surprising that a connection can be drawn between inflammation of the uterus and prolonged luteal phases pp (Opsomer et al., 2000; Mateus et al., 2002; Strüve et al., 2013). However, a repeated intrauterine infusion of LPS to heifers also induced an increase in plasma concentrations of $PGF_{2\alpha}$ metabolite and premature luteolysis compared with control heifers (Lüttgenau et al., 2016). Also in heifers, the estrous cycle was shortened

after the intrauterine infusion of a viable *E. coli* strain which was isolated from the uterus of a cow with metritis (Gilbert et al., 1990). When LPS was given to cows one-time intravenously, the plasma PGE_2 concentration was nearly double ($P \leq 0.05$) after 1 h compared to control cows and the plasma concentration of $PGF_{2\alpha}$ metabolites was around tenfold higher ($P \leq 0.05$) after 30 minutes (Herzog et al., 2012). In this context, not only the absolute concentration of PGE_2 seems to be important for its effect on the corpus luteum but the proportion to $PGF_{2\alpha}$. The $PGE_2/PGF_{2\alpha}$ ratio was higher in the luteal stage of the bovine estrous cycle than in the follicular stage and in estrus (Murakami et al., 2001). This exemplifies how complex the interplay of the endometrium, the immune and hormonal system and the estrous cycle really is.

2.4.3.2.4. Prostaglandin I_2

PGI_2, also known as prostacyclin, is another potent pro-inflammatory prostaglandin. PGI_2 is a vasodilatator and inhibits platelet aggregation (Gryglewski, 2008). It is derived from PGH_2 by the action of prostacyclin synthase (PTGIS) mainly by endothelial cells (Ingerman-Wojenski et al., 1981; Spisni et al., 2001).

2.4.3.2.5. Prostaglandin D_2

PGD_2 is a prostaglandin mainly produced by mast cells promoting allergic reactions (Lewis et al., 1982; Pettipher, 2008). Besides its involvement in several aspects of inflammation, it is suggested that PGD_2 also plays an important role in reproductive events such as implantation in humans (Saito et al., 2002). In cows, mRNA of the lipocalin-type prostaglandin D_2 synthase (PTGDS) was significantly higher expressed in case of CE/SCE and after ovulation, showing its involvement in both inflammatory processes and physiological reproductive events (Gabler et al., 2009).

2.4.3.3. Matrix metallopeptidase 1

The first MMP that was discovered was called MMP1, also known as interstitial collagenase (Gross and Lapiere, 1962). Matrix metalloproteinases (MMPs) are a family of endoproteinases involved in the degradation of extracellular matrix of tissue that is undergoing processes such as reparation and remodelling (Shapiro, 1998). This can be of physiological nature as well as it is always part of pathological events such as inflammation. Regarding inflammatory processes, MMPs are not only responsible for tissue breakdown. They also influence the innate immune system by the interaction with cytokines (Parks et al., 2004; Van Lint and Libert, 2007). Therefore, it seems very likely that MMPs are also involved in the inflammatory processes of bovine endometritis. Supporting this assumption, an *in vitro* study showed the upregulation of *MMP1* mRNA expression in bovine endometrium exposed to LPS (Swangchan-Uthai et al., 2012).

2.4.3.4. Protein tyrosine phosphatase, receptor type C

Protein tyrosine phosphatases (PTPs) are a group of enzymes known to function as specific signaling molecules important for cellular functions such as cell differentiation, growth, apoptosis and adhesion (Soulsby and Bennett, 2009). PTPRC is a specific receptor type, also known as cluster of differentiation 45 (CD45). Being expressed as antigen on the surface of leukocytes, it can be used as an indicator for an activated immune system (Thomas, 1989). In the bovine endometrium, severe inflammation on day 15 pp coincided with a significant increase of the endometrial mRNA expression of *PTPRC* relative to day 60 pp when inflammation had completely resolved (Chapwanya et al., 2012).

2.4.3.5. Platelet-activating factor receptor

Intercellular signaling during acute inflammation is also regulated by the platelet-activating factor (PAF), a phospholipid, which influences a wide range of cell types including endometrial cells and key cells of the innate immune system mediated by its specific receptor PTAFR (Ishii et al., 2002; Zimmerman et al., 2002). It was shown that PTAFR is mainly localized in the luminal and glandular epithelium of the bovine endometrium and its staining was markedly increased in pregnant cows (Tiemann et al., 2001).

2.5. The endometrial immune system during the bovine puerperium

During pregnancy, the bovine endometrial immune system is in a quiet state (Hansen, 2011). Subsequent, it seems to be highly beneficial if the cow can rapidly shift to an upregulation around the time of calving and early puerperium when the risk of bacterial contamination of the uterus is high. Towards the end of the puerperium, a downregulated endometrial immune system seems to be supportive for fertility.

Although the difference of pathological and physiological inflammation is not well understood, inflammatory processes on a molecular and cellular basis without clinical signs might be classified as a physiological rather than a pathological part of the early puerperium. It is assumed that a depressed immune system around that time favors the development of uterine diseases (Chapwanya et al., 2012; LeBlanc, 2012).

An early downregulation of the endometrial innate immune system had a negative influence on the immune status at the end of the puerperium and on reproductive performance. Cows that developed RFM and CE had higher periparturient (15 days prepartum, day of calving and 15 days pp) serum IL10 contents which inhibit pro-inflammatory processes (Islam et al., 2013). The proportion of neutrophils at the day of calving and in the early puerperium (0 and 7 days pp) was negatively correlated with neutrophil proportion at 5 or 7 weeks pp (Gilbert

and Santos, 2016). The same study showed that the group of cows with a low endometrial proportion of neutrophils at 7 days pp (< 40 %) had a lower likelihood of pregnancy than the group with higher proportions (Gilbert and Santos, 2016). Furthermore, cows with a low endometrial mRNA expression of the pro-inflammatory *TNF* one week after calving developed endometritis (Galvão et al., 2011).

In this context, it has to be considered that the metabolism of a dairy cow changes dramatically during the periparturient period, which negatively influences the functionality of the immune system (Esposito et al., 2014). With the start of lactation, the nutritional requirements of a modern dairy cow exceed her intake potential, which results in a state of negative energy balance. Body fat reserves are mobilized to provide energy resulting in elevated blood levels of non-esterified fatty acids (NEFA) and beta-hydroxybutyric acid (Busato et al., 2002; Accorsi et al., 2005), which are known to disrupt several immune functions (Contreras and Sordillo, 2011). For example, NEFA had a negative influence on neutrophil oxidative burst activity *in vitro* and *in vivo* associated with a decreased ability of neutrophils to fight bacterial infections (Hoeben et al., 2000; Ster et al., 2012). In multiparous cows, high blood concentrations of NEFA one week prior to calving increased the odds for CE and SCE (Kaufmann et al., 2010).

This shows the conflictive demands and influences on the bovine uterine immune system in the early puerperium and provides indication why especially high producing dairy cows are of great risk of an impaired immune system and the development of inflammatory uterine diseases after parturition (Sheldon et al., 2009b). However, the role of the metabolic status and its influence on the innate immunity of the bovine endometrium in connection with the development of uterine diseases is a comprehensive topic (Wankhade et al., 2017; Sheldon et al., 2018) and will not be presented in detail at this point.

The physiological upregulation of the innate immune system is expected to decline in the second half of the puerperium. When primiparous cows were sampled with the cytobrush technique on day 10, 17, 24, 31, 38 and 45 pp, the overall proportion of endometrial PMN peaked around days 10-24 pp and decreased afterwards (Gabler et al., 2010). This was confirmed when the proportion of PMN was influenced in a cubic way (decrease, increase, decrease) in cows sampled on day 0, 3, 9, 15, 21 and 28 days after calving (Prunner et al., 2014a). In another study, the endometrial PMN content was not different between day 0, 7 and 21 pp, but significantly lower on day 35 and 49 pp, if compared to the earlier days of sampling (Gilbert and Santos, 2016). Histological analysis of the bovine endometrium also revealed a higher number of immune cells 15 days pp, which was significantly decreased by day 30 pp and had completely resolved by day 60 pp (Chapwanya et al., 2012).

Comparing the early and late pp period, the overall endometrial mRNA expression of pro-inflammatory cytokines also declines. In healthy and fertile cows, the endometrial mRNA expression of *IL1B* was lower during weeks 3-7 pp if compared to days 0-7 pp (Herath et al., 2009b). The same study showed that the mRNA expression of *IL1A* and *IL6* was lower in endometrial samples from the later time point compared to the earlier time point in infertile cows (Herath et al., 2009b). In cows with a mixed clinical uterine health status, the mRNA expression of *CXCL5*, *IL1B*, *IL6*, *IL8*, *TNF* and *PTGS2* was significantly lower on day 31 pp if compared to day 17 pp in the endometrium (Gabler et al., 2010). The endometrial mRNA content of e.g. *IL1A*, *IL6* and *IL8* was significantly lower in a group of cows at 9 weeks pp compared to a group of cows at 2 weeks pp, also with a mixed clinical uterine health status within the groups (Chapwanya et al., 2009). A genome-wide transciptomic profile of bovine endometrial biopsies (no information given regarding the clinical uterine health status) using mRNA sequencing also revealed an activation of the immune response on day 15 pp that undergoes a functional change towards tissue proliferation and regeneration on day 30 pp (Foley et al., 2012).

A downregulation of the endometrial immune system in the second half of the puerperium until the time of AI seems to be beneficial for the development of an embryo and therefore the establishment of a pregnancy. In fact, significantly higher numbers of total embryos as well as transferable embryos were flushed from the uterus at day 7 after superovulation if the proportion of endometrial PMN was 0 % compared to > 0 % immediately before first AI (Drillich et al., 2012). However, considering that spermatozoa provoke an immune response in the uterus (Schuberth et al., 2008), it is suggested that after AI the proportion of PMN has to be rated differently again. The best first service conception rate was reached in the group of cows that had > 0 % to 15 % at 4 h after AI if compared to 0 % and > 15 % (Kaufmann et al., 2009). This assumption about a positive impact of an endometrial immune reaction after AI on the development of an embryo was supported by the study mentioned above (flushing of embryos at day 7 after superovulation). The highest number of transferable embryos was flushed from the group of cows with a proportion of endometrial PMN of 0 % immediately before AI, but with > 0 % at day 7 after AI (Drillich et al., 2012).

In summary, the activity of the immune system always has to be interpreted depending on the time point pp. If an appropriate upregulation of the immune system continues to later stages of the puerperium there is a transition from a physiological to a more pathological status that affects fertility (LeBlanc, 2012).

2.6. Main strategies for the therapy of bovine uterine inflammation

Currently, two major therapeutic strategies for the treatment of bovine uterine inflammation are used – antibiotics (intrauterine or systemic) and $PGF_{2\alpha}$ (systemic).

2.6.1. Antibiotics

Bovine uterine inflammation is often caused by bacterial infections, therefore the use of antibiotics has long been one main strategy for its abatement (Thurmond et al., 1993; Dohmen et al., 1995; Sheldon and Noakes, 1998; Lima et al., 2014; Pohl et al., 2018).

For the cure of metritis, a positive effect after the systemic administration of the antibiotic ceftiofur could be shown in a recent meta-analysis (Haimerl et al., 2017). The reproductive performance was better in cows with puerperal metritis that received ceftiofur subcutaneously than in an untreated metritis group (Piccardi et al., 2016). Furthermore, when cows were diagnosed with (muco)purulent vaginal discharge on days 7-28 pp after calving and therefore combined a group of cows with metritis and with CE, a single intrauterine infusion with cephapirin improved reproductive performance (Runciman et al., 2009).

In case of CE on day 27-42 pp, an intrauterine treatment with cephapirin was associated with a significant improvement of the first service conception rate (Denis-Robichaud and Dubuc, 2015; Tison et al., 2017), although a second genital examination 2 weeks after the treatment failed to show a clinical cure (Tison et al., 2017). Another study also found a positive effect of intrauterine cephapirin on days 28-42 pp on the first service conception rate of cows with cytological endometritis (Denis-Robichaud and Dubuc, 2015). In this study, the definition for cytological endometritis was ≥ 6 % PMN (cytobrush technique) in combination with the occurrence of purulent vaginal discharge (Metricheck), which would classify these cows as having CE according to Sheldon et al. (2006). Similar, an intrauterine treatment with cephapirin to cows with CE on days 27-33 pp shortened the time span to the next establishment of pregnancy but there was no benefit of treatment when conducted earlier than 4 weeks pp (LeBlanc et al., 2002b). However, in an evidence-based evaluation about the use of antibiotics in postpartum dairy cows with CE, it was concluded that a single intrauterine infusion of cephapirin in cows less than 4 weeks pp is appropriate to treat CE, but that other antibiotics (systemically or intrauterine administered) as a treatment or a preventive approach did not result in any improvement of the reproductive performance (Lefebvre and Stock, 2012).

Regarding cows with SCE, clinical trials describing treatments of SCE with antibiotics a rare at this moment. A single intrauterine infusion with cephapirin on days 20-33 pp improved the reproductive performance of cows with SCE compared to a control group (Kasimanickam et al., 2005b). Similar, but for a different species, a single intrauterine treatment with cephapirin

on day 40 pp also improved the reproductive performance of buffaloes that were diagnosed with SCE on day 35 pp (Nehru et al., 2019).

Regardless its efficiency, the potential development of bacterial resistances (Cristino, 1999; Oliver and Murinda, 2012) and the possible residues in animal products are considered a possible issue when using antibiotics in food-producing animals (McEvoy, 2016).

2.6.2. Prostaglandin $F_{2\alpha}$

Another traditional and frequently applied therapeutic strategy is the use of a systemic $PGF_{2\alpha}$ (analogue) injection for cows with a responsive corpus luteum (Jackson, 1977). It is assumed that $PGF_{2\alpha}$ supports the self-cleaning mechanisms of the uterus and the discharge of the infected content by inducing estrous and myometrial contractions (Weems et al., 2006). Independent of the uterine health status, an improvement of the first-service conception rates of cows was shown after the treatment with a single dose of $PGF_{2\alpha}$ on days 14-28 pp (Young et al., 1984), but not shown after the treatment with a single dose of $PGF_{2\alpha}$ on days 14-16 pp (McClary et al., 1989). Anyway, the effectiveness of the often applied treatment of bovine uterine inflammation that uses the hormonal effects of $PGF_{2\alpha}$ or its analogues remains unclear.

In case of metritis, 2 doses of $PGF_{2\alpha}$ 8 h apart on day 8 pp increased the conception at first service in primiparous cows (Melendez et al., 2004). In a different study, 2 doses of $PGF_{2\alpha}$ 8 h apart on day 8 pp (in combination with an intramuscular injection of ceftiofur for 5 days starting day 3 pp) improved the pregnancy rate after the first two services compared to an untreated control group (Jeremejeva et al., 2012). However, with this experimental setup it is impossible to judge if this effect results from $PGF_{2\alpha}$ or from the antibiotic. In a similar setup, 2 doses of $PGF_{2\alpha}$ 12 h apart on day 8 pp (with the same treatment plan for ceftiofur) had no influence on uterine inflammatory signs diagnosed by cytological examination on weeks 5-7 pp compared to an untreated control group (Jeremejeva et al., 2010).

Regarding postpartum dairy cows with CE, it was concluded that there is no scientific evidence that supports the therapeutic effectiveness of $PGF_{2\alpha}$ in an evidence-based evaluation (Lefebvre and Stock, 2012). An administration of $PGF_{2\alpha}$ at both 5 and 7 weeks pp, did not affect the probability of cure of CE at week 8 pp and also did not improve the effect of CE on reproductive performance (Dubuc et al., 2011). Moreover, a meta-analysis showed no improvement of the reproductive performance of cows with endometritis if treated with $PGF_{2\alpha}$ (Haimerl et al., 2013).

Dubuc et al. (2011) also investigated the effect of $PGF_{2\alpha}$ at both 5 and 7 weeks pp on the cure of SCE at week 8 pp and could not show a positive effect. Supporting these findings, the treatment with $PGF_{2\alpha}$ on day 21, 35 and 49 pp did not affect the prevalence of SCE either

on day 35 pp or on day 49 pp (Galvão et al., 2009). An administration of $PGF_{2\alpha}$ at both 5 and 7 weeks pp also did not reduce the negative effects of SCE on the reproductive performance (Dubuc et al., 2011). The treatment of SCE with $PGF_{2\alpha}$ twice on day 25±3 DIM and 39±3 DIM improved the cytological endometrial health only temporary on day 32±3 DIM, but not until day 46±3 DIM and had no positive influence on fertility (Lima et al., 2013). However, a single treatment with $PGF_{2\alpha}$ on days 20-33 pp led to higher pregnancy rates in cows with SCE (Kasimanickam et al., 2005b).

Similar to the worries about the use of antibiotics in veterinary medicine, there are concerns on the part of the public about residues of hormones in animal products with a negative impact on the consumers (McEvoy, 2016).

2.7. Alternative strategies for the therapy or prevention of bovine uterine inflammation

The controversy about the traditional therapeutic options for bovine uterine diseases and their possible ineffectiveness reveals the necessity to further search for alternative strategies for the treatment and prevention of this condition.

2.7.1. Vaccination

An alternative strategy for the prevention of bovine uterine diseases could be vaccination. However, the results of research are inconsistent. The subcutaneous application of inactivated bacterial components and/or protein subunits of *E. coli*, *F. necrophorum* and *T. pyogenes* to Holstein heifers was found to significantly reduce the incidence of puerperal metritis and reproductive performance was improved (Machado et al., 2014). In contrast, a herd-specific subcutaneous vaccine containing inactivated whole bacterial cells of *T. pyogenes*, *E. coli*, *Streptococcus uberis*, *Bacteroides sp*. and *Peptostreptococcus sp*. did neither effectively reduce the risk of puerperal metritis and CE nor influenced the reproductive performance in first lactating dairy cows (Freick et al., 2017).

2.7.2. Immunostimulants

Another possible approach is the infusion of the bovine uterus with immunostimulants such as oyster glycogen, leukotriene B4 or low concentrations of LPS (Dhaliwal et al., 2001). All three can stimulate the migration of PMN to the uterine lumen (Klucinski et al., 1990; Hussain and Daniel, 1992; Subandrio and Noakes, 1992; Zerbe et al., 1996). When 100 µg of LPS was administered to the uterus of RBC with unclear discharge at estrous, bacterial infections were cleared within one estrous cycle and the following conception rate was improved (Singh

et al., 2000). However, the efficacy of such immunostimulants has not been determined on a large scale and they were not established as therapeutics for bovine uterine inflammation.

2.7.3. Lactic acid bacteria (probiotics)

A more recent alternative approach for the prevention and/or treatment of bovine uterine diseases is the use of specific lactic acid bacteria (LAB). Among others, the group of LAB comprises pathogens and commensals of the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus* and *Enterococcus*. LAB are Gram-positive and catalase negative bacteria united by the ability to produce lactic acid as the major end-product of carbohydrate fermentation.

Specific LAB can be also referred to as probiotics that are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO, 2002; Ljungh and Wadstrom, 2006). In human and veterinary medicine, this positive influence of specific LAB on the health of treated individuals has been shown in many different studies, e.g. for the health of the gastrointestinal tract of humans and pigs and the urogenital tract of women (Homayouni et al., 2014; Lievin-Le Moal and Servin, 2014; Yang et al., 2015; Jacouton et al., 2017).

The role of LAB in the female genital tract and their influence on genital health has been studied intensively especially for women (Reid and Burton, 2002). Lactobacilli are the most prevalent and often numerically dominant microorganisms in the vaginal microbiota of healthy woman (Borges et al., 2014; Garcia-Velasco et al., 2017). They can be administered to the human vagina for the prevention or treatment of vaginal infections supporting urogenital health (Ng et al., 2018).

The last decade brought a rising interest towards the properties of specific LAB also in the field of buiatrics (Signorini et al., 2012; Pellegrino et al., 2019) and in the past few years, researchers started to investigate the effects of specific LAB on bovine genital health and reproductive performance of dairy cows with promising first results (Ametaj et al., 2014; Deng et al., 2015a; Deng et al., 2015b; Genis et al., 2018).

2.8. *Lactobacillus* spp. and their probiotic properties

Subordinated to the family of *Lactobacillaceae*, species of the genus *Lactobacillus* are rod-shaped bacteria or coccobacilli that include a high number of GRAS (Generally Recognized As Safe) species. *Lactobacillus* (*L.*) spp. are non-sporeforming, generally aero-tolerant or anaerobic, aciduric or acidophilic (Cai et al., 2012).

There are several characteristics awarded to *Lactobacillus* spp. that explain the potential positive influence on the health of their host and therefore their classification as probiotics. *Lactobacillus* spp. can represent a barrier to infection by suppressing the population of bacterial pathogens by the competition for nutrition and the production of organic acids, hydrogen peroxide (H_2O_2) and bacteriocins lethal to pathogens (**Fig. 1**) (Aroutcheva et al., 2001; Juarez Tomas et al., 2002; Tachedjian et al., 2017). They can also protect their host from the detrimental effects of pathogens by the competition for the adherence to epithelial cells and the production of a protective biofilm over the epithelial cell surface (**Fig. 1**) (Boris and Barbes, 2000; Rolfe, 2000).

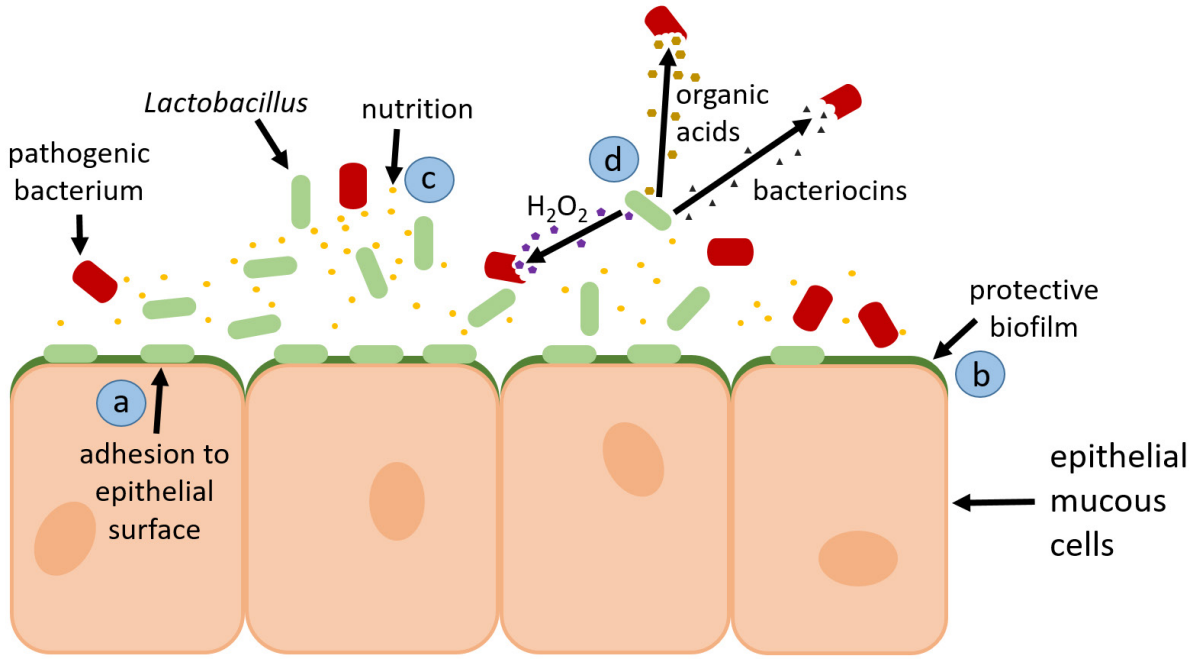

Figure 1. The bacterial genus *Lactobacillus* and its possible ways to suppress pathogenic bacteria on mucosal epithelial surfaces. (a) The adhesion of *Lactobacillus* to the mucosal epithelial surface prevents pathogenic bacteria from adhere to epithelial mucous cells. (b) The production of a protective biofilm by *Lactobacillus* on the mucosal epithelial surface prevents pathogenic bacteria from adhering to epithelial mucous cells. (c) The competition for nutrition leaves pathogenic bacteria with less nutrients for their metabolism. (d) The production of hydrogen peroxide (H_2O_2), organic acids and bacteriocins harms or kills pathogenic bacteria.

2.8.1. Occurrence of *Lactobacillus* spp. in the bovine genital tract

Lactobacillus spp. were found in samples from the bovine vagina by cultivation (Otero et al., 1999; Otero et al., 2000; Rodriguez et al., 2011) and by sequencing of the 16S ribosomal ribonucleic acid (16S rRNA) (Wang et al., 2013; Swartz et al., 2014; Laguardia-Nascimento et al., 2015).

In samples from the bovine uterus, *Lactobacillus* spp. were detected by cultivation (Eduvie et al., 1984; Messier et al., 1984; Williams et al., 1988; Gärtner et al., 2015), by sequencing of the 16S rRNA (Machado et al., 2012b; Bicalho et al., 2017b; Wang et al., 2018) and by Fourier-transform infrared spectroscopy (Wagener et al., 2015).

2.8.2. Influence of *Lactobacillus* spp. when administered to the bovine uterus

There are only two studies that focused on the effects of *Lactobacillus* spp. when administered to the bovine uterus. For both studies, non-autochthonous *Lactobacillus* spp. were chosen, which means that they were not previously isolated from a bovine uterus (Kummer et al., 1997; Genis et al., 2018).

The older study demonstrated that two selected viable lactobacilli strains were able to colonize the bovine uterus for up to 12 days and provoked a cellular infiltration of the endometrium with immune cells (Kummer et al., 1997).

The recent study showed that when a mixture of LAB, including two species of *Lactobacillus*, (*L. rhamnosus, L. reuteri* and *Pediococcus* [*P.*] *acidilactici*) was administered to the bovine uterus one day after calving, there was no difference in the endometrial mRNA expression of pro-inflammatory *CXCL8*, *IL1B*, *IL6* and *TNF* on day 1, 3 and 6 after calving (Genis et al., 2018). The same study showed that the prevalence of metritis on day 6 after calving was also not influenced by the intrauterine administration of the mixture of LAB on day 1 after calving.

2.9. *Lactobacillus buchneri*

L. buchneri is a microorganism which is widely spread in nature and well studied due to its variable capabilities in food and feed production (Heinl and Grabherr, 2017). It is associated with different food products, e.g. specific cheese (Zhang et al., 2016; Azizi et al., 2017) and pickle juice (Zeng et al., 2010) and it was isolated from the human intestinal tract (Koll et al., 2010). *L. buchneri* also plays an important role as starter culture for fermentation processes in animal feed, especially in different types of silage such as sugarcane silage (Silva et al., 2019), grass silage (Heinl et al., 2012), corn silage (da Silva et al., 2018) and alfalfa silage (Schmidt et al., 2009). Silage that was inoculated with *L. buchneri* had a better aerobic stability and fewer yeast and mold (Taylor et al., 2002; Kung et al., 2003).

Probiotic properties were shown for *L. buchneri* when it (in an appropriate dose) improved the intestinal microbiota and prevented diarrhea in weaning rabbits (Zhou et al., 2018). Another connection to animal health was drawn when the deprivation of *Lactobacillus* spp.,

including *L. buchneri*, in both the mammary and gut microbiota was associated with bovine mastitis (Ma et al., 2016).

L. buchneri (now registered as *L. buchneri* DSM 32407) was isolated from a healthy bovine uterus (Gärtner et al., 2015). The same study showed that *L. buchneri* DSM 32407 had no negative influence on the survival and vitality of co-cultured endometrial epithelial cells and that its immunostimulatory properties were the lowest in comparison with two other autochthonous species of *Lactobacillus* (*L. ruminis*, *L. amylovorus*) in cell culture experiments with bovine endometrial cells.

L. buchneri was also isolated from the vagina of cows and also heifers, which are rarely associated with endometritis (Otero et al., 1999; Stykova et al., 2013).

3. Aim of study

The main goal of the present study was to accomplish a broader understanding of the bovine endometrial immune reactions on a molecular basis regarding the postpartum period depending on (a) the uterine health status and (b) the intrauterine administration of a probiotic bacterial strain including its subsequent effect on the fertility.

Therefore, it was subdivided into two studies:

For **both** studies, the uteri of Holstein dairy cows were sampled in weekly intervals on days 24-30 pp, 31-37 pp, 38-44 pp and 45-51 pp.

The objective of the **first** study was to evaluate the mRNA expression pattern of 16 pro-inflammatory candidate genes (specific cytokines, specific prostaglandin synthases, *MMP1* and *PTAFR*) in the bovine endometrium during days 24-51 pp in weekly intervals (1) to show the relation to the actual uterine health status (healthy [H], SCE and CE) at each time point of sampling and (2) to analyze possible long-term effects of the initial uterine health status on days 24-30 pp. Additionally, an analysis of cultivable bacteria was performed on days 24-30 pp and 45-51 pp.

The objective of the **second** study was the evaluation of the influence of an intrauterine administration of a probiotic *Lactobacillus* sp. in the second half of the bovine puerperium and its effect on the endometrial immune reactions and the subsequent reproductive performance of dairy cows. Therefore, *L. buchneri* DSM 32407 was selected on the basis of the results of a preliminary *in vitro* study and either *L. buchneri* DSM 32407 or a placebo were administered to the uterus of clinically healthy cows in the luteal phase on days 24-30 pp. The intrauterine administration of *L. buchneri* DSM 32407 was analyzed for its influence on (1) the reproductive performance and the uterine health status of clinically healthy cows (H and SCE) and (2) the endometrial proportion of PMN and the endometrial mRNA expression of 17 pro-inflammatory candidate genes (specific cytokines, specific prostaglandin synthases, *MMP1* and *PTPRC*) in cows with SCE. In addition, the influence of an intrauterine administration of *L. buchneri* DSM 32407 (at various days pp) to clinically healthy cows on the histopathology of endometrial biopsy samples was evaluated. An analysis of cultivable bacteria was performed on days 24-30 pp, 31-37 pp and 45-51 pp.

4. Subsuming the published work

Publication 1:

Puerperal influence of bovine uterine health status on the mRNA expression of pro-inflammatory factors

Peter S., Michel G., Hahn A., Ibrahim M., Lübke-Becker A., Jung M., Einspanier R., Gabler C.

Published **2015** in *Journal of Physiology and Pharmacology*, 66, 449-462

Publication 2:

Influence of intrauterine administration of *Lactobacillus buchneri* on reproductive performance and pro-inflammatory endometrial mRNA expression of cows with subclinical endometritis

Peter S., Gärtner M.A., Michel G., Ibrahim M., Klopfleisch R., Lübke-Becker A., Jung M., Einspanier R., Gabler C.

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PUERPERAL INFLUENCE OF BOVINE UTERINE HEALTH STATUS ON THE MRNA EXPRESSION OF PRO-INFLAMMATORY FACTORS

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After parturition, uterine bacterial infections lead to inflammatory processes such as subclinical/clinical endometritis with high prevalence in dairy cows. Endometrial epithelial cells participate in this immune response with the production of pro-inflammatory factors. The objective of the present study was to evaluate the endometrial mRNA expression pattern of pro inflammatory factors during a selected postpartum (pp) period. Dairy cows with three different uterine health conditions on days 24-30 pp (healthy: n = 11, subclinical endometritis: n = 10, clinical endometritis: n = 10) were sampled using the cytobrush technique. Subsequently, each cow was sampled 3 more times in weekly intervals (days 31-37 pp; days 38-44 pp; days 45-51 pp). Samples were subjected to mRNA analysis performed by RT-qPCR. Additionally, an analysis of cultivable bacteria was performed at the early/late stage of the selected puerperal period. mRNA expression of 16 candidate genes was analyzed by using two different approaches. The first approach referred to the initial grouping on days 24-30 pp to reveal long-term effects of the uterine health on the subsequent puerperal period. The second approach considered the current uterine health status at each sampling to elucidate the impact of different points in time. Long-term effects seem to appear for chemokines, prostacyclin synthase and prostaglandin D2 synthase. If related to the current uterine health, the majority of candidate genes were significantly higher expressed in endometritic cows on days 45-51 pp in contrast to earlier stages of the puerperium. Microbiological analysis revealed the significantly higher prevalence of *Trueperella pyogenes* findings in cows with clinical endometritis on days 24-30 pp, but no correlations were found on days 45-51 pp. In conclusion, a strong immune response to subclinical/clinical endometritis in the late puerperium may be related to the negative impact of these conditions on reproductive performance in dairy cows.

Key words: *endometritis, dairy cow, puerperium, gene expression, chemokines, metallopeptidase-1, platelet activating factor, prostaglandin E_2*

INTRODUCTION

Endometritis in the postpartum period is one of the most prevalent disorders in high producing dairy cows (1, 2). This is associated with a negative impact on reproductive performance resulting in decreased conception rates, prolonged calving intervals, reduced milk yield and higher culling rates (3-5). A main cause for endometritis is the invasion of aerobic and anaerobic bacteria into the bovine uterus during and after parturition due to the disintegration of anatomical barriers (6-8). Endometrial luminal epithelial cells react by recognizing invading pathogens (9) and support the immune system with the production of pro-inflammatory factors such as cytokines and chemokines (10).

Chemokines are chemotactic proteins that play a central role in the initiation of the innate immune response regulating the migration of immune cells to the site of infection. This early recruitment of polymorphonuclear neutrophils (PMN) is essential for the containment of pathogens (11). The chemokine (C-X-C motif) ligands 1 (CXCL1), CXCL2, CXCL3 and CXCL5 are strong chemoattractants that all bind to the chemokine receptor 2 (CXCR2) and stimulate the recruitment and activation of neutrophils (12, 13). CXCL5 could already be emerged as a time-dependent modulator in the course of the postpartum period and is involved in immunoregulatory processes in case of subclinical or clinical endometritis (14, 15). Another potent chemoattractant of PMN that also binds to CXCR2 is interleukin 8 (IL8) (16). Its mRNA expression was observed to be dependent on the health status of the bovine endometrium along with other pro-inflammatory cytokines such as *IL1A*, *IL1B*, *IL6* and tumor necrosis factor (*TNF*) (15, 17, 18).

Besides chemokines and cytokines, prostaglandins (PGs) are another group of important inflammatory mediators in addition to their contribution to a diversity of physiological reproductive events. In previous studies, the expression of enzymes of the PG synthesis such as prostaglandin-endoperoxide synthase 2 (*PTGS2*), prostaglandin E synthase 3 (*PTGES3*) and prostaglandin D2 synthase (*PTGDS*) was linked to the health status of the puerperal bovine uterus (14, 19).

Furthermore, a potent phospholipid mediator of intercellular signaling in case of inflammation is the platelet-activating factor (PAF) (20). It is synthesized by a broad range of cell types and

activates a variety of cells including endometrial and immune cells by binding to the corresponding receptor (PTAFR) (21, 22).

In combination with the activation of potent pro-inflammatory factors, pathological events such as bacterial invasion of the uterus require repair mechanisms for the tissue. Matrix metallopeptidase 1 (MMP1) is an enzyme involved in the remodelling of tissue stimulated by pro-inflammatory cytokines (23).

The capability of the puerperal bovine endometrium for a sufficient immune response to uterine infection, cellular mechanisms and the involvement of pro-inflammatory factors are still a matter of interest and intensive research.

Therefore, the objective of the present study was to elucidate the mRNA expression pattern of candidate genes during the postpartum (pp) period in bovine endometrial samples taken at four points in time starting on days 24-30 pp. Two statistical approaches were performed with the obtained data: 1) analyzing possible long-term effects of the initial uterine health condition (healthy, subclinical endometritis and clinical endometritis) and 2) analyzing the influence of the actual bovine uterine health status at each point in time of sampling.

MATERIALS AND METHODS

Examination and classification of cows

Lactating Holstein cows included in this study were kept on a dairy farm in Brandenburg (Germany) housing around 200 cows in accordance with the guidelines of the National Animal Welfare Legislation.

At the first examination (Exam 1 [E1]) on days 24-30 pp, cows were carefully examined by inspection of the vulva, vaginoscopy, transrectal palpation and ultrasonography (Tringa Linear, Esaote, Germany) of the uterus and ovaries. Only cows (n = 31; 7 primiparous and 24 multiparous) that were not showing signs of estrus (presence of a Graafian follicle and/or discharge of clear and cohesive mucus from the vulva) were included in this study.

At E1, cows were divided into 3 groups dependent on the health status of the uterus (24).

With signs of clinical endometritis (purulent or mucopurulent uterine discharge detectable in the vagina), cows were enrolled in the first group (Clinical Endometritis [CE]; n = 10).

The second group included cows with subclinical endometritis (Subclinical Endometritis [SCE]; n = 10). These cows had no (muco)purulent discharge in the vagina, but the content of PMN in the cytological sample was ≥ 5% (19).

No (muco)purulent discharge and a PMN content of <5% classified cows as healthy (Healthy [H]; n = 11) and they were enrolled in the third group.

Each cow was examined three more times in weekly intervals in the same manner as done at E1 (days 24-30 pp). These examinations were performed on days 31-37 pp (Exam 2 [E2]), on days 38-44 pp (Exam 3 [E3]) and on days 45-51 pp (Exam 4 [E4]). After the examination of each cow, endometrial samples were collected at E1, E2, E3 and E4 as described below.

The first approach of analysis was to evaluate a long-term effect of the uterine health status at E1 on the subsequent mRNA expression pattern of the candidate genes. Therefore, cows were classified into the health groups at E1 as described above and this initial grouping remained for E2, E3 and E4.

The second approach of analysis considered the fact that the uterine health status of most cows changed after E1. Therefore, the cows were reclassified into groups considering their current uterine health at each point in time of sampling after E1. These are the same points in time of examination (E2, E3 and E4), but for the analysis the denotation was changed to Exam 2 Current Health (E2CH on days 31-37 pp), Exam 3 Current Health (E3CH on days 38-44 pp) and Exam 4 Current Health (E4CH on days 45-51 pp). This new labeling was done to distinguish this second approach of analysis from the first approach.

The stage of the estrous cycle was specified ultrasonographically. At E1, a corpus luteum was detectable in 22 cows (luteal phase). Without the presence of a corpus luteum and a Graafian follicle at E1, cows were considered to be prior to their first estrus phase after parturition (n = 9). Considering the status of the estrous cycle at the following points in time of sampling, only 9 cows were ultrasonographically classified as not being in the luteal phase due to the absence of a corpus luteum and the presence of a Graafian follicle. Seven of these cows were classified as being in pro-estrus (three cows at E2/E2CH, three cows at E3/E3CH and one cow at E4/E4CH) and two cows as being in estrus (one cow at E2/E2CH and one cow at E3/E3CH).

The cows showing signs of clinical endometritis at E1 were injected intra muscular with 25 mg $PGF_{2\alpha}$ (Dinolytic, Pfizer, Berlin, Germany) at E1 and E3 after sampling.

Collection of endometrial samples

Endometrial epithelium samples were obtained by using the cytobrush technique (19, 25). Briefly, the vulva was cleansed with dry paper towel. A sterile cytobrush (Celltip, Servoprax, Wesel, Germany), screwed on a 55 cm long rod, was inserted into the uterine body through a sterile metallic catheter, which was protected by a plastic cover (Hygienic Sheath, Minitube, Tiefenbach, Germany) to avoid contaminations from the vulva and vagina. Using a new cytobrush each time, separate samples were taken from each cow first for cytological analysis, second for microbiological analysis (at E1 and E4/E4CH) and third for isolation of RNA.

Cytological slides were prepared on the farm by rolling the cytobrush on a clean glass microscope slide. After transportation to the laboratory, the air-dried cytology slides were fixed and stained with the Hemacolor staining set (Merck, Darmstadt, Germany) following the manufacturer's protocol. All slides were examined in a meandering pattern by light microscopy (Zeiss, Axioskop, Oberkochen, Germany) at ×1000 magnification with oil. A total of 300 cells (endometrial epithelial cells, PMNs and lymphocytes) were counted to determine the percentage of PMN. Cows without (muco)purulent discharge were defined as having SCE with a PMN content of ≥ 5% or as being healthy (H) with a PMN content of <5% (19).

The cytobrush for microbiological analysis was placed in an Amies medium containing tube (Heinz Herenz, Hamburg, Germany) and transported at room temperature to the laboratory.

For mRNA analysis, one cytobrush was placed in a cryotube, which was immediately immersed into liquid nitrogen and stored at -80°C until use.

Microbiological analysis

The cytobrush samples were analyzed by aerobic and anaerobic cultivation after direct inoculation of the cytobrush with the suitable agar plates and also after the enrichment in a medium with subsequent cultivation on agar (all agar purchased from Oxoid, Wesel, Germany).

For the detection of aerobic bacteria, Columbia blood agar (5% sheep blood), Gassner agar and Brilliance UTI Clarity agar were inoculated with the cytobrush within 12 hours after sampling for 24-48 hours (aerobic, 36°C). For the evaluation of the presence of obligate anaerobic bacteria, the inoculation was

carried out on Columbia blood agar (5% sheep blood) with added L-cysteine (Merck), haemin (Sigma-Aldrich, Hamburg, Germany), vitamin K1 (Roche, Mannheim, Germany) and lysed sheep blood 0.5% (Oxoid) and on an additionally plate with the same agar and added gentamicin (Hexal, Holzkirchen, Germany), both for 48-72 hours (anaerobic, 36°C). Chocolate agar was used for cultivation of fastidious bacteria for 24-48 hours (36°C, microaerobic, 7% CO_2). In addition, an enrichment culture with brain heart infusion broth (Oxoid) was started from each cytobrush at 37°C for 24 hours, which was transferred to Columbia blood agar (5% sheep blood).

The identification of the species was carried out by Gram stain, oxidase and catalase tests according to standard protocols (26) and API strips (bioMérieux, Nürtingen, Germany).

Extraction of total RNA and reverse transcription

Total RNA was extracted from cytobrush samples by using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). The first steps of the manufacturer's protocol were slightly modified by adding the lysis buffer directly to each cytobrush and subsequent thoroughly vortexing to disrupt cells. The cytobrush was removed with clean forceps and the remaining sample was then processed as described in the manufacturer's protocol. The yield of obtained total RNA was quantified by spectrophotometry at the wavelength of 260 nm. Samples were stored at –80°C until further use.

To validate the integrity of RNA, 1 µl of total RNA was loaded onto a RNA 6000 Nano Chip using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) following the manufacturer's protocol.

100 ng total RNA per sample was used to generate single stranded cDNA by using 200 U RevertAid Reverse Transcriptase and 2.5 µM random hexamer primers (both Thermo Scientific, Schwerte, Germany) in a total volume of 60 µl (27). Before reverse transcription, a DNase treatment was performed for the removal of genomic DNA (28). Samples without addition of reverse transcriptase were also prepared as negative controls to confirm the absence of any genomic DNA or contaminations. Samples were stored in aliquots at -20°C until further analysis.

Real-time polymerase chain reaction (PCR)

To assess the mRNA expression of the candidate genes, quantitative real-time PCR was performed as previously reported (27) following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (29). The used primer pairs were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and are listed in *Table 1* . Unpublished primer pairs were first evaluated by a gradient-PCR to determine the optimal annealing temperature and obtained PCR products were submitted to commercial DNA sequencing (GATC Biotech, Konstanz, Germany) for confirmation of 100% homology to the published bovine sequences (27).

A total reaction volume of 10 µl per sample for real-time PCR contained 1 µl cDNA, 0.4 µM of each primer (forward and reverse; details given in *Table 1*) and 5 µl 2× SensiMix SYBR Low-ROX (Bioline, Luckenwalde, Germany). Amplification was carried out using the Rotor Gene 3000 (Corbett Research, Mortlake, Australia) and the following protocol. A denaturation step of 95°C for 10 min was followed by a three-step amplification in 45 cycles (except 25 cycles for *18S rRNA*): denaturation at 95°C for 15 s, annealing for 20 s (temperatures are indicated in *Table 1*) and extension at 72°C for 30 s. A subsequent melting curve program (50-99°C) with continuous fluorescence measurement was carried out to confirm specific amplification followed by a final cooling step to 40°C. A dilution series with known concentrations of the purified specific PCR products was amplified simultaneously to generate a standard curve. Rotor Gene 6.1 software (Corbett Research) was used to calculate the contents of specific mRNA in comparison with the standard curves.

Statistical analysis

Using the geNorm tool, 18S ribosomal RNA (*18S rRNA*) and suppressor of zeste 12 homolog (*SUZ12*) were chosen to normalize the expression of the genes of interest for each sample (30) and inter-run calibration was performed based on 10 inter-run calibrator samples (31). Normalized and calibrated values were used to generate box plots, which are presenting the median values with 50% of all data within the box.

Normal distribution was tested with the Shapiro-Wilk test. Neither the percentages of the PMN nor the values of any of the genes of interest were normally distributed.

Normalized and calibrated values were analyzed by the Mann-Whitney U test to compare samples from healthy cows with samples from cows with either subclinical or clinical endometritis at each point in time of examination. The level of significance was set at $P < 0.05$.

Fold changes were calculated as the approximate ratio of the mean value of normalized mRNA expression for the SCE or CE group to the mean value of the H group, respectively.

Correlations between the content of PMN with the selected factors as well as between each of the factors was calculated by the Spearman-rho test with the level of significance set at $P < 0.01$.

The Fisher's exact test was used to calculate the prevalence of *Trueperella pyogenes* and *Escherichia coli* in the group with purulent vaginal discharge (CE) in relation to the groups without purulent vaginal discharge (H and SCE) with $P < 0.05$.

All statistical evaluations and the generation of the box plots were performed using IBM SPSS Statistics 20.0 (SPSS, Chicago, USA).

RESULTS

Microbiological analysis

The evaluation of the aerobic cultivation of the cytobrush samples taken at E1 and E4CH revealed that specific pathogenic bacteria (e.g. *E. coli*, *T. pyogenes*) could not always be detected in the SCE and CE group.

At E1, *E. coli* was cultivable from only few samples of all groups. In detail, *E. coli* was detected in one sample of the H group, in three samples of the SCE group and in one sample from a cow with CE. *T. pyogenes* was not detected in the SCE group at E1, but in one sample of the H group and in four samples from cows with CE, which signifies a higher prevalence of *T. pyogenes* findings in cows with CE ($P = 0.027$).

At E4CH, no specific pathogenic bacteria were cultivable except *E. coli* in one cow of the SCE group (along with *Aerococcus* sp. and *Lactobacillus* sp.) and in one cow of the H group.

All five cows that were tested positive for *E. coli* at E1, even if they had SCE or CE, were healthy at the following examinations. In contrast, all five cows that had *T. pyogenes* cultivated from their cytobrush sample at E1, showed signs of subclinical or clinical endometritis at the following examinations with only one exception at E3CH when this cow was classified as healthy.

Additionally, aerobic cultivation revealed the sporadic presence of *Histophilus somni* in the SCE group in one cow at

Table 1. Selected gene transcripts, primer sequences and annealing temperatures used for RT-qPCR with resulting amplicon length.

Gene	Primer sequence	Reference/ GenBank accession no.	Fragment size	Annealing temperature
18S rRNA	For 5'-GAG AAA CGG CTA CCA CAT CCA A-3'	(27)	337 bp	61ºC
	Rev 5'-GAC ACT CAG CTA AGA GCA TCG A-3'			
SUZ12	For 5'-TTC GTT GGA CAG GAG AGA CC-3'	NM_001205587	286 bp	60ºC
	Rev 5'-GTG CAC CAA GGG CAA TGT AG-3'			
CXCL1/2	For 5'-GAC CTT GCA GGG GAT TCA CCT C-3'	(54)	125 bp	60ºC
	Rev 5'-CGG GGT TGA GAC ACA CTT CCT G-3'			
CXCL3	For 5'-GCC ATT GCC TGC AAA CTT-3'	(54)	189 bp	56ºC
	Rev 5'-TGC TGC CCT TGT TTA GCA- 3'			
CXCL5	For 5'-TGA GAC TGC TAT CCA GCC G- 3'	(15)	193 bp	61ºC
	Rev 5'-AGA TCA CTG ACC GTT TTG GG- 3'			
CXCR2	For 5'-AAC AGA CTC TGC CCC ATG TC-3'	(54)	151 bp	60ºC
	Rev 5'-AGT GAC AGA GCG ACC AAT CC-3'			
IL1A	For 5'-TCA TCC ACC AGG AAT GCA TC-3'	(19)	300 bp	59ºC
	Rev 5'-AGC CAT GCT TTT CCC AGA AG-3'			
IL1B	For 5'-CAA GGA GAG GAA AGA GAC A- 3'	(55)	236 bp	56ºC
	Rev 5'-TGA GAA GTG CTG ATG TAC CA- 3'			
IL6	For 5'-TCC AGA ACG AGT ATG AGG-3'	(55)	236 bp	56ºC
	Rev 5'-CAT CCG AAT AGC TCT CAG-3'			
IL8	For 5'-CGA TGC CAA TGC ATA AAA AC-3'	(15)	153 bp	56ºC
	Rev 5'-CTT TTC CTT GGG GTT TAG GC-3'			
PTGS1	For 5'-CAG ATG CGG AGT TTC TGA GTC G-3'	(27)	313 bp	60ºC
	Rev 5'-GGG TAG TGC ATC AGC ACG G-3'			
PTGS2	For 5'-CTC TTC CTC CTG TGC CTG AT-3'	(27)	359 bp	60ºC
	Rev 5'-CTG AGT ATC TTT GAC TGT GGG AG-3'			
PTGIS	For 5'-CTG TTG TCC CCA ACC AGG-3'	NM_174444	311 bp	60ºC
	Rev 5'-CTG TCC AGC ACA GGC ATG-3'			
PTGES3	For 5'-TGC AAA GTG GTA CGA TCG G-3'	(19)	253 bp	61ºC
	Rev 5'-TAA CCT TGG CCA TGA CTG G-3'			
PTGDS	For 5'-TGA GAC GCG GAC CTT ACT G-3'	(19)	193 bp	61ºC
	Rev 5'-CTG GGA GCG GCT GTA GAG-3'			
TNF	For 5'-CAA GTA ACA AGC CGG TAG CC-3'	(56)	354 bp	60ºC
	Rev 5'-GCT GGA AGA CTC CTC CCT G -3'			
MMP1	For 5'-GAT GAT GAT GAA TGG TGG ACC-3'	(57)	347 bp	60ºC
	Rev 5'-TCC ACT TCT GGG TAC AAG GG-3'			
PTAFR	For 5'-CAA CCA GGG TGA CTG GAT TC-3'	NM_001040538	338 bp	59ºC
	Rev 5'-GGA AAA TGT GGA TGG TGA GG-3'			

E1 and in one cow at E4CH, of *Streptococcus uberis* in one cow of the H group at E4CH and of *Staphylococcus* sp. in one cow of the CE group at E1.

The result of the anaerobic cultivation showed no cultivable bacteria in the H and SCE groups (except *Clostridium perfringens* in one SCE cow at E4CH). At E1, *Prevotella* sp. and *P. melaninogenica* were cultivable each in one cow with CE associated with the presence of *T. pyogenes*.

Current uterine health status at each sampling

The current uterine health status of each of the cows at each sampling is shown in *Fig. 1*.

Subsequent to E1, the number of cows in the groups dependent on their current uterine health was distributed as follows (H/SCE/CE): E2CH (n = 17/3/11), E3CH (n = 20/5/6) and E4CH (n = 16/8/7).

Most of the cows that were classified healthy at E1 were found in that same uterine health group continuously later in the puerperium. The majority of cows that had SCE at E1 were found in the healthy group at E2CH. In contrast, all of the five cows that were found in the SCE group at E3CH were also found in that group one week later. None of the cows continuously had subclinical endometritis. Most of the cows with CE at E1 were diagnosed with SCE or CE in the following puerperal period.

mRNA expression analysis

Endometrial epithelial mRNA expression of all selected candidate genes was detected in all uterine health groups and at all points in time of sampling. However, mRNA expression for *PTGS1*, *PTGS2*, *PTGIS*, *IL1A*, *IL6*, *TNF* and *MMP1* could not be found in each sample. In the following text, mainly significant differences in normalized mRNA expression are described.

The status of the estrous cycle from totally 124 examinations (luteal phase: n = 103, prior to first ovulation: n = 12, pro-estrus: n = 7, estrus: n = 2) did not have a statistical significant influence on the mRNA expression values.

mRNA expression of key enzymes of the prostaglandin synthesis

No differential mRNA expression for the analyzed enzymes of the prostaglandin synthesis in the endometrial epithelium was

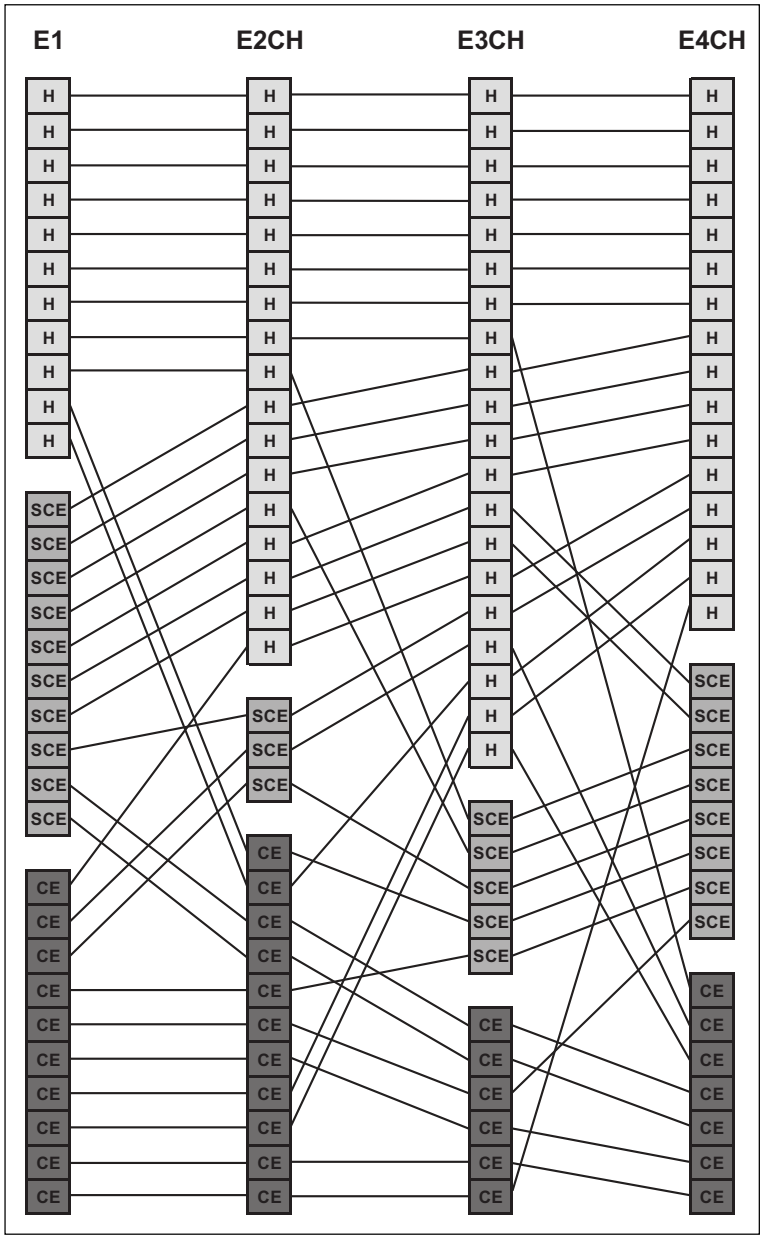

Fig. 1. Change of the uterine health status (H: healthy cow; SCE: cow with subclinical endometritis; CE: cow with clinical endometritis) of each of the cows over the period of sampling. E1 - days 24-30 pp; E2CH - days 31-37 pp; E3CH - days 38-44 pp; E4CH - days 45-51 pp.

detected on days 24-44 pp if the current uterine health status was considered. However, differences were found at the last examination (E4CH) for the mRNA expression of *PTGS1* and *PTGS2*. A fourfold higher expression (P = 0.042) was observed for *PTGS1* mRNA in the SCE group and for *PTGS2* mRNA in the CE group (P = 0.023), both compared with healthy cows (*Fig. 2A* and *2B*).

The mRNA expression of *PTGES3* did not reach any statistical differences between the groups of current uterine health at any point in time (*Fig. 2C*), also not for days 31-51 pp if evaluated referring to the initial grouping (E2, E3, E4; data not shown).

Following up the same cows dependent on the initial grouping at E1, no significant differences in *PTGIS* contents were found at E2 and E4. However at E3, *PTGIS* mRNA expression was eightfold higher (P = 0.009) in the CE group if compared with the H group (*Fig. 3A*). A similar expression pattern was found for *PTGDS* mRNA at the same time of sampling (ninefold higher; P = 0.001) with an additional threefold higher expression (P = 0.049) in the SCE group in comparison to the H group (*Fig. 3B*). At E2, the *PTGDS* transcript amount was also twofold higher (P = 0.011) in cows with CE in relation to healthy cows. Considering the actual health status at E4CH, *PTGDS* mRNA was higher expressed in both SCE (tenfold higher; P = 0.012) and CE (fivefold higher; P = 0.009) groups compared with cows of the H group.

mRNA expression of chemokines and their receptor

Sequences for *CXCL1* and *CXCL2* are to 99% homologous. Obtained sequences for the amplicons are to 100% homologous to *CXCL1* and *CXCL2*. Therefore, the primers named *CXCL1/2* were specific for *CXCL1* as well as for *CXCL2*.

At E1, *CXCL1/2* was fivefold higher (P = 0.011) expressed in SCE cows compared with H cows (*Fig. 4A*). Retaining the grouping from E1, the mRNA expression was sixfold higher (P = 0.029) in endometrial epithelium cells from cows with CE in

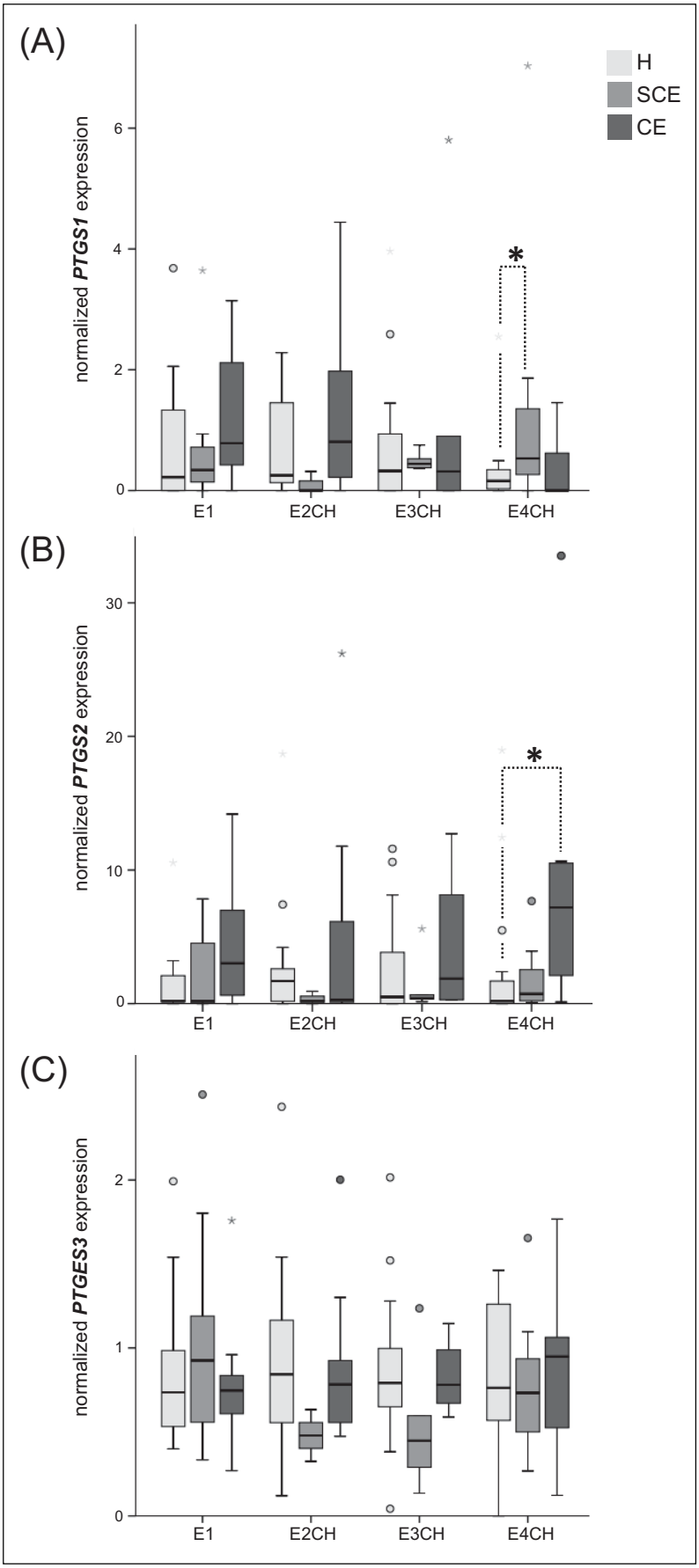

Fig. 2. Normalized mRNA expression of (A) *PTGS1*, (B) *PTGS2* and (C) *PTGES3* in bovine endometrial cytobrush samples harvested from dairy cows depending on the current status of uterine health (H: healthy, SCE: subclinical endometritis, CE - clinical endometritis) on days 24-30 pp (E1), on days 31-37 pp (E2CH), on days 38-44 pp (E3CH) and on days 45-51 pp (E4CH). Bold asterisks over dotted lines indicate significant differences between the groups ($P < 0.05$). Extreme values are diagrammed as asterisks, outliers as circles. Extreme values are not shown for *PTGS1* (9.02 and 9.76) obtained from two cows of the H group at E2CH.

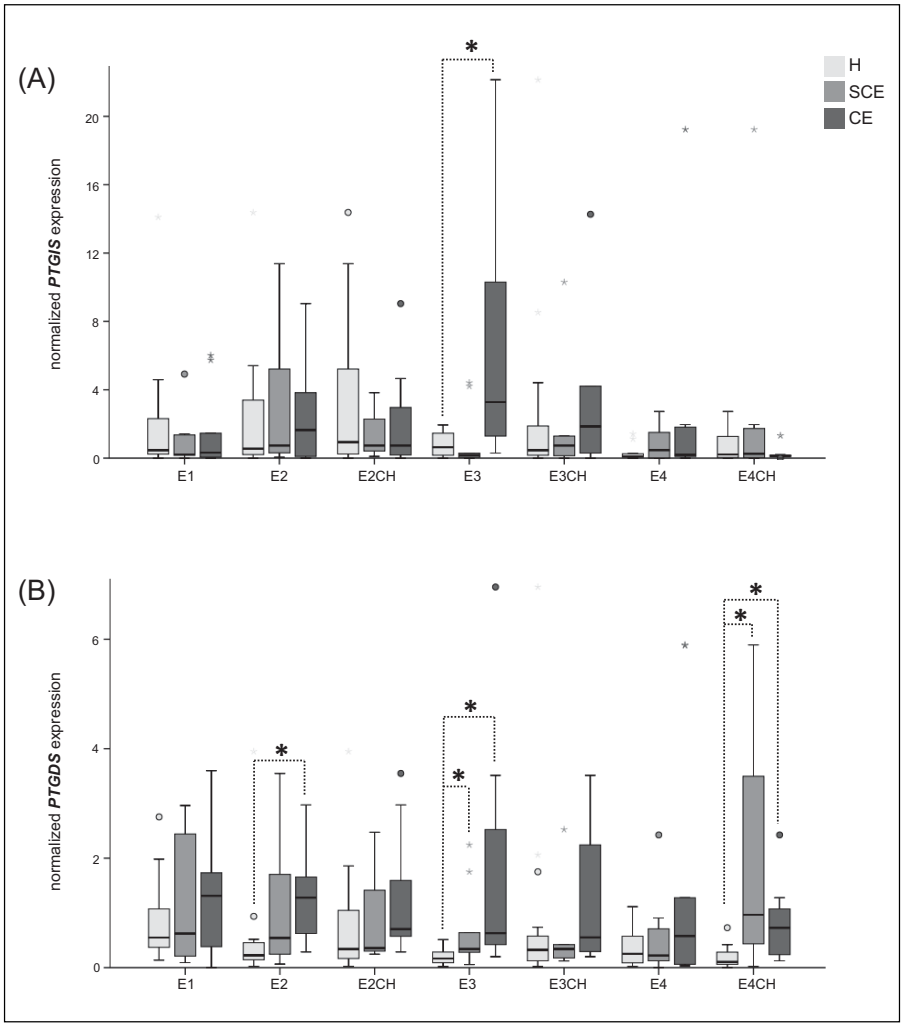

Fig. 3. Normalized mRNA expression of (A) *PTGIS* and (B) *PTGDS* in bovine endometrial cytobrush samples harvested from dairy cows depending on the status of uterine health (H: healthy, SCE: subclinical endometritis, CE: clinical endometritis) on days 24-30 pp (E1), on days 31-37 pp (E2/E2CH), on days 38-44 pp (E3/E3CH) and on days 45-51 pp (E4/E4CH). Bold asterisks over dotted lines indicate significant differences between the groups ($P < 0.05$). Extreme values are diagrammed as asterisks, outliers as circles.

comparison to the H group at E4. If the cows were classified dependent on their current health status at the time of sampling, the pattern for *CXCL1/2* mRNA additionally showed a significant correlation to the uterine health at E4CH both regarding SCE cows (threefold higher; P = 0.012) and CE cows (about 20-fold higher; P = 0.009) compared with the H group.

CXCL3 reached a twofold higher expression (P = 0.002) in CE cows compared with H cows at E1 and almost similar at E4CH (threefold higher; P = 0.013) considering the actual uterine health status (*Fig. 4B*).

Comparing transcript amounts of the CE group to the H group, *CXCL5* mRNA was significantly higher expressed at E1 (fivefold higher; P = 0.011), at E2CH (sixfold higher; P = 0.02), at E4 (sevenfold higher; P = 0.029) and at E4CH (20-fold higher; P = 0.016) (*Fig. 4C*). No differences were observed for *CXCL3* and *CXCL5* if comparing the SCE group to the H group.

The *CXCR2* mRNA expression pattern showed significant differences ($P < 0.05$) between the same groups as shown for *CXCL1/2*, with the addition that *CXCR2* was 30-fold higher expressed (P = 0.014) in SCE cows in relation to H cows at E3CH (*Fig. 4D*).

mRNA expression of interleukins

The endometrial epithelial mRNA expression of the analyzed interleukins was only correlated to the current uterine health status. A long-term effect of the health status was not observed when diagnosed at E1.

IL1A mRNA was correlated to the health status at E1, E3CH and E4CH (*Fig. 5A*). In comparison to the H group it was expressed about six- to sevenfold higher in cows with SCE, both at E1 (P = 0.02) and E3CH (P = 0.042). Later at E4CH, the mRNA expression was 100-fold higher (P = 0.011) in CE cows in comparison to the H group, but no significant differences for the SCE group were observed.

IL1B mRNA was higher expressed in cows with SCE at the early (E1; 15-fold higher; P = 0.006) and late (E4CH; 20-fold higher; P = 0.002) stage of the selected puerperial period. In addition and similar to *IL1A* expression, the highest fold change was observed also at E4CH in samples from cows with CE (200-fold higher; P = 0.003) in relation to the H group (*Fig. 5B*).

The transcript amounts of *IL6* were twofold higher (P = 0.023) in SCE cows at E3CH and sixfold higher (P = 0.022) in cows with CE at E4CH, both related to the H group (*Fig. 5C*).

The *IL8* mRNA expression pattern showed significant differences between the same groups at the same points in time of sampling as shown for *IL1A*, with the addition of a higher content of *IL8* mRNA in uterine samples from SCE cows compared with H cows at E4CH (15-fold higher; P = 0.023) (*Fig. 5D*). The expression was 25- and 10-fold higher in SCE cows at E1 (P = 0.006) and E3CH (P = 0.012), respectively. Comparable to the *IL1A* and *IL1B* expression pattern, the highest

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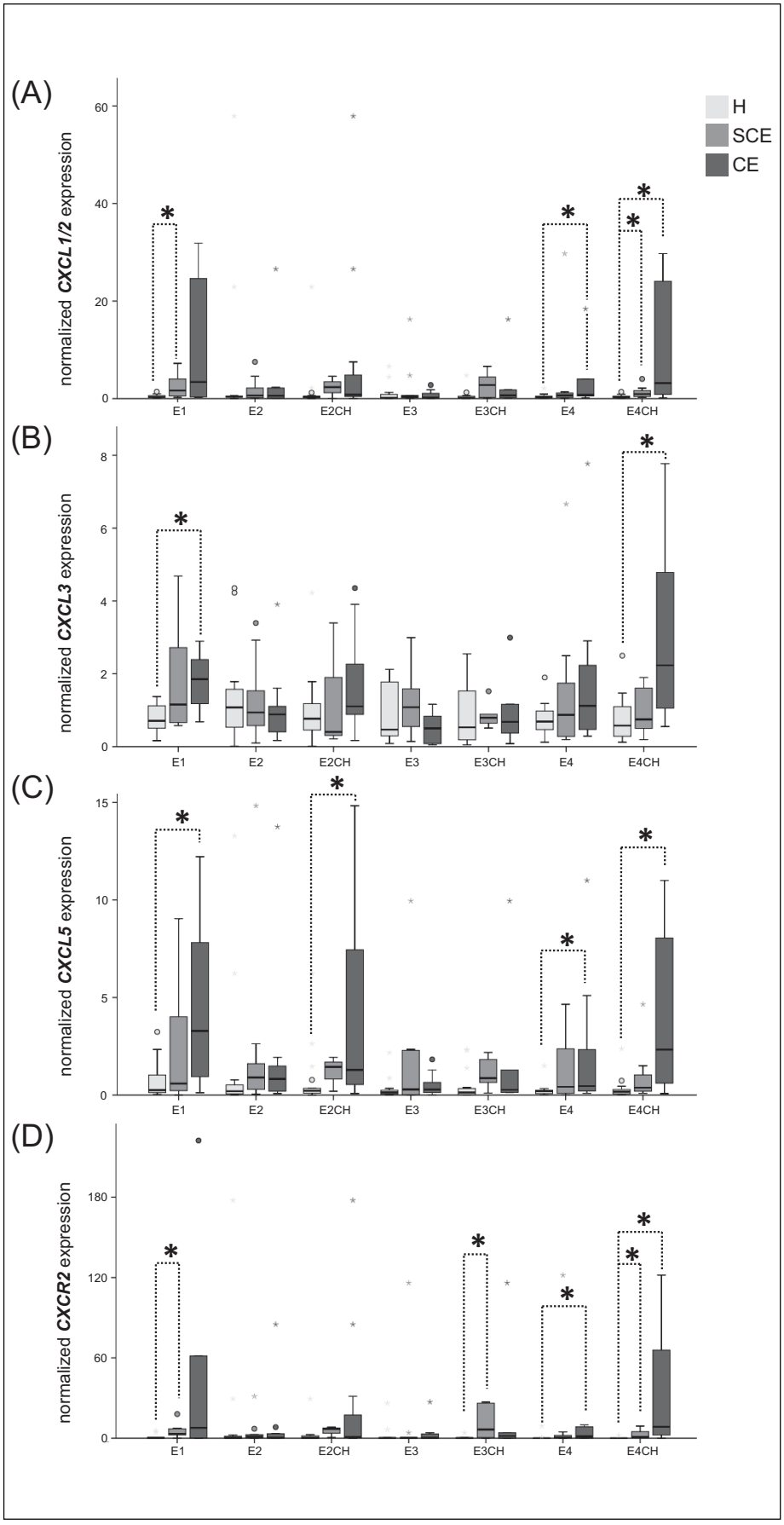

Fig. 4. Normalized mRNA expression of (A) *CXCL1/2*, (B) *CXCL3*, (C) *CXCL5* and (D) *CXCR2* in bovine endometrial cytobrush samples harvested from dairy cows depending on the status of uterine health (H: healthy, SCE: subclinical endometritis, CE: clinical endometritis) on days 24-30 pp (E1), on days 31-37 pp (E2/E2CH), on days 38-44 pp (E3/E3CH) and on days 45-51 pp (E4/E4CH). Bold asterisks over dotted lines indicate significant differences between the groups ($P < 0.05$). Extreme values are diagrammed as asterisks, outliers as circles. Extreme values are not shown for *CXCL1/2* (134.53) and *CXCR2* (321.89) obtained from a cow in the CE group at E4/E4CH and *CXCL5* (24.24) obtained from a cow in the SCE group at E4 and in the CE group at E4CH.

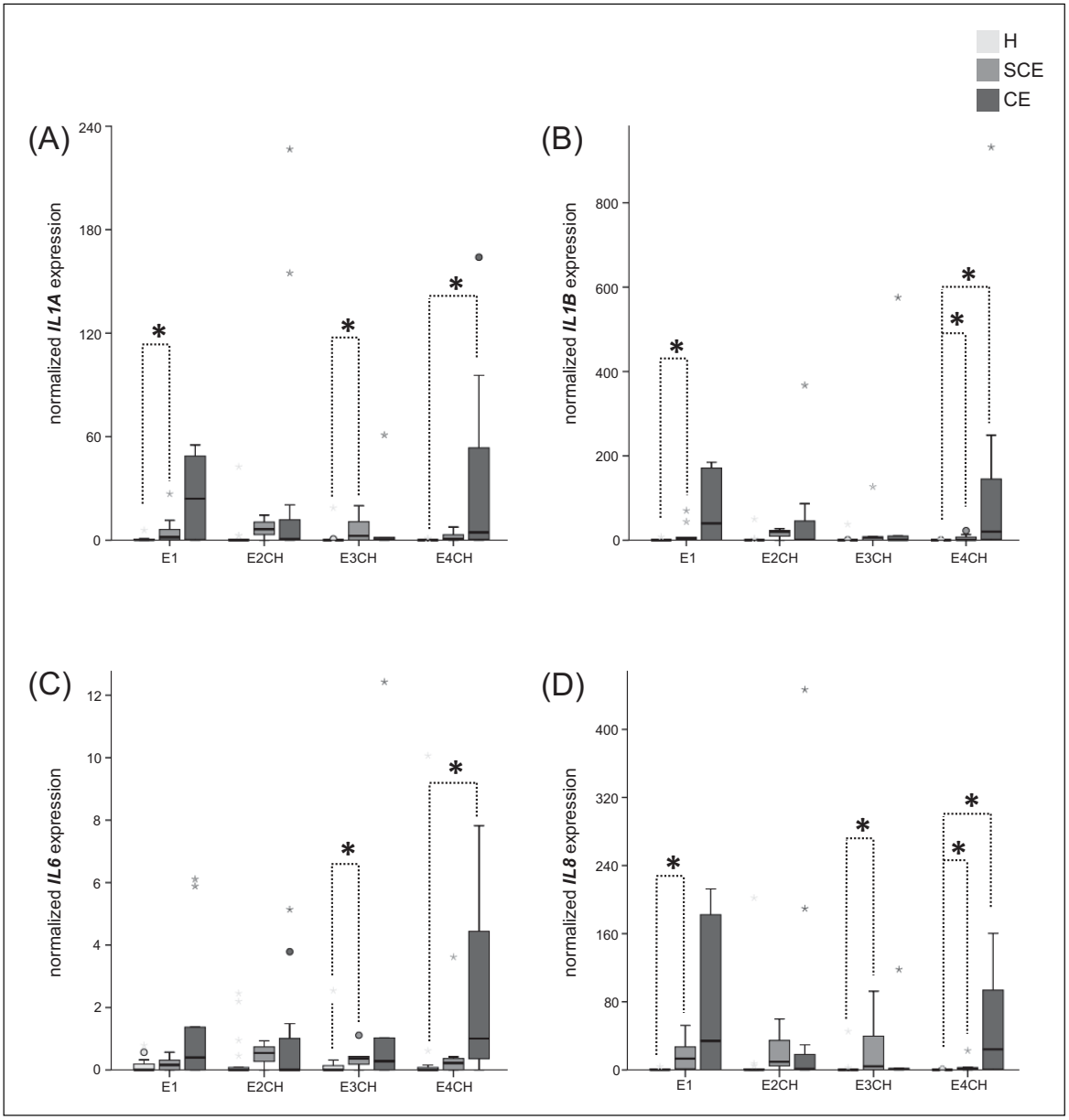

Fig. 5. Normalized mRNA expression of (A) *IL1A*, (B) *IL1B*, (C) *IL6* and (D) *IL8* in bovine endometrial cytobrush samples harvested from dairy cows depending on the current status of uterine health (H: healthy, SCE: subclinical endometritis, CE: clinical endometritis) on days 24-30 pp (E1), on days 31-37 pp (E2CH), on days 38-44 pp (E3CH) and on days 45-51 pp (E4CH). Bold asterisks over dotted lines indicate significant differences between the groups ($P < 0.05$). Extreme values are diagrammed as asterisks, outliers as circles. Extreme values are not shown for *IL1A* (323.80) and *IL1B* (1897.99) obtained from a cow with CE at E1 and for *IL1B* (1901.71) from a cow with CE at E2CH. Extreme values are also not shown for *IL6* (16.83) and *IL8* (940.72) obtained from a cow in the CE group at E4CH.

fold change (120-fold) in the CE group in relation to healthy cows was observed at E4CH ($P = 0.002$).

mRNA expression of tumor necrosis factor (TNF), matrix metallopeptidase 1 (MMP1) and platelet-activating factor receptor (PTAFR)

For the transcript amount of these selected candidate genes significant differences were also noted if the current uterine health status was considered.

The mRNA expression of *TNF* in luminal endometrial epithelium was affected at E3CH and E4CH (*Fig. 6A*). At E3CH, *TNF* was higher ($P = 0.034$) expressed in cows with SCE and at E4CH, the mRNA content of *TNF* was 15-fold higher ($P = 0.005$) only in the CE group in comparison to the H group.

MMP1 mRNA expression was dependent on the current health status starting on days 31-37 pp. Compared with the H group, a higher mRNA expression was detected in CE cows at E2CH (60-fold higher; $P = 0.01$), at E3CH (20-fold higher; $P = 0.019$) and at E4CH (150-fold higher; $P = 0.024$) and in

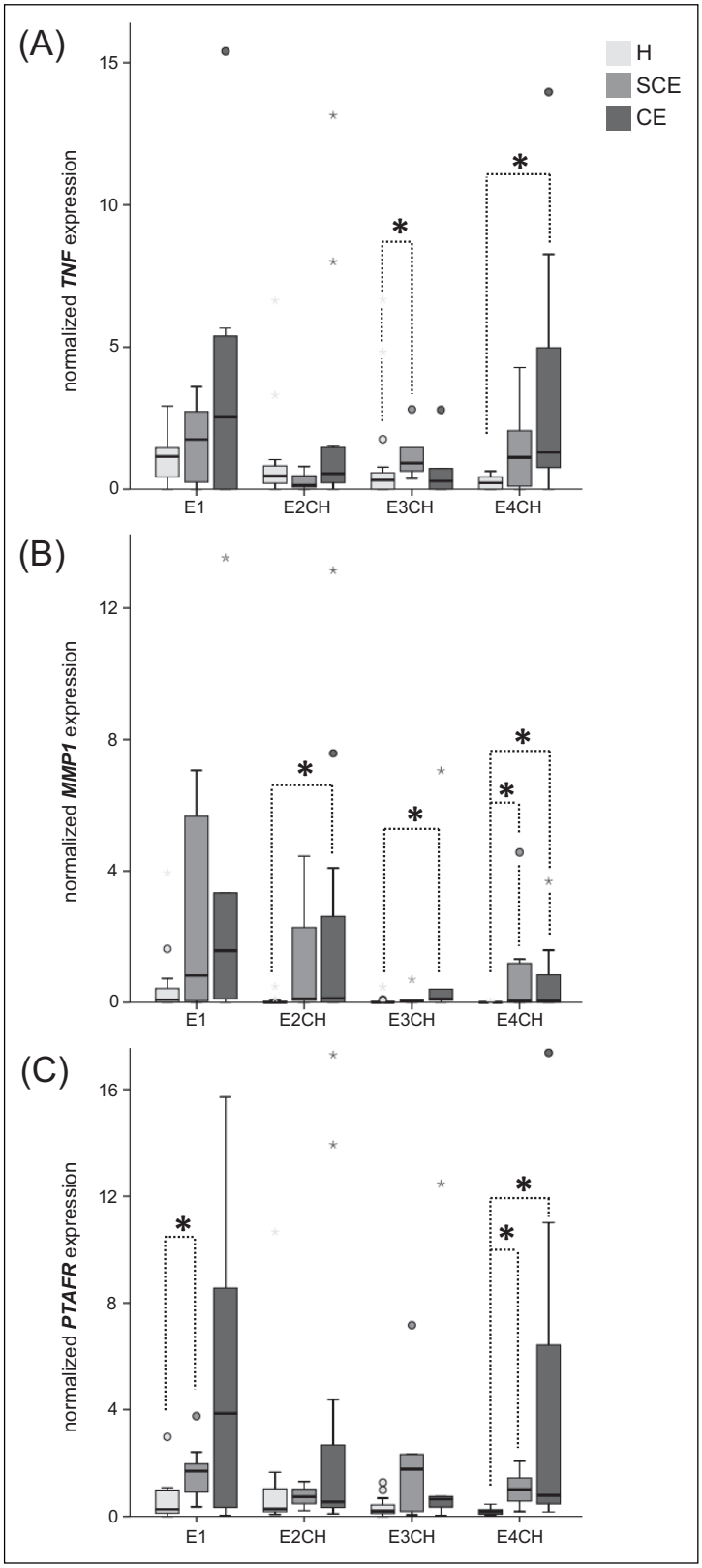

Fig. 6. Normalized mRNA expression of (A) *TNF*, (B) *MMP1* and (C) *PTAFR* in bovine endometrial cytobrush samples harvested from dairy cows depending on the current status of uterine health (H: healthy, SCE: subclinical endometritis, CE: clinical endometritis) on days 24-30 pp (E1), on days 31-37 pp (E2CH), on days 38-44 pp (E3CH) and on days 45-51 pp (E4CH). Bold asterisks over dotted lines indicate significant differences between the groups ($P < 0.05$). Extreme values are diagrammed as asterisks, outliers as circles. One extreme values is not shown for *MMP1* (107.35) obtained from a cow with CE at E1.

addition in SCE cows (180-fold higher; P = 0.047) at E4CH (*Fig. 6B*).

In comparison to the H group, *PTAFR* was twofold higher (P = 0.017) expressed in SCE cows at E1 (*Fig. 6C*). At E4CH, a direct correlation of *PTAFR* mRNA expression to the uterine health was observed with a higher expression in the endometrial epithelium in both SCE cows (fivefold higher; P = 0.001) and CE cows (25-fold higher; P = 0.003) in comparison to the H group.

Table 2. Significant correlation coefficients between the content of PMN with the mRNA expression of the selected factors as well as between each of the factors in endometrial cytobrush samples during days 24-51 pp presented as a matrix format ($P < 0.01$). When no value was indicated, no significant correlation was observed.

	PMN	*PTGS1*	*PTGS2*	*PTGIS*	*PTGES3*	*PTGDS*	*CXCL1/2*	*CXCR2*	*CXCL3*	*CXCL5*	*IL1A*	*IL1B*	*IL6*	*IL8*	*TNF*	*MMP1*	*PTAFR*
PMN			0.280			0.360	0.552	0.704	0.323	0.545	0.675	0.655	0.484	0.634	0.378	0.493	0.587
PTGS1				0.409		0.407											0.311
PTGS2	0.280					0.311	0.338	0.312	0.240	0.314	0.366	0.357	0.453	0.340	0.375	0.319	0.366
PTGIS		0.409				0.284					- 0.252	- 0.276		- 0.354			
PTGES3																	
PTGDS	0.360	0.407	0.311	0.284				0.420		0.420	0.306	0.402	0.237	0.258	0.323	0.350	0.500
CXCL1/2	0.552		0.338					0.802	0.694	0.779	0.807	0.803	0.610	0.835	0.483	0.471	0.718
CXCR2	0.704		0.312			0.420	0.802		0.462	0.738	0.791	0.877	0.598	0.843	0.473	0.506	0.740
CXCL3	0.323		0.240				0.694	0.463		0.618	0.526	0.487	0.413	0.509	0.436	0.368	0.467
CXCL5	0.545		0.314			0.420	0.779	0.738	0.618		0.702	0.718	0.523	0.699	0.553	0.500	0.696
IL1A	0.675		0.366	- 0.252		0.306	0.807	0.791	0.526	0.702		0.851	0.604	0.872	0.530	0.602	0.715
IL1B	0.655		0.357	- 0.276		0.402	0.803	0.877	0.487	0.718	0.851		0.645	0.887	0.476	0.515	0.660
IL6	0.484		0.453			0.237	0.610	0.598	0.413	0.523	0.604	0.645		0.624	0.370	0.485	0.484
IL8	0.634		0.340	- 0.354		0.258	0.835	0.843	0.509	0.699	0.872	0.887	0.624		0.459	0.552	0.608
TNF	0.378		0.375			0.323	0.483	0.473	0.436	0.553	0.530	0.476	0.370	0.459		0.389	0.591
MMP1	0.493		0.319			0.350	0.471	0.506	0.368	0.500	0.602	0.515	0.485	0.552	0.389		0.519
PTAFR	0.587	0.311	0.366			0.500	0.781	0.740	0.467	0.696	0.715	0.660	0.484	0.608	0.591	0.519	

Correlation analysis

Significant correlations are listed in *Table 2*. The percentage of PMN was highest correlated to mRNA expression of *CXCR2*, *IL1A*, *IL1B* and *IL8*. *PTGS1*, *PTGIS* and *PTGES3* were the only investigated factors whose mRNA expression did not show significant correlation to the number of PMN.

Especially the mRNA content of the factors that mainly attract PMNs showed high correlations among each other (*IL1A*, *IL1B*, *IL8*, *CXCL1/2* and *CXCR2*) with the highest correlation between *IL1B* and *IL8* mRNA expression. In contrast, the attractants for PMNs had none or only weak to moderate correlations to the enzymes of the synthesis of prostaglandins. *IL1A*, *IL1B* and *IL8* were negatively correlated to *PTGIS*.

The enzymes of the synthesis of PGs also showed either none or mostly only weak to moderate correlations among each other and to other factors. For *PTGES3*, no correlations at all could be observed. The only enzyme of PG synthesis that is correlated to all of the other groups (chemokines, interleukins, *TNF*, *MMP1* and *PTAFR*) is *PTGS2*.

DISCUSSION

The influence of the immune system on reproductive processes in the female was reported for many species (32-35). In the bovine, an upregulation of the local immune system for the clearance of invaded bacteria and lochia as well as for the repair of the epithelium is considered as a physiological process in the early postpartum period as part of involution of the uterus. This process often seems to be inefficient and the transition from a physiological to a more pathological status is associated with a shifted duration and severity of the immune response to later stages of the puerperium (10).

Several former studies already correlated the mRNA expression of pro-inflammatory factors to the uterine health, but mainly focused on only one point in time of the puerperium. Cows with clinical or subclinical endometritis had a higher mRNA expression of pro-inflammatory factors on days 21-27 pp (15, 19), on days 28-35 pp (36) and on days 28-41 pp (18). However, the present study design allowed a repeated sampling of the same cows. Therefore, it was possible to differentiate the expression patterns of pro-inflammatory factors at different points in time during a longer period of the puerperium (days 24-51 pp). This provided additional information especially about later stages of the puerperium.

The results show that in contrast to earlier points in time, almost all of the analyzed factors (except *PTGIS* and *PTGES3*) were affected by the uterine health status on days 45-51 pp. Interestingly, the fold changes were mostly higher if compared with earlier stages. This suggests that the severity of inflammation might differ during the time course of the puerperium. Clinical and subclinical endometritis on days 45-51 pp seem to cause a more severe inflammatory response than at earlier stages. This is supported by the findings of Galvao *et al.* (17) who also found an increase of *IL1B*, *IL6* and *IL8* mRNA expression in uterine biopsies from cows with endometritis around the same stage of the puerperium (week 7 after calving), even though there were no correlations to the uterine health at earlier stages. This implies that gene analysis around days 45-51 pp might be more informative when correlating pro-inflammatory factors to subclinical and/or clinical endometritis. When evaluating the effect of new applicable therapy strategies for bovine uterine diseases, this impact of the point in time of the puerperium on the mRNA expression pattern should be considered.

In this context, especially the enzymes for the PG synthesis seem to be of special interest. Although *PTGS2* mRNA expression showed a puerperal time-dependency with a significant peak on day 17 pp (14), another study did not reveal a dependency on the expression of *PTGS2* mRNA to the uterine health for the earlier puerperal stage (days 21-27 pp) (15). However, the results of the present study show that *PTGS1*, *PTGS2* and *PTGDS* were affected on days 45-51 pp. This suggests that the inflammatory response seems to be delayed on the part of PG production. Although enzymes of the synthesis of PGs did not show a significant dependency on the uterine health status on days 24-30 pp, the mRNA expression of *PTGDS* was affected on days 31-44 pp and of *PTGIS* on days 38-44 pp in cows with CE if referring to the initial grouping. Similar long-term effects failed to appear for most of the other analyzed factors. However, it has to be considered that cows with CE were injected with $PGF_{2\alpha}$ after the sampling on days 24-30 pp (E1) and on days 38-44 pp (E3). Therefore, an effect of the first $PGF_{2\alpha}$ injection on the increased mRNA expression of *PTGDS* and *PTGIS* cannot be excluded.

A special role among the PGs regarding reproductive events is awarded to PGE_2. A dysregulated too high production in combination with its luteotrophic effect (37) might lead to disrupted luteolysis and a prolonged luteal phase (38). High

concentrations of PGE_2 in the peripheral blood plasma, stimulated by the administration of a high dose of TNF, prolonged the estrous cycle in dairy cows (39). Extended postpartum luteal phases can be linked to uterine diseases (40), probably caused by the switch of the secretion from $PGF_{2\alpha}$ to PGE_2 after bacterial infection (41), whereas the absolute concentrations might be less decisive than the resulting higher $PGE_2/PGF_{2\alpha}$ ratio (42). Baranski *et al.* (43) found a significantly higher content of PGE_2 in cytobrush samples from cows with clinical endometritis on days 21-28 pp. Another study showed significantly higher concentrations of PGE_2 in the uterine fluid of cows with severe clinical endometritis compared with mild endometritis cases (44). In addition, a significantly stimulated PGE_2 output from endometrial tissue in the luteal phase or early pregnancy after the addition of pro-inflammatory IL1A as being a potent stimulator on PG production in bovine endometrial stromal cells was observed (45-47). In this context, peroxisome proliferator activated receptors were shown to be mediators for PGE_2 release from porcine endometrial explants harvested during the luteal phase of the estrous cycle or during the time of implantation (48).

However, a dependency on the mRNA expression of *PTGES3* on the uterine health status during the whole period (days 24-51 pp) was not observed in the present study. Thereby, it has to be considered that there are three different synthases for PGE_2. While PTGES1 can especially be linked to inflammation, female reproduction and tissue repair, PTGES2 and PTGES3 are more constitutive and unaffected by pro-inflammatory stimuli (49). Therefore, *PTGES1* mRNA expression analysis on days 24-51 pp should be of prospective interest.

The results of the microbiological analysis suggest that a sampling at a later stage of the puerperium is not expedient. On days 45-51 pp, almost no pathogenic bacteria at all could be detected, even if cows had purulent vaginal discharge. Therefore, no correlations to the health groups were found in strong contrast to the mRNA expression analysis. Consistent with the finding of Westermann *et al.* (50) who showed a significant increased number of samples positive for *T. pyogenes* in relation to increasing vaginal discharge score, *T. pyogenes* was found more often in cows with CE in the present study, but only on days 24-30 pp. Interestingly, cows positive for *T. pyogenes* mainly remained in either the SCE or CE group afterwards. In contrast, all cows positive for *E. coli* on days 24-30 pp switched to the H group. This is comparable to the results of Sens and Heuwieser (51) whose study revealed the higher odds of having >18% PMN on 21-27 days in milk (DIM) for cows with a *T. pyogenes* infection at 10 ± 1 DIM, but that an *E. coli* infection at 10 ± 1 DIM had no effect on the prevalence of SCE later in the puerperium. Thereby it has to be considered that the detected *E. coli* may belong to rather non-pathogenic strains than to endometrial pathogenic *E. coli* (52). In addition, there were also no bacteriological findings with significant prevalence for the SCE group. This supports the statement of McDougall *et al.* (53) that the quantification of the percentage of PMN in endometrial cytology is better suitable to predict reproductive performance than conventional intra-uterine bacteriology or the scoring of vaginal contents.

In conclusion, the attempt of revealing possible long-term effects of the initial uterine health status on days 24-30 pp on the subsequent mRNA expression pattern of the candidate genes showed effects on chemokines, *PTGDS* and *PTGIS*. If correlated to the current uterine health at each sampling, mRNA expression analysis of pro-inflammatory factors suggests a different severity of inflammatory response during days 24-51 pp with the strongest immune response at the later stage (on days 45-51 pp). In contrast, microbiological analysis of uterine samples was not meaningful around that time of the puerperium.

The present study contributes to a better understanding of inflammatory processes in the puerperal bovine endometrium, which might help with the development of new strategies of treatment for endometritis. However, the supposed differences in the severity of inflammatory response shown by the mRNA expression patterns should be substantiated by analyzing protein levels in a prospective study.

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Conflict of interests: None declared.

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Influence of intrauterine administration of *Lactobacillus buchneri* on reproductive performance and pro-inflammatory endometrial mRNA expression of cows with subclinical endometritis

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Potential beneficial effects of lactic acid bacteria on the genital health of cows become of particular interest when considering the importance of an optimal uterine health status for the success of breeding in dairy farming. Therefore, the aim of the present study was to analyse the influence of an intrauterine administration of the *Lactobacillus buchneri* DSM 32407 on reproductive performance, uterine health status, endometrial mRNA expression of pro-inflammatory factors of cows with signs of subclinical endometritis (SCE). *L. buchneri* DSM 32407 (n = 56; [LAC]) or a placebo (n = 60; [PLA]) was administered on day 24–30 postpartum. Endometrial cytobrush samples of cows with SCE were taken before the administration and at three following weeks (n = 16 cows each for LAC/SCE and PLA/SCE). A higher proportion of cows of the LAC and LAC/SCE group was pregnant after the first service and median days to conception for cows pregnant on day 200 pp were shorter. Three weeks after the administration, the endometrial mRNA expression of *CXCL1/2*, *CXCL3*, *CXCR2*, *IL1B*, *IL8* and *PTPRC* was lower in the LAC/SCE group compared with the PLA/SCE group. These findings suggest that the presence of *L. buchneri* DSM 32407 contributes to a uterine environment that results in a better reproductive performance.

Bovine subfertility is one of the major reasons for enormous economic losses in the dairy industry[1]. Impaired reproductive performance such as prolonged intervals from calving to conception can be associated with uterine diseases of the postpartum period, e.g. subclinical endometritis (**SCE**)[2,3].

Current treatments of **SCE** include antimicrobials and prostaglandin (PG) $F_{2\alpha}$[4,5]. However, unwanted consequences of the administration of antimicrobials are residues in milk and meat[6] and the potential spread of bacterial resistances[7]. Furthermore, the efficacy of a hormonal therapy with $PGF_{2\alpha}$ remains controversial. Administration of a single dose or 2 treatments with $PGF_{2\alpha}$ at 35 and 49 (±3) days in milk (DIM) did not affect the prevalence of **SCE** and purulent vaginal discharge[8,9]. The reproductive performance was neither improved by antibiotic nor $PGF_{2\alpha}$ treatment[9,10]. Therefore, an alternative strategy for the effective treatment of **SCE** to improve the fertility rates without the unwanted implications of the use of antimicrobials would be of great benefit.

In human medicine, an alternative to the use of antimicrobials for the treatment of bacterial vaginosis in women is the use of lactobacilli[11]. Characteristics of the Gram-positive lactobacilli are the capacity to produce acetic and lactic acid, hydrogen peroxide and bacteriocins, which are rated to be beneficial for the suppression of pathogenic bacteria[12].

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From samples of the bovine uterus, several *Lactobacillus* spp. were cultivable[13–16] and also detected by metagenomic pyrosequencing of the 16S rRNA gene[17,18]. It was shown that co-culturing of bovine endometrial epithelial cells with *L. buchneri* (now registered as *L. buchneri* DSM 32407) up to a multiplicity of infection (MOI) of 10 did not affect the viability of epithelial cells[16]. In addition, the mRNA expression or release of pro-inflammatory factors was not influenced for up to 6 h and 48 h, respectively. In contrast, the presence of *L. ruminis* and *L. amylovorus* provoked a pro-inflammatory response of the epithelial cells. An early study indicated that lactobacilli have an immunostimulatory effect on the endometrium[19]. In that study, after the intrauterine administration of two live *Lactobacillus* spp., an infiltration with mostly mononuclear cells into the endometrium was observed and a colonization of the endometrium by the selected lactobacilli strains for up to 12 days was noted. However, the impact of an intrauterine administration beyond the endometrial infiltration with immune cells remains unclear, especially referring to the uterine health status.

One possibility to draw conclusions on the uterine health status is the analysis of the mRNA expression of pro-inflammatory factors such as interleukins, chemokines, and enzymes of the PG synthesis. An elevation of the mRNA expression of such selected factors in case of **SCE** was found in several studies[20–24]. In the present study, the exact same time points of the puerperium were chosen for sampling as in a previous study where the levels of mRNA expression of such factors showed correlations with **SCE**[24]. Therefore, the mRNA expression analysis of pro-inflammatory factors seemed suitable to support the evaluation of endometrial health.

The objective of the present study was to clarify the influence of the intrauterine administration of *L. buchneri* DSM 32407 on the reproductive performance of clinically healthy cows (**SCE** or healthy). Therefore, cows were divided into two groups on days 24–30 postpartum (pp) and either the autochthonous *L. buchneri* DSM 32407 or a placebo was administered intrauterine. In addition, all cows with **SCE** were monitored for their uterine health status and the endometrial mRNA expression pattern of selected pro-inflammatory factors on a weekly basis during the following days 31–51 pp.

Materials and Methods

Preparation of *L. buchneri* solutions for intrauterine administration. The strain *L. buchneri* DSM 32407 was isolated from a uterus of a healthy cow and stored long-term at −80 °C in 15% (v/v) glycerol in MRS broth (according to DeMan, Rogosa and Sharpe; Sigma-Aldrich, Steinheim, Germany)[16]. This stock was used for enrichment by cultivation under aerobic conditions in MRS broth (Sigma-Aldrich) at 37 °C for 48 h until the suspension reached an optical density of one at the wavelength of 600 nm. After centrifugation for 10 min at 15000 × g and resuspension in MRS broth with 50% (v/v) glycerol, 200 µl aliquots were stored at −80 °C until further use.

36–48 h prior to the preparation of the solutions for intrauterine administration, aliquots were thawed at room temperature. To calculate the number of colony forming units (cfu)/ml of the aliquots, serial dilutions were cultivated on Rogosa SL agar (Sigma-Aldrich) under microaerophilic conditions (Anaerocult C, Merck, Darmstadt, Germany) at 37 °C. Thawed aliquots were stored at 4 °C until the preparation of the solutions for intrauterine administration.

It was observed that the number of cfu/ml was stable for up to 48 h in the thawed aliquots (stored at 4 °C) and for 8 h in the prepared solutions for intrauterine administration (stored at room temperature).

L. buchneri solutions for intrauterine administration consisted of $1.5–2 \times 10^{10}$ cfu in 20 ml 0.9% (w/v) isotonic saline solution (B. Braun, Melsungen, Germany) drawn up into a 20 ml plastic syringe (Injekt, B. Braun). Bacteria were prepared at the day of administration (08:00 a.m.), transported to the farm at room temperature and administered latest on the same day after a maximum of 8 h.

Examination and enrollment of cows in this study. Lactating Holstein cows included in this study were kept at the Lehr- und Versuchsanstalt für Tierzucht und Tierhaltung e. V. in Groß Kreutz (Brandenburg, Germany), housing around 200 cows in freestall facilities with slotted floors and cubicles in accordance with the guidelines of the National Animal Welfare Legislation. Animal experimental procedures were approved by the relevant authorities of the state Brandenburg, Germany (Landesamt für Umwelt, Gesundheit und Verbraucherschutz; V3-2347-2-2012 and V3-2347-19-2013).

On days 24–30 pp (**E**xam **1** [**E1**]), cows were examined by inspection of the vulva, vaginoscopy, transrectal palpation and ultrasonography (Tringa Linear, Esaote, Köln, Germany) of the uterus and ovaries to determine their uterine health status and stage of the oestrous cycle as reported previously[24].

All cows with an initial status of a clinical healthy uterus (no (muco)purulent discharge detectable in the vagina)[25] were included in this study (n = 116; 30 primiparous and 86 multiparous). Cows with signs of clinical endometritis (**CE**) [(muco)purulent uterine discharge detectable in the vagina] at **E1** were excluded from the study.

If a corpus luteum was present, cows were considered as being in the luteal phase (n = 95). The absence of a corpus luteum and also a Graafian follicle defined cows to be either prior to their first ovulation after parturition or 1–3 days after ovulation (n = 21). Cows showing signs of oestrus (presence of clear and cohesive mucus discharged from the vulva and/or the presence of a Graafian follicle) were excluded to prevent that the applied bacteria would be flushed out by the mucus.

Intrauterine administration of *L. buchneri* or placebo and collection of luminal endometrial epithelial samples. After the examination at **E1**, samples from the endometrial epithelium were obtained with the cytobrush technique from the uterine body as reported previously[20,26]. Three cytobrush samples were collected. The first cytobrush was used for cytological analysis by rolling the cytobrush on a clean glass microscope slide directly after sampling at the farm. The second cytobrush was collected for the isolation of total RNA and was placed in a cryotube, which was immediately immersed into liquid nitrogen and stored at −80 °C until

further use. The third collected cytobrush was used for bacteriological analysis and therefore was placed in an Amies medium containing tube (Heinz Herenz, Hamburg, Germany) and transported to the laboratory at room temperature.

After the sampling, cows were randomly divided into two groups. The prepared *L. buchneri* solutions were administered intrauterine to cows of the first group (n = 56; *Lactobacillus buchneri* group [**LAC**]). 20 ml of isotonic saline solution 0.9% (w/v) per cow as a placebo were administered intrauterine to the cows of the second group (n = 60; placebo group [**PLA**]). Briefly, the metallic catheter passing the cervix for cytobrush sampling from the uterine body was hold in place after the collection of endometrial samples. A sterilized 62 cm long polytetrafluoroethylene tube with an internal diameter of 2 mm (Rotilabo, Carl Roth) was inserted through the catheter for the aseptic administration of the prepared *L. buchneri* solutions or the placebo.

After the transportation to the laboratory, slides for cytological analysis were prepared and evaluated as reported previously[24]. If the content of polymorphonuclear neutrophils (PMN) in the cytological sample was < 5%, the uterine health status of the cows was defined as healthy (**H**; n = 83). If the content was ≥5%, cows were classified as having **SCE** (n = 33)[20,27]. Within the **LAC** group (n = 56), 40 cows were classified as **H** and 16 cows were diagnosed with signs of **SCE**. Within the **PLA** group (n = 60), 43 cows were classified as **H** and 17 cows were diagnosed with **SCE**. Reproduction performance data of all cows treated with either *L. buchneri* or placebo were recorded and considered for statistical analysis.

Monitoring of uterine health status and collection of luminal endometrial epithelial samples after E1. Cows with **SCE**, both from the **LAC** and the **PLA** group, were further monitored for their uterine health status and sampled for mRNA expression analysis except for one cow from the **PLA** group because of technical reasons. This results in a distribution of 16 cows per group (**LAC/SCE** and **PLA/SCE**).

Each of these cows was examined and sampled three more times in the same manner as described above at **E1** in weekly intervals on days 31–37 pp (**E**xam **2** [**E2**]), on days 38–44 pp (**E**xam **3** [**E3**]), and on days 45–51 pp (**E**xam **4** [**E4**]).

Microbiological analysis. At **E1**, **E2** and **E4**, a cytobrush sample was taken for microbiological analysis from 31 cows of the **LAC** group (**LAC/SCE** n = 16 and **LAC/H** n = 15) and from 17 cows of the **PLA** group (**PLA/SCE** n = 15 and **PLA/H** n = 2), respectively. Within the **PLA/SCE** group, the sample at **E2** could not be taken from one cow for technical reasons. The samples were analysed by aerobic and anaerobic cultivation after direct inoculation of the cytobrush with the suitable agar plates and also after the enrichment in a medium with subsequent cultivation on agar as described previously[24].

Biopsy sampling and histopathological examination. Non-pregnant cows (n = 8), which were not in the phase around oestrus and without any signs of clinical endometritis, were sampled for histopathological examination. Briefly, the vulva was cleansed with dry paper towel. The biopsy instrument (Institute for the Reproduction of Farm Animals, Bernau, Germany) was inserted into the uterine body through the cervix. Endometrial tissue samples were taken from the uterine body, followed by intrauterine administration of $1.5–2 \times 10^{10}$ cfu of *L. buchneri* in 20 ml 0.9% (w/v) isotonic saline solution (**B/LAC**; n = 5) or placebo (20 ml 0.9% (w/v) isotonic saline solution; **B/PLA**; n = 3) in each cow, respectively. The application procedure was the same as described above. Biopsy samples from each cow were taken again one week later.

Pieces of endometrial tissue were immediately immersed into tubes containing Bouin's solution for transportation. Samples were embedded in paraffin. Two sections of 2 µm thickness each 1000 µm apart were prepared, stained with hematoxylin and eosin, and examined for the presence of immune cells, integrity of the mucosa and proliferation by an observer blinded to the administration of *L. buchneri* or placebo.

Breeding management. The voluntary waiting period was 60 days pp for most cows included in this study. However, 14 cows (**LAC** n = 9 and **PLA** n = 5) were inseminated earlier. Heat detection was done by observation once daily (10:00 a.m.) and by cow activity measured automatically with a respactor (X-ponder, Nedap, Groenlo, Netherlands) and a pedometer (Acto, Insentec, Marknesse, Netherlands). Cows were artificially inseminated within 24 h when heat was detected with deep frozen semen from bulls with similar fertility. Pregnancy diagnosis was performed by transrectal palpation and ultrasonography 30 days after insemination.

Isolation of total RNA and reverse transcription. Total RNA from cytobrush samples harvested from cows with signs of **SCE** was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), stored at −80 °C, and the integrity of the obtained total RNA was validated as previously reported[24].

For the removal of genomic DNA, a DNase treatment was performed before reverse transcription[28]. Single strand cDNA was generated from 100 ng total RNA per sample with the addition of 200 U RevertAid Reverse Transcriptase and 2.5 µM random hexamer primers (both Thermo Scientific, Schwerte, Germany) in a total volume of 60 µl[29]. For the confirmation of the absence of any genomic DNA or contaminations, samples without reverse transcriptase were also prepared as negative controls. Generated cDNA was stored in aliquots at −20 °C until further analysis.

Quantitative polymerase chain reaction (qPCR). qPCR was performed to evaluate the mRNA expression of the candidate genes as reported previously[29] following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines[30]. The genes of interest that were measured by this method are chemokine ligand 1/2 (*CXCL1/2*), *CXCL3*, *CXCL5*, chemokine receptor 2 (*CXCR2*), interleukin 1 alpha (*IL1A*), interleukin 1 beta (*IL1B*), *IL6*, *IL8*, *IL10*, interleukin 1 receptor antagonist (*IL1RN*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), prostaglandin E_2 synthase 1 (*PTGES*), *PTGES3*, prostaglandin D_2 synthase (*PTGDS*), tumour necrosis factor (*TNF*), matrix metallopeptidase 1 (*MMP1*), and protein tyrosine phosphatase, receptor type C

(*PTPRC*). Primer pairs were synthesized by Eurofins Genomics (Ebersberg, Germany) and details are given in Supplement Table 1. A gradient-PCR was performed to determine the optimal annealing temperature of unpublished primer pairs and obtained amplicons were subjected to commercial DNA sequencing (GATC Biotech, Konstanz, Germany) to confirm 100% homology to the published bovine sequences[29].

Using the Rotor Gene 3000 (Corbett Research, Mortlake, Australia), amplification of 1 µl cDNA per sample was carried out in the presence of 0.4 µM of each primer (forward and reverse) and 5 µl 2 × SensiMix SYBR Low-ROX (Bioline, Luckenwalde, Germany) in a total reaction volume of 10 µl. Denaturation at 95 °C for 10 min was followed by a three-step amplification in 45 cycles: denaturation at 95 °C for 15 s, annealing for 20 s (temperatures depicted in Supplement Table 1), and extension at 72 °C for 30 s. Subsequently, a melting curve program (50–99 °C) with continuous fluorescence measurement confirmed specific amplification. For the generation of a standard curve, a dilution series with known concentrations of the purified amplicons was amplified simultaneously. In comparison with these standard curves, transcript amounts of specific mRNA were calculated using the Rotor Gene 6.1 software (Corbett Research).

Statistical analysis. Reproductive performance was described by proportion of cows sold/culled, proportion of cows pregnant, days to first service, conception at first service, days to conception, and services per pregnancy. Survival curves for the proportion of cows pregnant on day 200 pp in relation to the number of days pp were generated using the Kaplan-Meier survival analysis. For the comparison of the Kaplan-Meier survival curves, three different statistical tests were used. The Breslow test tends to perform best towards the early time points and the Log Rank test to the late time points of the investigated period after calving. The Tarone-Ware test tends to perform best for the middle of this period.

The Fisher's exact test was used to calculate the incidence of cultivable bacteria in the **LAC** group in relation to the **PLA** group at **E2** and **E4**.

The obtained mRNA expression values of the genes of interest was normalized using the geNorm tool[31]. Succinate dehydrogenase complex, subunit A (*SDHA*) and suppressor of zeste 12 homolog (*SUZ12*) were chosen as reference genes. Inter-run calibration was performed based on 10 inter-run calibrator samples using formula 15'[32]. Box plots were generated presenting the median values with 50% of all data within the box. Outliers (circles; values between 1.5- and 3.0-fold the interquartile range) and extreme values (asterisks; values beyond 3.0-fold the interquartile range) were included in statistical analysis.

Normal distribution was tested with the Shapiro-Wilk test. Neither the percentages of the PMN nor the normalized values of mRNA expression of the genes of interest or the data for reproductive performance were normally distributed. The Mann-Whitney U test was used to analyse values of the mRNA expression comparing samples from the **LAC/SCE** group with samples from the **PLA/SCE** group at **E2**, **E3** and **E4** and for the data of reproduction comparing the **LAC** group with the **PLA** group and the **LAC/SCE** group with the **PLA/SCE** group. Fold changes of the mRNA expression levels were calculated as the approximate ratio of the mean value of mRNA expression for the **LAC/SCE** group to the mean value of the **PLA/SCE** group.

All statistical evaluations and the generation of the box plots were performed using IBM SPSS Statistics 20.0 (SPSS, Chicago, USA) and the level of significance was set at $P \leq 0.05$. A tendency to significance was considered when P was <0.10.

Results

Reproduction performance data. Several data of the descriptive reproductive performance considering all treated cows (**SCE** and **H**) are shown in Table 1. The proportion of cows sold/culled before pregnancy was 25% both in the **LAC** group and in the **PLA** group. Two cows of the **PLA** group were sold before breeding and 27 cows were culled due to problems as follows: the locomotor system (**LAC** group: 1 cow; **PLA** group: 4 cows), the udder (**LAC** group: 11 cows; **PLA** group: 7 cows), the fertility (**LAC** group: 2 cows), or a low milk yield (**PLA** group: 2 cows). The proportion of cows becoming pregnant was 75% both in the **LAC** and in the **PLA** group. The number of cows inseminated at least once was 96 in total, including 9 cows that were culled before conception (**LAC** group: 4; **PLA** group: 5).

The median days to first service were lower and the first service conception rate was higher in the **LAC** group compared with the values of the **PLA** group. The **LAC** group had less services per pregnancy and significant shorter median days to conception compared with the data in the **PLA** group.

Similar differences were observed when only considering cows with signs of **SCE** (Table 1). 8 cows of the **SCE** group were culled due to problems as follows: the locomotor system (**LAC/SCE** group: 1 cow), the udder (**LAC/SCE** group: 5 cows; **PLA/SCE** group: 3 cows). The days to first service were similar. However, a higher proportion of cows of the **LAC/SCE** group was pregnant after the first service and less services per pregnancy were required compared with the **PLA/SCE** group. The median days to conception for cows pregnant on day 200 pp were 90 days shorter in the **LAC/SCE** group ($P = 0.001$) compared with the **PLA/SCE** group.

Kaplan-Meier survival analysis was performed to show survival curves for the proportion of cows pregnant on day 200 pp in relation to the number of days pp (Fig. 1A). Cows pregnant after day 200 pp were censored. The proportion of cows censored was 7.1% (3 out of 42) for the **LAC** group and 20% (9 out of 45) for the **PLA** group. Comparing these groups, cows from the **LAC** group became pregnant significantly earlier using the Breslow test ($P = 0.035$) and the Tarone-Ware test ($P = 0.047$), whereas the Log Rank test ($P = 0.06$) showed a tendency to significance. The Kaplan-Meier survival analysis is also presented for the cows with signs of **SCE** to show the proportion of cows pregnant on day 200 pp (Fig. 1B). The cows from the **LAC/SCE** group became significantly earlier pregnant compared with the **PLA/SCE** group using the Breslow test, the Tarone-Ware test and the Log Rank test ($P = 0.001$ each).

Variable	LAC group	PLA group	*P*-value	LAC/SCE group	PLA/SCE group	*P*-value
Number of cows	56	60		16	17	
Number of cows inseminated	46	50		12	17	
Number of cows sold/culled before pregnancy	14 (25%)	15 (25%)		6 (37.5%)	2 (11.8%)	
Number of cows pregnant	42 (75%)	45 (75%)		10 (62.5%)	15 (88.2%)	
Median days to first service	67	81	0.157	63	65	0.107
Conception at first service	45.7%	30%		60%	13.3%	
Services per pregnancy	1.9	2.4	0.087	1.7	3.3	0.012
Number of cows pregnant on day 200 pp	39	36		10	10	
Median days to conception (of cows pregnant on day 200 pp)	103	133	0.035	74	164	0.001

Table 1. Descriptive reproductive performance outcomes of 116 cows as well as of 33 cows with signs of **SCE** after the intrauterine administration of $1.5–2 \times 10^{10}$ cfu of *L. buchneri* DSM 32407 diluted in 20 ml isotonic saline solution 0.9% (**LAC** group) or 20 ml isotonic saline solution 0.9% as a placebo (**PLA** group) on days 24–30 pp.

Uterine health status for the LAC/SCE and PLA/SCE groups at E2, E3, and E4. The number of cows in the **LAC/SCE** group (n = 16 at **E1**) dependent on their current uterine health status (**H/SCE/CE**) was distributed at the later time points of monitoring as follows: **E2** (n = 10/5/1), **E3** (n = 12/2/2), and **E4** (n = 12/3/1). The distribution for the **PLA/SCE** group (n = 16 at **E1**) was as follows (**H/SCE/CE**): **E2** (n = 13/1/2), **E3** (n = 13/1/2), and **E4** (n = 11/2/3).

After day 31 pp, the uterine health status of most cows was diagnosed as **H** in both groups. However, there were still 5 cows with signs of **SCE** in the **LAC/SCE** group in comparison to only 1 cow in the **PLA/SCE** group at **E2**. At **E3**, all of these cows were diagnosed as **H**.

Two cows of the **PLA/SCE** group showed continuously signs of **CE** at **E2**, **E3**, and **E4**. In both groups, all other cows showing signs of **SCE** or **CE** at **E3** and/or **E4** developed these diseases after being healthy before, except for one cow of the **LAC/SCE** group that had signs of **CE** at **E2**, **SCE** at **E3**, and was healthy at **E4**.

Content of PMN in endometrial cytobrush samples and microbiological analysis. At **E1**, the content of PMN in the cytological preparations from endometrial cytobrush samples varied from 5–73.3%. At the later time points, the majority of samples had a percentage of PMN of <5%, both in the **PLA/SCE** and **LAC/SCE** groups (Fig. 2).

No significant differences were observed comparing the content of PMN of the **PLA/SCE** group with the **LAC/SCE** group at **E2**, **E3**, and **E4**. However, the number of PMN tended to be higher ($P = 0.071$) in the **LAC/SCE** group compared with the **PLA/SCE** group at **E2** (Fig. 2).

At **E1**, **E2** and **E4**, cultivable bacteria were *Trueperella pyogenes*, *Escherichia coli*, *Histophilus somni* and *Streptococcus uberis* from few samples of both the **PLA/SCE** and the **LAC** group. *Klebsiella pneumoniae* was only cultivable from one sample of **LAC/SCE** group. The number of samples that were positive for each bacterial species for these time points are presented in Supplement Table 2.

A significant difference was only observed for *H. somni* at **E2** ($P = 0.032$) with a higher number of positive samples in the **PLA/SCE** group compared with the **LAC** group.

Evaluation of the mRNA expression analysis. Endometrial epithelial mRNA expression of all selected candidate genes was detected at all investigated time points in the postpartum period. However, mRNA expression for *IL1A*, *IL6, IL10, TNF*, *MMP1* and *PTPRC* could not be found in each sample.

At **E2**, **E3** and **E4**, most of the cows (**PLA/SCE** and **LAC/SCE**) were during their luteal phase, 1–3 days after ovulation or still prior to their first ovulation. However, seven cows were found during pro-oestrus at different time points (two at **E2**, two at **E3**, and three at **E4**) and two cows were found during oestrus at **E3**. The different oestrous cycle stages did not have a statistical significant influence on the mRNA expression values.

In the following text, mainly significant differences in normalized mRNA expression are described.

mRNA expression of *CXCL1/2*, *CXCL3*, *CXCL5*, and *CXCR2*. Primers named *CXCL1/2* are specific for *CXCL1* as well as for *CXCL2*[33]. At **E4**, *CXCL1/2* and *CXCL3* mRNA was seven- and threefold more highly expressed in the **PLA/SCE** group compared with the **LAC/SCE** group, respectively (Fig. 3A–B). In contrast, the mRNA expression pattern of *CXCL5* did not show significant differences between the **PLA/SCE** and **LAC/SCE** groups at all time points (Fig. 3C). However, a tendency ($P = 0.07$) was observed at **E2** when the *CXCL5* mRNA expression was higher in the **LAC/SCE** group compared with the **PLA/SCE** group.

Similar to *CXCL1/2* and *CXCL3*, the mRNA expression of *CXCR2* in luminal endometrial epithelium samples was affected at **E4**. *CXCR2* mRNA was 34-fold more highly expressed in the **PLA/SCE** group compared with the **LAC/SCE** group (Fig. 3D).

mRNA expression of *IL1A*, *IL1B*, *IL6*, *IL8*, *IL10*, and *IL1RN*. No significant differences were observed for the contents of *IL1A* mRNA in cytobrush samples of the **PLA/SCE** group compared with the samples of the **LAC/SCE** group during all time points (Fig. 4A). In addition, a decrease of the *IL1A* mRNA expression from

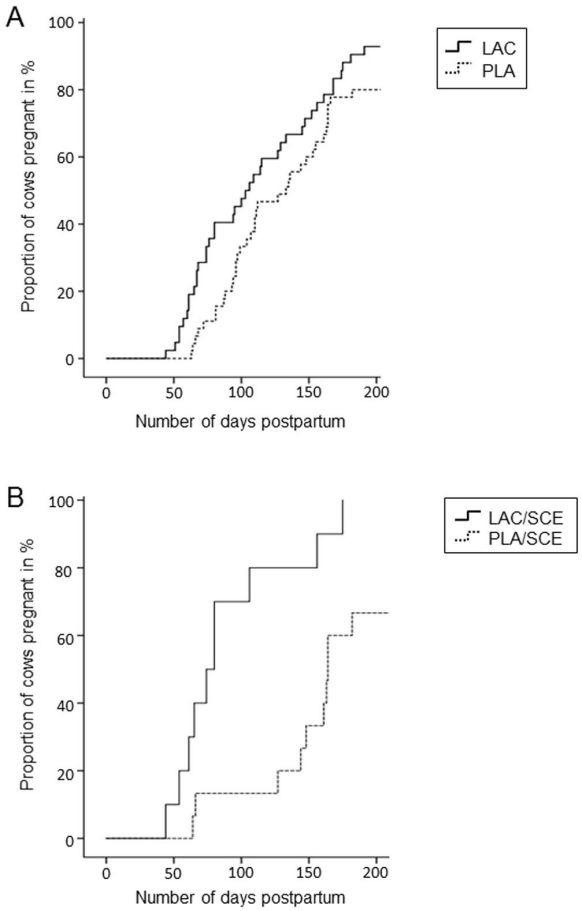

Figure 1. Kaplan-Meier survival curves for cows pregnant at day 200 pp. **(A)** The curves show the proportion of cows pregnant (in %) in relation to the number of days pp for the **LAC** group (n = 42; solid line) and the **PLA** group (n = 45; dotted line). The proportion of cows censored (not pregnant at day 200 pp) was 7.1% for the **LAC** group and 20.0% for the **PLA** group. *P* values: Breslow test ($P = 0.035$), Log Rank test ($P = 0.06$) and Tarone-Ware test ($P = 0.047$). **(B)** The curves show the proportion of cows pregnant (in %) in relation to the number of days pp for the **LAC/SCE** group (n = 16; solid line) and the **PLA/SCE** group (n = 17; dotted line). The proportion of cows censored (not pregnant at day 200 pp) was 0.0% for the **LAC/SCE** group and 33.3% for the **PLA/SCE** group. *P* values: Breslow test, Log Rank test and Tarone-Ware test ($P = 0.001$ each).

E1 to the later time points was observed. In contrast, the mRNA expression of *IL1B* at **E4** was higher ($P = 0.05$) in samples from cows of the **PLA/SCE** group compared with the samples obtained from the **LAC/SCE** group (Fig. 4B).

IL6 mRNA content was similar during the investigated period of the puerperium in all groups (Fig. 4C).

However, *IL8* mRNA was about 50-fold more highly expressed in endometrial samples from cows of the **PLA/SCE** group compared with the **LAC/SCE** group at **E4** (Fig. 4D).

IL10 and *IL1RN* transcript amount did not differ significantly between the different treatment groups (Fig. 4E–F). However, *IL1RN* mRNA expression tended to be higher ($P = 0.07$) at **E4** in samples of the **PLA/SCE** group compared with samples of the **LAC/SCE** group.

mRNA expression of *TNF*, *MMP1*, and *PTPRC*. The mRNA expression of *TNF* in endometrial epithelium cells was affected at **E2** by the treatment (Fig. 5A). *TNF* mRNA was threefold more highly expressed in samples obtained from cows of the **LAC/SCE** group compared with samples of the **PLA/SCE** group.

MMP1 mRNA contents did not differ between the **PLA/SCE** group compared with the **LAC/SCE** group at all time points (Fig. 5B).

Furthermore, *PTPRC* mRNA was threefold more highly expressed in the endometrial samples of the **PLA/SCE** group compared with the samples of the **LAC/SCE** group at **E4** (Fig. 5C).

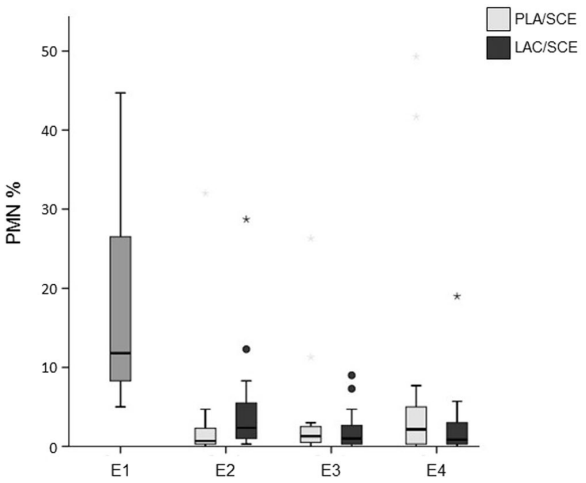

Figure 2. Percentages of PMN in bovine endometrial cytobrush samples harvested from dairy cows on days 24–30 pp (E1; n = 32), on days 31–37 pp (E2), on days 38–44 pp (E3), and on days 45–51 pp (E4). At E2, E3 and E4 cows were divided into the **PLA/SCE** (n = 16) and the **LAC/SCE** (n = 16) groups. Extreme values are diagrammed as asterisks, outliners as circles. Extreme values are not shown for one cow at E1 (73.3%) and for one cow of the **PLA/SCE** group at E2 (65%).

mRNA expression of *PTGS2*, *PTGES*, *PTGES3*, and *PTGDS*. No significant differences of the mRNA expression were found in the transcript amounts in samples obtained from the **LAC/SCE** group compared with samples of the **PLA/SCE** group at **E2**, **E3** and **E4** for the analysed key enzymes of the PG synthesis as follows: *PTGS2*, *PTGES*, *PTGES3*, and *PTGDS* (Fig. 5D,G). However, the **LAC/SCE** group tended to have a higher *PTGDS* mRNA expression ($P = 0.095$) at **E3** compared with the **PLA/SCE** group (Fig. 5G).

Histopathology of biopsy samples. No infiltration with immune cells was observed in all biopsy samples taken before the intrauterine administration of the *L. buchneri* solutions or the placebo, respectively. In addition, the integrity of the mucosa and the status of proliferation were without pathological findings (Fig. 6A,B).

One week after the intrauterine administration of the *L. buchneri* solutions or the placebo, respectively, all biopsy samples of the **B/PLA** group and one sample of the **B/LAC** group were judged identical to the samples that were taken previously to the administration. In the **B/LAC** group, a minimal granulocytic infiltration was observed in one sample, a minimal lymphocytic infiltration was observed in two samples, and a moderate mainly granulocytic infiltration was observed in one sample. This sample also showed a moderate multifocal degeneration (Fig. 6C,D). The integrity of the mucosa and the status of proliferation were without pathological findings in all other samples from the **B/LAC** group.

Discussion

In a former study was shown that the strain *L. buchneri* DSM 32407 did neither influence the viability of endometrial epithelial cells nor provoke a pro-inflammatory response in contrast to other *Lactobacillus* strains[16]. Pathogenic (*Trueperella pyogenes*) or potential pathogenic strains (*Bacillus pumilus*) caused cell death even within 16–24 h[33,34]. Therefore, the strain *L. buchneri* DSM 32407 seems suitable to serve as a probiotic strain within the bovine uterus to modulate the immune response and the reproductive performance of dairy cows. The results of the present study support the hypothesis that lactobacilli improve the genital health resulting in better fertility rates, which are negatively influenced by inflammatory processes in the bovine uterus caused by pathogenic bacteria[35–37].

A beneficial impact on reproductive performance, in particular on the interval from calving to conception, was observed after the intrauterine administration of *L. buchneri* DSM 32407. This might be associated to the down-regulated local immune system on days 45–51 pp, an indication for a healthy uterus at a time of the puerperium closer to first insemination[24]. Supporting this assumption of a positive impact on reproductive performance, one other study revealed that the presence of lactobacilli in the bovine uterus is related to a better pregnancy rate[17]. The percentage of cows positive for lactobacilli at 35 days in milk (DIM) was significantly higher in cows that were pregnant by 200 DIM compared with the group of cows that were not pregnant by that time. Interestingly, a similar effect was observed in a recent study that investigated the human endometrial microbiota. It showed that women with a *Lactobacillus*-dominated-microbiota (>90%) have higher chances of implantation, pregnancy and live birth after *in vitro* fertilization[38]. In this context, a positive effect of intrauterine lactobacilli on fertility might also be related to stimulatory effects on the blastocyst around the time of implantation. *In vitro* experiments showed that *L. acidophilus* culture supernatant positively influenced the growth and development of bovine embryos[39].

Such influence on bovine genital health was additionally observed in recent studies. The weekly administration of a mixture of lactobacilli (LAB) into the vagina from two weeks before until four weeks after parturition (six treatments) decreased the occurrence of purulent vaginal discharge in dairy cows at week three pp[40]. In the same study, survival analysis revealed shorter calving to pregnancy intervals for the group of multiparous cows treated intravaginally with LAB. In two related studies, the same mixture of LAB were administered intravaginally only

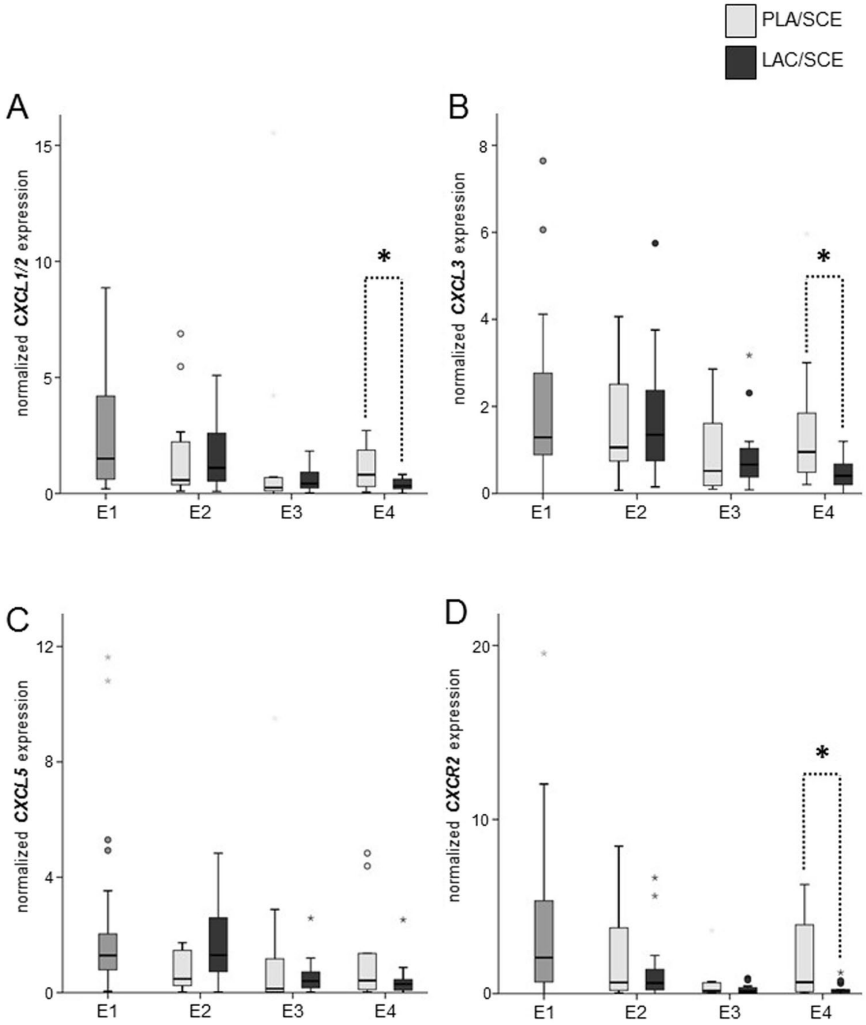

Figure 3. Normalized mRNA expression of (**A**) *CXCL1/2*, (**B**) *CXCL3*, (**C**) *CXCL5* and (**D**) *CXCR2* in bovine endometrial cytobrush samples harvested from dairy cows on days 24–30 pp (E1; n = 32), on days 31–37 pp (E2), on days 38–44 pp (E3), and on days 45–51 pp (E4). At E2, E3 and E4 cows were divided into the **PLA/SCE** (n = 16) and the **LAC/SCE** (n = 16) groups. Bold asterisks over dotted lines indicate significant differences between the groups ($P \leq 0.05$). Extreme values are diagrammed as asterisks, outliners as circles. Extreme values are not shown for *CXCL1/2* obtained from three cows at E1 (17.69, 18.75, and 19.87) and from one cow of the **PLA/SCE** group at E4 (26.63); for *CXCL3* obtained from one cow at E1 (22.67); for *CXCL5* obtained from one cow of the **PLA/SCE** group at E2 (13.56) and E4 (21.72) and for *CXCR2* obtained from one cow of the **PLA/SCE** group at E2 (28.62), E3 (110.76), and E4 (109.09).

three or two times. Treated cows had a faster uterine involution and a lower incidence of uterine infections, cows with three treatments resumed ovarian cyclicity earlier and cows with two treatments had fewer days open[41,42]. However, a recent study showed that the number of intrauterine counted lactobacilli did not differ compared with controls when lactobacilli were intravaginal administered[43]. In contrast to the mentioned studies, the present study focused on the treatment of cows with signs of **SCE**, which have impaired reproductive performance and are difficult to detect[44]. In addition, the present study revealed that only one intrauterine application improved the reproductive performance tremendously. It was shown that cows, even healthy or with signs of **SCE**, showed a better reproductive performance after the treatment.

The improved fertility may be obtained because an intrauterine administration of *L. buchneri* DSM 32407 initially stimulates the local immune system. One week after the administration, at a time when the uterine health status of most cows recovered to health, cows of the **LAC/SCE** group tended to have higher percentages of PMN in endometrial samples. These findings are supported by an earlier study that observed the endometrial infiltration with immune cells for up to 12 days after the intrauterine administration of lactobacilli to clinically healthy cows and stated a possible stimulatory effect on endometrial defence mechanisms[19].

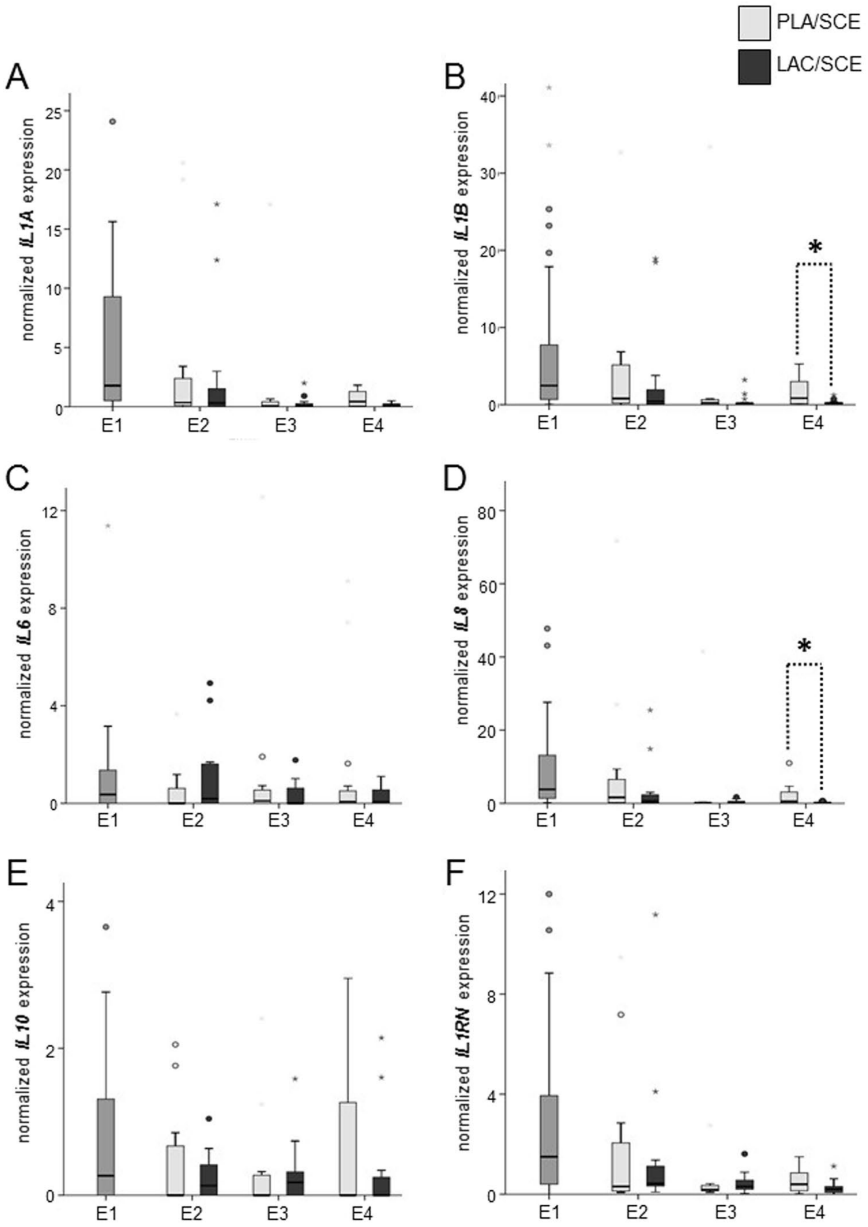

Figure 4. Normalized mRNA expression of (**A**) *IL1A*, (**B**) *IL1B*, (**C**) *IL6*, (**D**) *IL8*, (**E**) *IL10* and (**F**) *IL1RN* in bovine endometrial cytobrush samples harvested from dairy cows on days 24–30 pp (E1; n = 32), on days 31–37 pp (E2), on days 38–44 pp (E3), and on days 45–51 pp (E4). At E2, E3 and E4 cows were divided into the **PLA/SCE** (n = 16) and the **LAC/SCE** (n = 16) groups. Bold asterisks over dotted lines indicate significant differences between the groups ($P \leq 0.05$). Extreme values are diagrammed as asterisks, outliners as circles. Extreme values are not shown for *IL1A* obtained from one cow at E1 (198.24) and from one cow of the **PLA/SCE** group at E3 (64.07) and E4 (94.13); for *IL1B* obtained from one cow of the **PLA/SCE** group at E2 (79.07), E3 (549.78), and E4 (223.11); for *IL6* obtained from two cows of the **LAC/SCE** group at E2 (116.41) and E3 (13.87); for *IL8* obtained from two cows at E1 (167.56 and 144.01) and from one cow of the **PLA/SCE** group at E3 (112.67) and E4 (143.67); for *IL10* obtained from one cow at E1 (14.2) and from one cow of the **PLA/SCE** group at E4 (18.7) and for *IL1RN* obtained from one cow at E1 (20.89) and from one cow of the **PLA/SCE** group at E3 (30.99) and E4 (19.59).

The higher mRNA expression of *TNF* in the **LAC/SCE** group one week after the intrauterine administration of *L. buchneri* DSM 32407 supports these findings on a molecular basis. TNF is a pro-inflammatory cytokine that is produced by monocytes and/or macrophages infiltrating to the site of an inflammation[45]. It was found to be

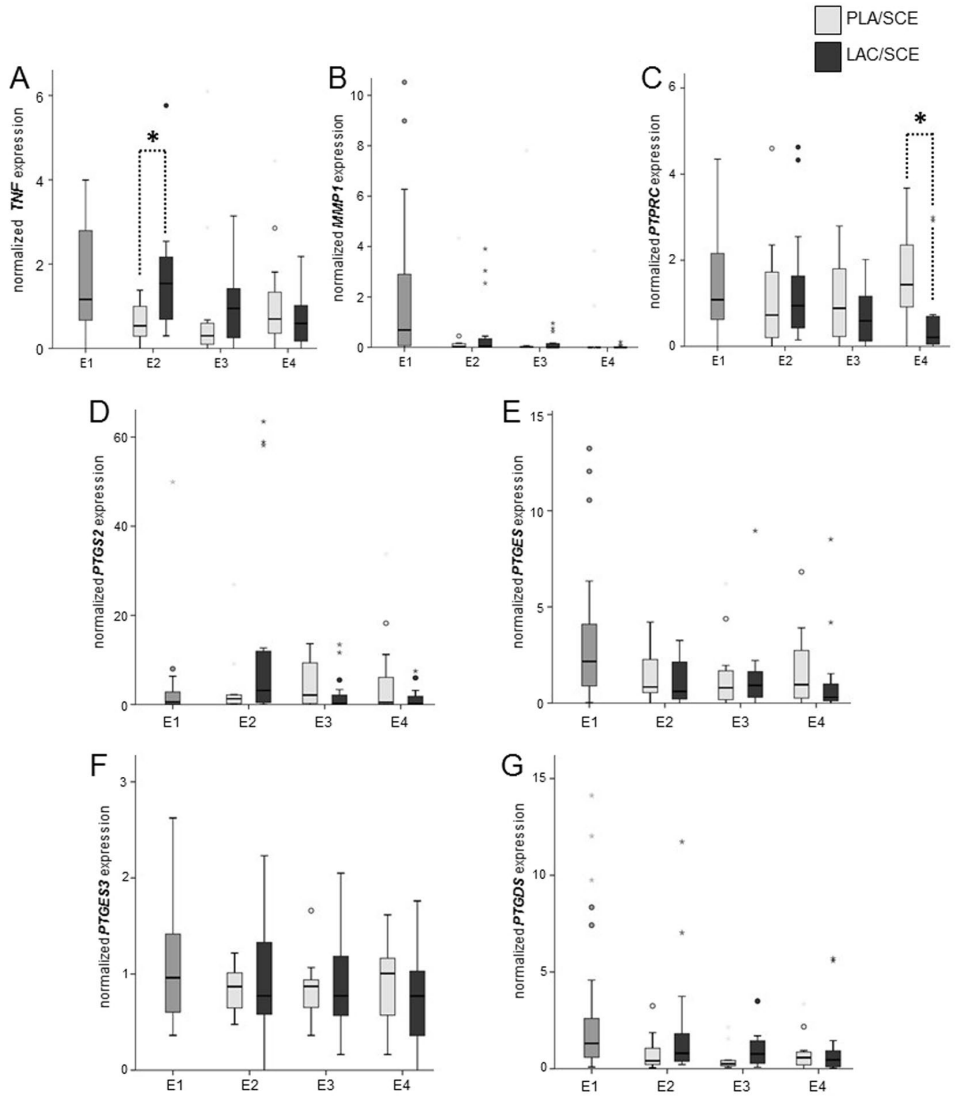

Figure 5. Normalized mRNA expression of (**A**) *TNF*, (**B**) *MMP1*, (**C**) *PTPRC*, (**D**) *PTGS2*, (**E**) *PTGES*, (**F**) *PTGES3* and (**G**) *PTGDS* in bovine endometrial cytobrush samples harvested from dairy cows on days 24–30 pp (E1; n = 32), on days 31–37 pp (E2), on days 38–44 pp (E3), and on days 45–51 pp (E4). At E2, E3 and E4 cows were divided into the **PLA/SCE** (n = 16) and the **LAC/SCE** (n = 16) groups. Bold asterisks over dotted lines indicate significant differences between the groups ($P \leq 0.05$). Extreme values are diagrammed as asterisks, outliners as circles. Extreme values are not shown for *TNF* obtained from two cows at E1 (14.83 and 11.06) and from one cow of the **PLA/SCE** group at E4 (13.44); for *MMP1* obtained from one cow at E1 (56.61); for *PTPRC* obtained from one cow at E1 (23.53) and from one cow of the **PLA/SCE** group at E2 (12.86); for *PTGS2* obtained from one cow at E1 (138.26); for *PTGES3* obtained from one cow at E1 (6.16) and for *PTGDS* obtained from one cow of the **PLA/SCE** group at E4 (23.04).

more highly expressed in the bovine endometrium during an inflammatory process, which reflects an activated immune system within the uterus[46]. The stimulation of cells with TNF results in the synthesis of CXCL5[47,48], which explains that *CXCL5* mRNA contents tended to be higher in the **LAC/SCE** group at this time point. CXCL5 is a chemoattractant responsible for mediating neutrophil recruitment during inflammation and infection and binds to CXCR2, which is present especially on the surface of immune cells, e.g. PMN[49,50].

In addition, the histopathology of endometrial biopsy samples of cows showed an infiltration with immune cells only in cows of the **B**/**LAC** group one week after the intrauterine administration. These biopsy data were supported by an *in vitro* study showing that *L. buchneri* DSM 32407 did not affect the viability of bovine endometrial epithelial cells in this short-time co-culturing experiments with a MOI up to 10^{16}. However, this study only

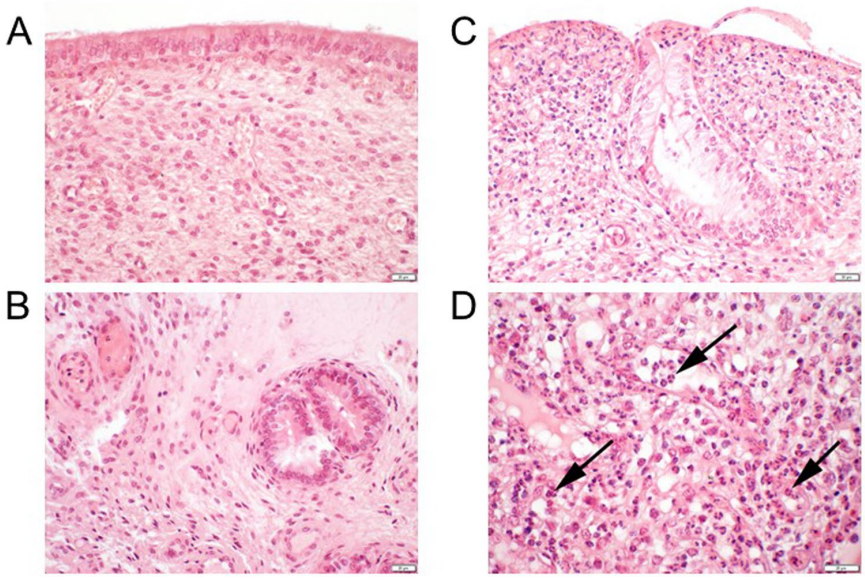

Figure 6. Biopsy samples of the endometrium of one cow from the **B/LAC** group. Hematoxylin and eosin staining; 2 µm thickness. (**A** and **B**) prior to the intrauterine administration of *L. buchneri* DSM 32407; no infiltration of immune cells, integrity of the mucosa and status of proliferation without pathological findings. (**C** and **D**) 7 days after the intrauterine administration of *L. buchneri* DSM 32407; moderate mainly granulocytic infiltration (arrows) and moderate multifocal degeneration.

focused on the short-term influence of *L. buchneri* DSM 32407, whereas the results of the present study provide more information about the long-term effects *in vivo*.

Although there is an indication of an activated local immune system, the mRNA expression levels of the other investigated pro-inflammatory factors were not significantly influenced seven days after the intrauterine administration of *L. buchneri* DSM 32407. However, it is possible that the mRNA expression of these factors increased earlier than seven days after the administration, previous to the influx of immune cells.

In the early puerperium, an up-regulation of the local immune system for the clearance of invaded pathogenic bacteria is regarded as a physiological process[51]. In this context, it was observed that cows with a high PMN infiltration within the uterus during the first week pp have a better fertility compared with cows, which a have lower PMN infiltration[52]. Bacterial contaminations of the bovine uterus are almost inevitable with a prevalence of around 90%[53] with pathogenic bacteria being the main cause for the development of endometritis[54,55]. The immunostimulatory effects for lactobacilli were also observed in earlier studies[19,56,57] and the present study also suggests that lactobacilli are supportive for the containment of these pathogenic bacteria. It was also shown that a distinct lactobacilli strain alone or in combination with other reduced an *E. coli* infection and affected the pro-inflammatory reaction in bovine endometrial cells *in vitro*[58,59].

In this context, there is indication that lactobacilli produce mitogenic and chemotactic factors. Culture supernatant of lactobacilli had strong pro-inflammatory properties, inducing the influx of PMN, the proliferation of macrophages and lymphocytes and the production of TNF by macrophages[60]. The competition with pathogens for limited nutrients also has to be taken into account[61].

An early containment of pathogens might subsequently reduce local inflammatory processes in the endometrium and has beneficial effects on the genital health status. Although no different effects of the administration of *L. buchneri* DSM 32407 or the placebo on the clinical uterine health status of the **LAC/SCE** and **PLA/SCE** groups were observed, the present results of the mRNA expression analysis support this assumption. Three weeks after the intrauterine administration of *L. buchneri* DSM 32407 or the placebo, the mRNA of *CXCL1/2, CXCL3*, *CXCR2*, *IL1B*, *IL8* and *PTPRC* was lower expressed in the **LAC/SCE** group compared with the **PLA/SCE** group. These pro-inflammatory factors were higher expressed in cows with signs of **SCE** and/or **CE** and thereby related to uterine health problems in a recent study at the same time point[24] and in previous studies at earlier time points of the puerperium[21,23,33]. The mRNA data of the present study thereby indicates that the endometrial immune system of cows treated with *L. buchneri* is downregulated three weeks after its administration to the uterus. Unfortunately, the present study design does not allow a satisfying explanation on why it takes three weeks to a noticeable effect on the immune system. In future studies, it would be of great benefit to investigate the endometrial microbiota by pyrosequencing before and after the administration of *L. buchneri* to better understand the time-dependent changes and possible correlations to the effects on the immune system.

In conclusion, *L. buchneri* DSM 32407 improved the reproductive performance of cows with **SCE** and healthy cows. This distinct lactobacillus strain seems to first have a stimulatory effect on the local immune system one week after its intrauterine administration to dairy cows on days 24–30 pp. This might be beneficial at that time point of the

puerperium, presumably supporting the elimination of pathogenic bacteria from the uterine lumen. Three weeks after the administration, the endometrial mRNA expression of several pro-inflammatory factors was down-regulated, suggesting the containment of local inflammation possibly related to the earlier containment of pathogenic bacteria, which in turn might be associated with better parameters for reproductive performance. However, to confirm this suggestion of improved fertility, a larger cohort of cows treated with *L. buchneri* DSM 32407 would be necessary.

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Author Contributions

S.P. contributed ideas, performed experiments, analyzed data, wrote the manuscript, and prepared the figures; G.M., M.A.G., M.I., R.K. and A.L.-B. performed experiments and edited the manuscript; M.J. and R.E. contributed ideas and edited the manuscript; C.G. conceived the study, designed experiments, analyzed data, and wrote the manuscript.

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Supplement Table 1: Selected gene transcripts, primer sequences and annealing temperatures used for RT-qPCR with resulting amplicon length.

Gene	Primer sequence	Reference / GenBank accession no.	Fragment size	Annealing temperature
SDHA	For 5'-GGG AGG ACT TCA AGG AGA GG-3'	1	219 bp	60 °C
	Rev 5'-CTC CTC AGT AGG AGC GGA TG-3'			
SUZ12	For 5'-TTC GTT GGA CAG GAG AGA CC-3'	2	286 bp	60 °C
	Rev 5'-GTG CAC CAA GGG CAA TGT AG-3'			
CXCL1/2	For 5'-GAC CTT GCA GGG GAT TCA CCT C-3'	1	125 bp	60 °C
	Rev 5'-CGG GGT TGA GAC ACA CTT CCT G-3'			
CXCL3	For 5'-GCC ATT GCC TGC AAA CTT-3'	1	189 bp	56 °C
	Rev 5'-TGC TGC CCT TGT TTA GCA-3'			
CXCL5	For 5'-TGA GAC TGC TAT CCA GCC G-3'	3	193 bp	61 °C
	Rev 5'-AGA TCA CTG ACC GTT TTG GG-3'			
CXCR2	For 5'-AAC AGA CTC TGC CCC ATG TC-3'	1	151 bp	60 °C
	Rev 5'-AGT GAC AGA GCG ACC AAT CC-3'			
IL1A	For 5'-TCA TCC ACC AGG AAT GCA TC-3'	4	300 bp	59 °C
	Rev 5'-AGC CAT GCT TTT CCC AGA AG-3'			
IL1B	For 5'-CAA GGA GAG GAA AGA GAC A-3'	5	236 bp	56 °C
	Rev 5'-TGA GAA GTG CTG ATG TAC CA-3'			
IL6	For 5'-TCC AGA ACG AGT ATG AGG-3'	5	236 bp	56 °C
	Rev 5'-CAT CCG AAT AGC TCT CAG-3'			
IL8	For 5'-CGA TGC CAA TGC ATA AAA AC-3'	3	153 bp	56 °C
	Rev 5'-CTT TTC CTT GGG GTT TAG GC-3'			
IL10	For 5'-TGT TGA CCC AGT CTC TGC TG-3'	NM_174088	279 bp	60 °C
	Rev 5'-TGG CTT TGT AGA CAC CCC TC-3'			
IL1RN	For 5'-ATG TGG TAC CCA TCG AAC CC-3'	4	298 bp	59 °C
	Rev 5'-AAC TTG GTG ACC TTG AGG GC-3'			
PTGS2	For 5'-CTC TTC CTC CTG TGC CTG AT-3'	6	359 bp	60 °C
	Rev 5'-CTG AGT ATC TTT GAC TGT GGG AG-3'			
PTGES	For 5'-TGC TGG TCA TCA AAA TGT ACG-3'	4	300 bp	58 °C
	Rev 5'-GCA GTT TCC CCA GGT ATG C-3'			

PTGES3	For 5'-TGC AAA GTG GTA CGA TCG G-3'	4	253 bp	61 °C
	Rev 5'-TAA CCT TGG CCA TGA CTG G-3'			
PTGDS	For 5'-TGA GAC GCG GAC CTT ACT G-3'	4	193 bp	61 °C
	Rev 5'-CTG GGA GCG GCT GTA GAG-3'			
TNF	For 5'-CAA GTA ACA AGC CGG TAG CC-3'	7	354 bp	60 °C
	Rev 5'-GCT GGA AGA CTC CTC CCT G -3'			
MMP1	For 5'-GAT GAT GAT GAA TGG TGG ACC-3'	8	347 bp	60 °C
	Rev 5'-TCC ACT TCT GGG TAC AAG GG-3'			
PTPRC	For 5'-CAA ATT TAA ATG TGA TGG CGG-3'	NM_001206523	286 bp	56 °C
	Rev 5'-TCG TCC ACC TGG AGT AAT CC-3'			

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Supplement Table 2: Number of samples positive after microbiological analysis of endometrial cytobrush samples from 31 cows of the **LAC** group and 17 cows of the **PLA** group at E1 (days 24-30 pp), E2 (days 31-37 pp) and E4 (days 45-51 pp).

		LAC group (31 samples)		**PLA** group (17 samples)	
		LAC/SCE (16 samples)	**LAC/H** (15 samples)	**PLA/SCE** (15 samples)	**PLA/H** (2 samples)
		Number of samples positive		Number of samples positive	
E1	*Trueperella pyogenes*	-	-	-	-
	Escherichia coli	2	1	4	-
	Histophilus somni	-	-	2	-
	Streptococcus uberis	1	-	-	-
	Klebsiella pneumoniae	-	-	-	-
E2	*Trueperella pyogenes*	-	1	-	-
	Escherichia coli	1	2	-	-
	Histophilus somni	-	-	3	-
	Streptococcus uberis	1	-	2	-
	Klebsiella pneumoniae	1	-	-	-
E4	*Trueperella pyogenes*	1	1	1	-
	Escherichia coli	1	-	3	-
	Histophilus somni	1	1	1	-
	Streptococcus uberis	-	-	1	-
	Klebsiella pneumoniae	-	-	-	-

5. Discussion

For the success in dairy farming, the cow's reproductive tract requires very special attention particularly during the time span from calving to conception.

The results of the present study help to attend to this topic with shedding light on (1) the mRNA expression of pro-inflammatory factors in healthy and inflamed bovine endometria pp and (2) the intrauterine influence of *L. buchneri* DSM 32407 on (a) the endometrial mRNA expression of pro-inflammatory factors of cows with SCE and (b) the subsequent reproductive performance of cows with SCE and a healthy endometrium.

5.1. Definition of subclinical endometritis

As mentioned before, thresholds for the percentage of PMN that define SCE range from 4 % to 18 % PMN (Kasimanickam et al., 2004; Galvão et al., 2009). It is to mention that these studies used different techniques and time points for the sampling and different criteria for the examination of the cows. Therefore, it is difficult to determine the optimal threshold.

To define SCE for the present study, the cytobrush technique and a threshold of 5 % PMN were chosen, which was confirmed by a review about SCE (Wagener et al., 2017a). Clinically healthy cows with no (muco)purulent vaginal discharge and a percentage of PMN ≥ 5 % were defined as having SCE, below 5 % as being healthy (**Peter et al., 2015**; **Peter et al., 2018**). In former studies, it was shown that this threshold is appropriate for the evaluation of mRNA expression patterns of pro-inflammatory factors in endometrial epithelial cells (Gabler et al., 2009; Fischer et al., 2010; Gärtner et al., 2016). Furthermore, in a recent study using the cytobrush technique, the cutoff PMN values for the diagnosis of SCE in dairy cows were set at 8 % for 21-33 DIM, at 6 % for 34-47 DIM and at 4 % for 48-62 DIM, with an overall 5 % for 21-62 DIM (Madoz et al., 2013). This study also observed that the percentage of PMN did not vary with the stage of estrous cycle.

For the cytobrush technique, it has to be considered that the endometrial location chosen for sampling (e.g. corpus uteri or uterine horns) might not be representative for the whole endometrium and therefore likely affects the exactness of SCE diagnosis (de Boer et al., 2014). When cows were sampled at 5 different endometrial sites (corpus uteri, left horn base and tip, right horn base and tip), the probability to detect a cow with SCE with a single sample was only 40 % for a threshold of 18 % PMN and only 51 % for a threshold of 5 % PMN (Pothmann et al., 2019). This study suggests that, when only one sample is taken, a result of < 5 % PMN might wrongly define a cow as healthy and therefore recommends a second sample. However, as these studies were published after the start of the sampling for

both studies (**Peter et al., 2015**; **Peter et al., 2018**), they could not be considered and the cytobrush samples were taken only from the uterine body as described before (Kasimanickam et al., 2004; Gabler et al., 2009) with ≥ 5 % PMN defining SCE and < 5 % PMN defining a healthy endometrium.

5.2. Endometrial pro-inflammatory mRNA expression profiles

5.2.1. Relation to the uterine health status in the late puerperium

As shown in several studies, an elevation of the endometrial mRNA expression of different pro-inflammatory factors can be found in cows with endometritis (SCE and/or CE) compared with healthy cows, e.g. on days 21-27 pp (Gabler et al., 2009; Fischer et al., 2010), on days 24-30 pp (Gärtner et al., 2016), on days 28-36 pp (Kasimanickam et al., 2014; Johnson et al., 2015) and on days 28-41 pp (Ghasemi et al., 2012). Genes of the innate immune system were also upregulated in cows with SCE on day 29 pp when measured with microarray analysis (Walker et al., 2015). This coincides with the results of the present study that show an elevated mRNA expression of specific pro-inflammatory factors in the endometrium of cows with SCE and/or CE during days 24-51 pp (**Peter et al., 2015**).

However, the mentioned studies mainly focused on one time point and not later than day 41 pp, whereas the present study evaluated the mRNA expression of pro-inflammatory factors and the genital health status during the second half of the puerperium in weekly intervals (days 24-51 pp) and therefore gives additional information especially about days 45-51 pp in comparison to earlier time points (**Peter et al., 2015**).

There is a lack of studies that also investigated the bovine endometrium in correlation to the uterine health status at later stages of the puerperium. A recent study showed that both Serum amyloid A (SAA) protein levels and mRNA expression on days 40-60 pp were positively correlated to the severity of endometritis based on clinical signs and histopathology in endometrial biopsies (Zhang et al., 2018). This suggests that SAA is influenced by endometrial inflammation in the late puerperium. However, *SAA* mRNA expression was not investigated in the present study.

Another study about the late pp period showed that cows diagnosed with SCE on day 60 pp had higher protein levels of IL6 and IL10 in uterine flush samples compared to healthy animals (Brodzki et al., 2015). This stays in contrast to the results of the present study, which did not show a difference of the endometrial mRNA expression of *IL6* in cows with SCE compared to healthy animals on days 45-51 pp (**Peter et al., 2015**). In the present study, the

mRNA content of *IL10* was not evaluated for the comparison of endometritic with healthy cows on days 45-51 pp.

However, to interpret the results of the present study, they should be linked to the knowledge about the dynamics of the endometrial innate immunity during the puerperium. As described before, the completion of a physiological puerperium in high-producing dairy cows takes about 6 to 7 weeks and the endometrial immune system shifts from a downregulated state during pregnancy to an upregulated state during the early puerperium and to a downregulated state towards the late puerperium, which can be seen as the physiological process.

This leads to assume that the differences within the innate immune responses of healthy or inflamed uteri become clearer the more advanced the pp period is. Matching this hypothesis, the present study showed that the number of pro-inflammatory factors upregulated in CE and/or SCE compared to H was highest on days 45-51 pp (**Peter et al., 2015**). This regards the mRNA expression of almost all of the investigated factors on days 45-51 pp and the fold changes were mostly higher in comparison to the earlier time points. This highly suggests the strongest relation between the uterine health status and the activity of the endometrial innate immune system at the end of the puerperium.

Similar results were observed for several antimicrobial peptides (AMPs), another part of the innate immune system, that were studied in healthy cows and cows with SCE or CE at exactly the same four time points pp using the same samples as in the present study (Ibrahim et al., 2016). Two AMPs from the bovine beta-defensin (DEFB) family and two AMPs from the S100A family showed this special endometrial mRNA expression pattern when comparing healthy cows to endometritic cows. Cows with SCE and/or CE had a significantly higher expression of *DEFB1* and *DEFB4A* only on days 45-51 pp and of *S100A8* and *S100A12* only on days 38-51 pp. Once more, these findings support the assumption that the differences within the innate immune responses of healthy or inflamed uteri become clearer with the progression of the pp period.

However, the mRNA expression of *PTGES3* and *PTGIS* was unaffected on days 45-51 pp regarding the current uterine health. The mRNA expression of *PTGES3* was even equally distributed at all the time points of sampling without any correlation to the uterine health status, the number of PMN or other pro-inflammatory factors (**Peter et al., 2015**). This is well explainable with the mainly constitutive production of this specific enzyme in cells (Kudo and Murakami, 2005). Interestingly, the mRNA expression of *PTGES3* was even lower in cows with SCE compared to healthy cows on days 21-27 pp (Gabler et al., 2009). Therefore, PTGES3 seems unsuitable as marker gene for bovine endometritis. In this context, it should

be kept in mind that different results could be expected for the mRNA expression of *PTGES*, which is induced by pro-inflammatory stimuli and tissue repair (Kudo and Murakami, 2005) and could represent an interesting enzyme for further studies about endometrial pro-inflammatory mRNA expression profiles. The intrauterine administration of *L. buchneri* DSM 32407 on days 24-30 pp to cows with SCE had no influence on the mRNA expression of *PTGES* in the three following weeks, whereas *L. buchneri* influenced the mRNA expression of other pro-inflammatory factors (**Peter et al., 2018**). However, further research is needed to evaluate PTGES as a potential biomarker for CE and SCE.

As the content of *PTGIS* mRNA was also not influenced by the current uterine health status at all the time points of sampling, not correlated to the number of PMN and even negatively correlated to the mRNA expression of *IL1A*, *IL1B* and *IL8*, PTGIS seems also inapplicable as marker gene for puerperal bovine endometritis (**Peter et al., 2015**). There is no other study that investigated the endometrial mRNA expression of *PTGIS* in the pp period of cows.

Overall, the results of the present study may indicate that the endometrial immune response of some cows with SCE and CE is insufficient at an earlier stage of the puerperium. And vice versa, that (even clinically healthy) cows can have an earlier appropriate upregulation of the endometrial immune system that helps to maintain a healthy uterus with a downregulated immune system at the end of the puerperium. This would explain why the strength of the immune response in relation to the uterine health status is more distinct at the end of the puerperal period.

5.2.2. Long-term effects of uterine health

PTGIS mRNA was higher expressed on days 38-44 pp in cows with CE compared to the H group if referring to the initial grouping on days 24-30 pp (**Peter et al., 2015**). This suggests a long-term effect of the initial uterine health status on days 24-30 pp on the subsequent mRNA expression of *PTGIS* two weeks later. The same applies for the mRNA transcript amounts of *PTGDS* one and two weeks after the initial grouping and of *CXCL1/2*, *CXCL5* and *CXCR2* three weeks after the initial grouping and therefore long-term effects could be suggested (**Peter et al., 2015**).

Regarding the investigated interleukins and TNF, no long-term effect of SCE and CE on days 24-30 pp was observed in the present study (**Peter et al., 2015**). In contrast, when cows were diagnosed with SCE or CE at 4 weeks pp, IL1B derived from uterine flush samples was elevated in cows with CE compared to healthy cows and cows with SCE at 4, 6 and 8 weeks pp (Kim et al., 2014). This suggests that CE at 4 weeks pp has a long-term effect on the protein level of IL1B two and four weeks later. Furthermore, higher mRNA contents of *IL1A*, *IL1B* and *TNF* were found on days 57-63 pp in cows that were diagnosed with RFM

and/or clinical metritis in comparison to cows that were clinically healthy within the first 21 days pp (Heppelmann et al., 2016). The results of all of these studies are inconsistent towards the question if puerperal uterine diseases have measurable long-term effects on specific endometrial interleukins and TNF.

However, the conclusions about long-term effects should be conclusions drawn carefully regarding the numerous group changes of uterine health that cows can make during the puerperium, especially concerning cows that change from H to SCE and/or CE (**Peter et al., 2015**). In the study of Heppelmann et al. (2016), cows with RFM and/or clinical metritis tended to have a higher prevalence of a histopathologic evidence of purulent endometritis later in the puerperium than cows without RFM and/or metritis. This should also be considered for the evaluation of possible long-term effects of RFM and/or metritis on the mRNA expression of the investigated factors on days 57-63 pp (Heppelmann et al., 2016).

In conclusion, for an evaluation of possible long-term effects of uterine diseases on the endometrial mRNA expression of specific factors, only samples from cows that (after the uterine disease) become and stay healthy for a certain amount of time should be included.

5.2.3. Influence on reproductive performance

As mentioned before, an upregulated endometrial immune system at the time of AI is suspected to interfere with fertility (Drillich et al., 2012).

Unfortunately, data of the reproductive performance were not statistically evaluated for the cows of the first study (**Peter et al., 2015**). But in the second study, cows with significantly lower endometrial mRNA contents of *CXCL1/2*, *CXCL3*, *CXCR2*, *IL1B*, *IL8* and *PTPRC* on days 45-51 pp had a considerably better first service conception rate and also the median days to conception (for cows pregnant on day 200 pp) were significantly ($P = 0.001$) shorter (**Peter et al., 2018**). However, when rating these results, it has to be kept in mind that the uteri of these cows were infused with *L. buchneri* DSM 32407 on days 24-30 pp, whose possible influence will be discussed later on.

Further supporting the assumption that infertility negatively correlates to an upregulation of the endometrial immune system, the endometrial mRNA expression of *IL1B*, *IL6*, *IL8* and *TNF* was higher in RBC with SCE compared to nonRBC (Kasimanickam et al., 2014) and endometrial *CXCL3* mRNA content was higher in RBC with healthy uteri compared with control heifers (Wagener et al., 2017b). However, this last study could not show any differences for the mRNA contents of *IL1A*, *IL1B*, *IL6*, *IL8* and *CXCL5* and interestingly, *PTGS2* mRNA was expressed even lower.

5.3. *L. buchneri* DSM 32407 in the bovine uterus

5.3.1. Influence on uterine health status and endometrial immune system

In the present study, the effect of the intrauterine administration of *L. buchneri* DSM 32407 on the uterine health status was monitored only for the cows that were diagnosed with SCE and in the luteal phase on days 24-30 pp (**Peter et al., 2018**). Cows outside the luteal phase with signs of estrus (swollen vulva, clear and cohesive vaginal discharge and/or a Graafian follicle) on days 24-30 pp were excluded to prevent that the solution with *L. buchneri* DSM 32407 would be flushed out by the mucus.

As the administration of bacteria to a living animal in untested amounts can be of potential risk for its health, *L. buchneri* DSM 32407 was selected on the basis of a preliminary study of Gärtner et al. (2015). The authors of this study isolated *L. buchneri* DSM 32407 from a healthy bovine uterus and showed that it had no negative influence on bovine endometrial cells *in vitro*.

Overall, it was shown that *L. buchneri* DSM 32407 also did not have a negative influence on the bovine uterine health status *in vivo*. Out of 16 cows in each group on days 24-30 pp, most cows were classified into the H group one week after the infusion of their uteri with either *L. buchneri* DSM 32407 or the placebo. However, on days 31-37 pp there were 5 cows with SCE in the *Lactobacillus* group and only 1 cow with SCE in the placebo group. This goes along with the number of PMN, which tended to be higher in the *Lactobacillus* group at this time point (**Peter et al., 2018**), which suggests that an infusion of the endometrium with *L. buchneri* DSM 32407 might have a stimulatory effect on the influx of PMN to the endometrium.

In an early study of Kummer et al. (1997), it was also shown that the endometrium was infiltrated with immune cells after two viable lactobacilli strains (*Lactobacillus* sp. and *L. casei*) were administered to the uterus of cows *in vivo*. 8 cows were slaughtered either 5 to 6 or 10 to 12 days after the intrauterine administration of the *Lactobacillus* strains. In comparison to a control group, it was histologically shown that the endometrium had a cellular infiltration with immune cells such as mononuclear cells, polymorphonuclear cells, mast cells and macrophages. The strongest infiltration was observed for mononuclear cells with a predominant portion of lymphocytes. It was also shown that the selected lactobacilli were able to colonize the bovine endometrium for up to 12 days (Kummer et al., 1997).

The present study provided similar results after the histopathological examination of endometrial biopsy samples that were taken directly before (no infiltration with immune cells) and 7 days after the uterine infusion with either *L. buchneri* DSM 32407 or the placebo. No

infiltration with immune cells was observed after the intrauterine administration of the placebo in 3 out of 3 samples. In contrast, a granulocytic and/or lymphocytic infiltration was observed in 4 out of 5 samples 7 days after the infusion of the uterus with *L. buchneri* DSM 32407 (**Peter et al., 2018**).

In accordance with the results for the number of PMN, one week after the infusion with *L. buchneri* DSM 32407 or the placebo on days 24-30 pp, the mRNA expression of *TNF* was significantly higher in endometrial cells obtained from cows of the *Lactobacillus* group. Following the same pattern, *CXCL5* transcript amounts tended to be higher (**Peter et al., 2018**). With the experimental setup of the present *in vivo* study it remains unclear if *L. buchneri* DSM 32407 itself, or specific factors secreted by it, induce the production of TNF. However, an indication that lactobacilli can activate cytokines by the secretion of specific factors was given by an *in vitro* study, when lactobacilli culture supernatants induced the production of TNF in murine macrophages and porcine kidney cells (Halper et al., 2003).

Regarding the other investigated pro-inflammatory factors, there was no such effect on the mRNA content one week after the intrauterine infusion with *L. buchneri* DSM 32407 (**Peter et al., 2018**). It remains unclear if their expression initially increased, but then already decreased before the second sampling. Regarding a possible fast increase, the preliminary *in vitro* study showed that co-culturing of bovine endometrial cells with *L. buchneri* DSM 32407 had no effect on the endometrial mRNA expression of *IL1A*, *IL6*, *IL8* and *PTGS2* at 2, 4 and 6 h (Gärtner et al., 2015). It had also no effect on the endometrial release of IL8 and $PGF_{2\alpha}$ at 24 and 48 h after the co-culturing (Gärtner et al., 2015). Therefore, the speculation about a fast increase of the mRNA expression of *IL1A*, *IL6*, *IL8* and *PTGS2* after the intrauterine administration of *L. buchneri* DSM 32407 is not supported by Gärtner et al. (2015). In the same study, *L. ruminis* and *L. amylovorus* were able to induce a significant increase of such factors. This also indicates that different *Lactobacillus* species have different immunostimulatory properties.

Overall, this shows that lactobacilli and/or their products can have an immunostimulatory effect and that they can activate cell defense mechanisms of the bovine endometrium. Interestingly, an enrichment of *Lactobacillus* in endometrial samples of cows on day 30 pp was associated with the occurence of SCE and therefore an increased endometrial proportion of PMN (Wang et al., 2018). However, it remains unclear if the enrichment of naturally occurring *Lactobacillus* was the actual cause for the occurrence of SCE as also an enrichment with *Acinetobacter* was shown for SCE cows in the study of Wang et al. (2018).

Further regarding the clinical uterine health status, two cows of the placebo group but none of the *L. buchneri* group showed signs of CE for three following weeks after the intrauterine

infusion on days 24-30 pp to cows with SCE (**Peter et al., 2018**). These results are not significant, but might provide indication that the intrauterine administration of *L. buchneri* DSM 32407 on days 24-30 pp could be negatively correlated with the subsequent occurrence of (muco)purulent vaginal discharge. However, a larger cohort of cows would be necessary to confirm or disprove this assumption.

The only other study that investigated the influence of an intrauterine administration of specific LAB (*L. rhamnosus*, *P. acidilactici*, *L. reuteri*) did not show a positive effect on the subsequent clinical uterine health status (Genis et al., 2018). However, the present study and the study of Genis et al. (2018) are not comparable regarding the condition of the uteri of the cows involved. In the present study, *L. buchneri* was administered on days 24-30 pp during the luteal phase (**Peter et al., 2018**), when the cervix was closed and the main part of uterine involution was completed. Therefore, a good retention and distribution of *L. buchneri* in the uterine lumen can be assumed. In the study of Genis et al. (2018), the mixture of LAB was administered on day 1 pp, when the involution of the uterus just started and the cervix was still open to allow lochia to discharge from the uterus. In this case, a rather bad retention and distribution of the administered combination of LAB can be assumed.

However, the same study showed that 2 intravaginal doses of the same mixture of LAB per week during the 3 weeks before calving reduced the prevalence of subsequent metritis (Genis et al., 2018). Similar, a reduction of purulent vaginal discharge at 3 weeks pp was shown when a mixture of LAB (*L. sakei* and *P. acidilactici*) was administered to the vagina once per week during -2, -1, +1, +2, +3, +4 weeks relative to the (expected) day of calving (Ametaj et al., 2014). The incidence of metritis was also lower when LAB (*L. sakei* and *P. acidilactici*) were administered intravaginally on week -2, -1 and +1 relative to the (expected) day of calving (Deng et al., 2015a). Interestingly, the same study showed that intravaginal LAB increased the concentration of secretory Immunoglobulin A in the vaginal mucus, which serves as the first line of defense against pathogenic bacteria (Mestecky and McGhee, 1987; Brandtzaeg, 2007). It can be suggested that this prevention of uterine infections was also the reason for a faster uterine involution and earlier resumption of ovarian cyclicity and contributed to the overall well-being of the cows resulting in a better feed intake and higher milk production during the transition period (Deng et al., 2015b; Deng et al., 2016). However, these results can not be compared to the present study regarding the time pp (periparturient vs. days 24-30 pp) and location (intravaginal vs. intrauterine) of the administration of the selected LAB (**Peter et al., 2018**).

The comparability of the mentioned studies with the present study is especially questioned regarding the results of another *in vivo* study. The results of this study demonstrate that a

mixture of selected LAB (*L. rhamnosus*, *P. acidilactici*, *L. reuteri*) administered to cows intravaginal are unable to neither reach the uterus nor regulate the endometrial mRNA expression of *CXCL8*, *IL1B*, *IL6* and *TNF* (Genis et al., 2017a), whereas an influence of *L. buchneri* on the endometrial immune system was clearly shown in the present study (**Peter et al., 2018**).This leaves to assume that the effect of selected and intravaginally administered LAB on uterine health must be caused by the containment of only vaginal pathogenic bacteria around the time of calving preventing them from colonizing the uterus, but that this way of administration is unlikely to have a sufficient effect on an already existing uterine colonization with pathogens.

In this context, it has also be taken into account that different strains of *Lactobacillus* have different abilities to adhere to the mucus of different parts of the reproductive tract, which is likely to also influence the ability to colonize different parts of the bovine reproductive tract. For example, *L. buchneri* 5/K (isolated from a bovine vagina) was strongly adherent and *L. buchneri* 24S8 (also isolated from a bovine vagina) was non-adherent to mucus of the bovine uterine horn, but both were strongly adherent to mucus of the bovine cervix (Stykova et al., 2013). This leaves open if *L. rhamnosus* CECT 278, *P. acidilactici* CECT 5915 and *L. reuteri* DSM 20016, which were isolated from humans and used in the study of Genis et al. (2017a), were maybe just not able to colonize the bovine uterus after an intravaginal administration, but that other strains maybe would have been. Although there is no research regarding this topic, it can be assumed that *L. buchneri* DSM 32407 is able to adhere to mucus from the bovine uterus as it was isolated exactly from there (Gärtner et al., 2015).

Regarding the results for the mRNA contents of pro-inflammatory factors, the present study impressively showed an advantageous long-term influence of an intrauterine infusion with *L. buchneri* DSM 32407 to cows with SCE on days 24-30 pp (**Peter et al., 2018**). On days 45-51 pp, three weeks after the administration of *L. buchneri* or the placebo, the endometrial mRNA expression of *CXCL1/2*, *CXCL3*, *CXCR2*, *IL1B*, *IL8* and *PTPRC* was significantly and the one of *IL1RN* in tendency lower in cows that received *L. buchneri* DSM 32407. These results are of particular significance if correlated to the results of the first publication, which showed that especially at this time point of the puerperium a downregulated endometrial immune system can be related to a healthy uterus (**Peter et al., 2015**).

It was also shown that the intrauterine administration of *L. buchneri* DSM 32407 has no influence on the mRNA expression of *PTGES* and *PTGES3* (**Peter et al., 2018**). In terms of the possible use of *L. buchneri* as an alternative strategy for the prevention or therapy of endometrial inflammation, this can be rated positive considering that PTGES and PTGES3

are capable to disturb a regular estrous cycle in case of an immoderate synthesis of PGE_2 (Magness et al., 1981; Reynolds et al., 1983).

5.3.2. Influence on reproductive performance

The administration of *L. buchneri* DSM 32407 to the uterus of cows without clinical signs of endometritis (H or SCE) on days 24-30 pp had a significantly positive influence on the mean interval from calving to conception (**Peter et al., 2018**). The shorter interval in the group of cows whose uterus was infused with *L. buchneri* DSM 32407 can be especially associated with a higher first service conception rate. With 60 % for the *Lactobacillus* group and only 13.3 % for the placebo group, this difference is exceptionally distinct in the group of SCE cows. Interestingly, these two groups had a very comparable mean interval from calving to first service with 63 days and 65 days, respectively (**Peter et al., 2018**). As the first AI was therefore relatively close to the sampling of the endometrium on days 45-51 pp, it supports the assumption that a down-regulated endometrial immune system at the time of AI (influenced by the intrauterine administration of *L. buchneri* DSM 32407) is highly beneficial for the implantation and survival of an embryo.

A study of Machado et al. (2012b) supports the assumption that lactobacilli can provide for a uterine environment that is beneficial for bovine reproductive performance. When uterine lavage samples were collected from cows at 35 DIM, the presence of lactobacilli in the bovine endometrium was rated positive for fertility. From the group of cows pregnant by 200 DIM, the percentage of cows tested positive for *Lactobacillus* spp. was higher in comparison to the group of cows that was unable to establish a pregnancy (Machado et al., 2012b). Interestingly and as mentioned before, the uterine microbiota of cows with SCE showed an enrichment of *Acinetobacter* and *Lactobacillus* when endometrial samples were examined on day 30 pp (Wang et al., 2018). However, from the setup of the study of Wang et al. (2018), it remains unclear if cows with a naturally occurring enrichment of *Lactobacillus* had a better subsequent reproductive performance even though they had SCE as it was shown for the intrauterine administration of *L. buchneri* on days 24-30 pp to cows with SCE (**Peter et al., 2018**).

There is no other study that investigated the intrauterine administration of lactobacilli during the second half of the puerperium on the reproductive performance of dairy cows. Only two other studies correlated an administration of lactobacilli to the bovine genital tract to the subsequent reproductive performance. In contrast to the present study (**Peter et al., 2018**), the cows of these studies were treated with LAB intravaginal instead of intrauterine (Ametaj et al., 2014; Deng et al., 2015b). Even though the intravaginal treatment with LAB improved the subsequent uterine health, which is known to have a strong influence on bovine fertility,

the results are inconsistent. The infusion of the vagina with LAB once per week on week -2, -1, +1, +2, +3 and +4 relative to the (expected) day of parturition did not improve the overall pregnancy rate (Ametaj et al., 2014), whereas the results of the second study showed that an intravaginal treatment done twice with LAB on week -2 and -1 resulted in fewer days open (Deng et al., 2015b). However, in the same study the treatment done three times on week -2, -1 and +1 did also not improve the subsequent reproductive performance.

In summary, this demonstrates that an intrauterine treatment of cows with *L. buchneri* DSM 32407 seems expedient for the subsequent fertility when conducted on days 24-30 pp, especially for cows with SCE, but that the efficiency of an intravaginal treatment with a mixture of specific LAB around the time of calving seems questionable. But as the data of a dairy herd's fertility depend on numerous factors, follow up studies with even higher number of cows would be necessary to confirm these preliminary results.

Furthermore, the effect of an intrauterine *L. buchneri* DSM 32407 treatment in cows with CE on the endometrial immune system and reproductive performance would be of special interest for prospective recommendations in dairy management.

5.4. Endometrial microbiology

When cows (H, SCE and CE) were sampled on days 24-30 pp and 45-51 pp, there was a significantly higher number of endometrial samples positive of *T. pyogenes* from cows with CE, but only at the early time point (**Peter et al., 2015**). This is comparable to the results of Machado et al. (2012a) showing that a uterine infection with *T. pyogenes* at 35±3 DIM is positively correlated to the occurrence of CE.

No other cultivable bacteria could be connected to the purulent vaginal discharge on days 45-51 pp either. This suggests that the cultivation of endometrial bacteria from cows with CE at the end of the puerperium does not support the clinical diagnosis.

Anyway, potentially pathogenic bacteria such as *E. coli* or *T. pyogenes* were also found in endometrial samples from healthy cows in both studies (**Peter et al., 2015**; **Peter et al., 2018**). These results are consistent with other studies that suggest a different pathogenicity of the isolates (Bicalho et al., 2010; Sheldon et al., 2010; Ibrahim et al., 2017).

A significant difference in cultivable bacteria after the administration of *L. buchneri* DSM 32407 was only observed for *Histophilus* (*H.*) *somni*. A significantly higher number of endometrial samples positive for *H. somni* was found on days 31-37 pp in the SCE group of cows that received the placebo in comparison to the group that received *L. buchneri* on days 24-30 pp (**Peter et al., 2018**). This could be an indication that *L. buchneri* DSM 32407 is able

to influence the occurrence of *H. somni* in the bovine uterus *in vivo*. *H. somni* is able to cause inflammatory diseases in the bovine genital tract as well as it was found to be part of the genital microbiota of healthy cows (Kwiecien and Little, 1991).

Although the present *in vivo* study could not show a significant influence of *L. buchneri* DSM 32407 on the occurrence of cultivable *E. coli*, the potential of lactobacilli to influence *E. coli* is supported by the results of three recent *in vitro* studies. After the infection of bovine endometrial epithelial cells with FimH positive and hemolytic *E. coli* (isolated from a cow with metritis) for 6 h, it was shown that the concurrent presence of specific amounts of *L. sakei*, *L. reuteri* or *P. acidilactici* can lower the number of colony forming units per milliliter of *E. coli* (Genis et al., 2016). In a second study, combinations of different LAB (*P. acidilactici*, *L. rhamnosus*, *L. reuteri* and *L. sakei*) was tested with the same cell culture model and it could be shown that the best combination to treat an *E. coli* infection composed of *L. rhamnosus*, *P. acidilactici* and *L. reuteri* in a ratio of 25:25:2 (Genis et al., 2017b). Furthermore, the pretreatment with *L. rhamnosus* contained *E. coli* induced cell damage in a different bovine endometrial epithelial cell culture model (Liu et al., 2016). In all three *in vitro* studies was also shown that the increase of endometrial mRNA expression or secretion of cytokines caused by the presence of *E. coli* can be downregulated by the presence of the selected LAB, which is likely due to the containment of *E. coli* (Genis et al., 2016; Liu et al., 2016; Genis et al., 2017b).

Furthermore, when cows were treated intravaginal with either a combination of LAB (*L. rhamnosus*, *P. acidilactici* and *L. reuteri*) or a sterile carrier twice per week during 3 weeks and culled afterwards, the counts for *E. coli* tended to be lower in vaginal swabs of cows treated with the LAB mixture (Genis et al., 2017a).

However, culture-dependent methods for microbiological analysis are unable to draw a complete picture of the bacterial diversity in endometrial samples. Therefore, a culture-independent method such as pyrosequencing should be seen as a more suitable method for further studies about the bovine genital microbiota, especially to evaluate the influence of an intrauterine administration of *L. buchneri* DSM 32407.

5.5. The potential role of *L. buchneri* DSM 32407 as alternative strategy for the therapy of subclinical endometritis

SCE impairs reproductive performance and represents a condition in need for alternative treatment options. The evaluation of *L. buchneri* DSM 32407 as alternative strategy for the therapy of SCE is especially justified by the disadvantages of the currently available main strategies, antibiotics and $PGF_{2\alpha}$.

Regardless its efficiency, it has long been known that the use of antibiotics always increases the potential development of bacterial resistances (Cristino, 1999; Oliver and Murinda, 2012) and therefore should be kept to an absolute minimum (Earnshaw et al., 2013). Bacterial resistances towards antibiotics can lead to therapy failure with a direct negative effect on animal health and welfare (Bengtsson and Greko, 2014). Already, pathogens causing endometritis such as *T. pyogenes* and *E. coli* are not always fully susceptible to common antibiotics (Brodzki et al., 2014) and the overall decreasing effectiveness of antibiotics due to increasing numbers of resistances is a worldwide problem in human and veterinary medicine. The potential spread of bacterial resistances of antibiotics between animals and people (Laxminarayan et al., 2013) leaves consumers increasingly concerned about the use of antibiotics in veterinary medicine (McEvoy, 2016). This is also a reason why, in Germany, the regulations for veterinary pharmacies were changed in February 2018 and the use of antibiotics is now more strongly regulated (TÄHAV). For example, ceftiofur (3rd generation cephalosporin) can only be used after providing an antibiogram first. This additional amount of work for the veterinarian and the cost for the laboratory work results in additional costs for the farmer, which would be omitted with the use of *L. buchneri* DSM 32407.

It should also be kept in mind that the effect of antibiotics, besides the desired killing of specific pathogens, can also be the killing of important commensal bacteria, which might lead to the overgrowth of e.g. pathogenic fungi. This demonstrates that the use of antibiotics can also be disadvantageous for the health of especially the skin and the oral, respiratory, gastrointestinal and vaginal tract (Ferrer et al., 2017).

Another problem of the treatment of farm animals with antibiotics or hormones are the possible residues in animal products such as milk and meat. This is why the Department for Health and Food Safety of the European Commission has established procedures for the setting of Maximum Residue Limits (MRLs) of veterinary drugs in edible animal tissue (Regulation (EC) No 470/2009) and a list of MRLs for permitted substances (Regulation (EU) No 37/2010). After the treatment of a cow with certain substances such as antibiotics, its milk has to be discarded and/or its meat is not usable for food production for a certain amount of time. This means an economic loss for the farmer in case of the use of antibiotics. However, the responsible German department (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit) decided that there is no restriction on the use of milk and meat of a cow after the intrauterine administration of *L. buchneri* DSM 32407. Although this decision was only temporary for the procedures of the present study, it may be expected that the Department for Health and Food Safety of the European Commission would decides likewise, which would be a great benefit of *L. buchneri* DSM 32407 over the use of antibiotics and hormones.

The results of the present study indicate that the intrauterine administration of *L. buchneri* DSM 32407 on days 24-30 pp to the female bovine genital tract of cows with SCE has a positive influence on the endometrial pro-inflammatory mRNA expression on days 45-51 pp (**Peter et al., 2018**). Therefore, the present study supports the hypothesis that an intrauterine administration of *L. buchneri* DSM 32407 is a potential alternative strategy for the therapy of SCE concerning the long-term effects. However, it should be expected that *L. buchneri* DSM 32407 first activates the endometrial immune system and therefore higher number of cows with SCE will be found one week after the treatment (**Peter et al., 2018**).

If *L. buchneri* DSM 32407 could also be a potential alternative strategy for the prevention of SCE can not be answered with the results of the present study. Further research with a higher number of cows is necessary to attend to this topic.

6. Conclusion

The present study suggests that the correlation of the endometrial mRNA expression of selected factors of the innate immune system to SCE and CE depends on the time point pp, with a stronger correlation towards the end of the puerperium. Conclusions about long-term effects of the initial uterine health status on days 24-30 pp on the subsequent mRNA expression of selected chemokines and synthases of prostaglandins should be drawn carefully as it was shown that not all cows convalesced equally. This likely had a short-term influence that could not be considered in this statistical approach.

Concerning the intrauterine administration of *L. buchneri* DSM 32407 to cows with SCE, the present study suggests a stimulating effect on the endometrial immune system one week later and a downregulating long-term effect on the endometrial mRNA expression pattern of selected pro-inflammatory candidate three weeks later. Furthermore, it was shown that the intrauterine administration of *L. buchneri* DSM 32407 to clinically healthy cows improves the subsequent reproductive performance, especially of cows suffering from SCE.

7. Summary

In the puerperium, inflammatory diseases of the bovine uterus are observed with a high prevalence in dairy cows. These diseases impair animal welfare, the reproductive performance of the herd and, as a result, the economic situation of dairy farms.

To evaluate bovine uterine inflammatory diseases on a molecular basis, the endometrial mRNA expression of specific factors can be used. Representatives for inflammation include e.g. different types of immune cells (e.g. polymorphonuclear neutrophils [PMN]), mediators (e.g. interleukins [ILs], tumor necrosis factor [TNF], chemokine ligands [CXCLs]), enzymes (e.g. prostaglandin [PG] synthases, matrix metallopeptidase 1 [MMP1]) and receptors (e.g. protein tyrosine phosphatase, receptor type C [PTPRC], platelet-activating factor receptor [PTAFR]).

For the therapy of bovine uterine inflammatory diseases, mainly antibiotics and the hormone $PGF_{2\alpha}$ are applied. However, there are disadvantages to both strategies, such as possible residues in animal products and the potential development of bacterial resistances or a questionable efficiency, respectively. This explains the ongoing search for alternatives, which includes research about *Lactobacillus* (*L.*) spp. as potential probiotics for the bovine reproductive tract.

The aim of the first study was the evaluation of the mRNA expression profiles of selected pro-inflammatory factors in the endometrium of dairy cows depending on their uterine health status (healthy [H], subclinical endometritis [SCE] and clinical endometritis [CE]) during the second half of the puerperium (days 24-51 postpartum [pp]).

The second study analyzed the uterine health, the endometrial PMN contents and the endometrial mRNA expression profiles of selected pro-inflammatory factors after the intrauterine administration of *L. buchneri* DSM 32407 on days 24-30 pp to cows with SCE. In addition, the impact of *L. buchneri* DSM 32407 on the histopathology of endometrial biopsy samples of clinically healthy cows (taken at various days pp) was evaluated. Furthermore, the influence of the intrauterine administration of *L. buchneri* DSM 32407 on days 24-30 pp to clinically healthy cows (H and SCE) on reproductive performance was investigated.

For both studies, cows were examined and sampled on a weekly basis during days 24-51 pp. Additionally, endometrial samples for the cultivation of bacteria were obtained in both studies.

The mRNA expression analysis of endometrial pro-inflammatory factors revealed the strongest correlation to the uterine health status on days 45-51 pp, the end of the puerperal period. In contrast to the earlier investigated time points, almost all of the selected factors

were significantly higher expressed in endometrial samples from cows with SCE and/or CE in comparison to healthy cows. This regards the mRNA expression of *CXCL1/2*, *CXCL3*, *CXCL5*, chemokine receptor 2 (*CXCR2*), *IL1A*, *IL1B*, *IL6*, *IL8*, prostaglandin-endoperoxide synthase 1 (*PTGS1*), *PTGS2*, prostaglandin D2 synthase (*PTGDS*), *TNF*, *MMP1* and *PTAFR*.

The intrauterine administration of *L. buchneri* DSM 32407 on days 24-30 pp to cows with SCE resulted in a significantly higher mRNA expression of *TNF* on days 31-37 pp. Furthermore, the mRNA expression of *CXCL1/2*, *CXCL3*, *CXCR2*, *IL1B*, *IL8* and *PTPRC* was significantly lower on days 45-51 pp in comparison to cows treated with a placebo. The histopathological evaluation showed an infiltration of immune cells into the endometrium one week after the intrauterine administration of *L. buchneri* DSM 32407. Clinically healthy cows treated with *L. buchneri* DSM 32407 had significantly shorter median days to conception compared to cows treated with a placebo (103 vs. 133 median days to conception; $P = 0.035$). This difference was even more significant when only cows with SCE were observed (74 vs. 164 median days to conception; $P = 0.001$).

In the first study, there was a significantly higher number of endometrial samples positive for cultivable *Trueperella* (*T.*) *pyogenes* on days 24-30 pp from cows with CE in comparison to cows from the H and SCE group. The second study showed a significantly higher number of endometrial samples positive for *Histophilus* (*H.*) *somni* on days 31-37 pp in the SCE group that received a placebo in comparison to the whole group (H and SCE) that received *L. buchneri* DSM 32407.

In conclusion, the first study revealed the strongest correlation of endometrial pro-inflammatory mRNA contents to the uterine health status (H, SCE and CE) on days 45-51 pp. This suggests that the mRNA expression analysis could be a powerful tool for the judgement of the severity of endometrial inflammation especially at this time point of the puerperium.

The second study showed that an intrauterine administration of *L. buchneri* DSM 32407 first had an immunostimulatory effect with a subsequent downregulation of the endometrial immune system on days 45-51 pp. Moreover, the intrauterine administration of *L. buchneri* DSM 32407 was highly beneficial for the following reproductive performance of clinically healthy cows (H and SCE). Overall, this indicates that an intrauterine administration of *L. buchneri* DSM 32407 on days 24-30 pp could contribute to a uterine environment that is associated with health on days 45-51 pp and an improved subsequent reproductive performance.

8. Zusammenfassung

Molekulare Einblicke in die bovine Endometritis und die intrauterine Verabreichung von *Lactobacillus buchneri* als potenzielle Behandlung der subklinischen Endometritis

Im Puerperium werden entzündliche Erkrankungen des bovinen Uterus mit einer hohen Prävalenz beobachtet. Diese Erkrankungen beeinträchtigen das Tierwohl, die Reproduktionsleistung der Herde und in der Folge die ökonomische Situation von Milchviehbetrieben.

Für die Untersuchung entzündlicher Erkrankungen des bovinen Uterus auf molekularer Ebene kann die endometriale mRNA-Expression ausgewählter Faktoren genutzt werden. Marker für eine Entzündung sind beispielsweise verschiedene Immunzelltypen (z.B. polymorphkernige neutrophile Granulozyten [PMN]), Entzündungsmediatoren (z.B. Interleukine [ILs], Tumornekrosefaktor [TNF]), Chemokin-Liganden [CXCLs]), Enzyme (z.B. Prostagladin [PG] Synthasen, Matrix-Metallopeptidase 1 [MMP1]) und Rezeptoren (z.B. Protein-Tyrosin-Phosphatase Rezeptor Typ C [PTPRC], Plättchenaktivierender Faktor-Rezeptor [PTAFR]).

Für die Therapie entzündlicher Erkrankungen des bovinen Uterus werden hauptsächlich Antibiotika und das Hormon $PGF_{2\alpha}$ genutzt. Jedoch gibt es Nachteile für beide Strategien, wie beispielsweise mögliche Rückstände in tierischen Produkten und die potenzielle Entwicklung bakterieller Resistenzen bzw. eine fragliche Wirksamkeit. Dies erklärt die anhaltende Suche nach Alternativen, die auch die Forschung über *Lactobacillus* (*L.*) spp. als potenzielles Probiotikum für den bovinen Reproduktionstrakt einschließt.

Das Ziel der ersten Studie war die Evaluierung der mRNA-Expressionprofile von ausgewählten pro-inflammatorischen Faktoren im Endometrium von Milchkühen in Abhängigkeit vom uterinen Gesundheitsstatus (gesund [H], subklinische Endometritis [SCE] und klinische Endometritis [CE]) während der zweiten Hälfte des Puerperiums (Tage 24-51 postpartum [pp]).

Die zweite Studie analysierte den uterinen Gesundheitsstatus, die endometrialen PMN-Gehalte und die endometrialen mRNA-Expressionsprofile von ausgesuchten pro-inflammatorischen Faktoren nach der intrauterinen Verabreichung von *L. buchneri* DSM 32407 an den Tagen 24-30 pp an Kühe mit SCE. Zusätzlich wurden die Auswirkungen von *L. buchneri* DSM 32407 auf die Histopathologie endometrialer Biopsieproben von klinisch gesunden Kühen (gewonnen an unterschiedlichen Tagen pp) untersucht. Darüber hinaus

wurde der Einfluss der intrauterinen Verabreichung von *L. buchneri* DSM 32407 an klinisch gesunde Kühe (H und SCE) an den Tagen 24-30 pp auf die Reproduktionsleistung geprüft.

Für beide Studien wurden die Kühe während der Tage 24-51 pp wöchentlich untersucht und beprobt. Zusätzlich wurden in beiden Studien Proben vom Endometrium für die Kultivierung von Bakterien gewonnen.

Die mRNA-Expressionsanalyse von endometrialen pro-inflammatorischen Faktoren zeigte die stärkste Korrelation zum uterinen Gesundheitsstatus an den Tagen 45-51 pp, dem Ende des Puerperiums. Im Gegensatz zu den früheren Zeitpunkten waren dort in den endometrialen Proben von Kühen mit SCE und/oder CE, im Vergleich zu gesunden Tieren, fast alle ausgewählten Faktoren signifikant höher exprimiert. Dies betrifft die mRNA-Expression von *CXCL1/2*, *CXCL3*, *CXCL5*, Chemokine Rezeptor 2 (*CXCR2*), *IL1A*, *IL1B*, *IL6*, *IL8*, Prostaglandin-Endoperoxidsynthase 1 (*PTGS1*), *PTGS2*, Prostaglandin D2-Synthase (*PTGDS*), *TNF*, *MMP1* und *PTAFR*.

Die intrauterine Verabreichung von *L. buchneri* DSM 32407 an den Tagen 24-30 pp an Kühe mit SCE resultierte in einer signifikant höheren mRNA-Expression von *TNF* an den Tagen 31-37 pp. Darüber hinaus war an den Tagen 45-51 pp die mRNA-Expression von *CXCL1/2*, *CXCL3*, *CXCR2*, *IL1B*, *IL8* und *PTPRC* signifikant niedriger im Vergleich zu Kühen, die mit einem Placebo behandelt wurden. Die histopathologische Untersuchung zeigte eine Infiltration des Endometriums mit Immunzellen eine Woche nach der intrauterinen Verarbreichung von *L. buchneri* DSM 32407. Klinisch gesunde Kühe, die mit *L. buchneri* DSM 32407 behandelt wurden, hatten signifikant kürzere mittlere Güstzeiten im Vergleich zu Kühen, die mit einem Placebo behandelt wurden (103 vs. 133 mittlere Güsttage; $P = 0.035$). Dieser Unterschied war noch signifikanter, wenn nur Kühe mit SCE betrachtet wurden (74 vs. 164 mittlere Güsttage; $P = 0.001$).

In der ersten Studie waren die endometrialen Proben von Kühen mit CE, im Vergleich zu denen von Kühen von der SCE- und H-Gruppe, an den Tagen 24-30 pp signifikant häufiger positiv für kultivierbare *Trueperella* (*T.*) *pyogenes*. Die zweite Studie zeigte an den Tagen 31-37 pp eine signifikant höhere Anzahl von Proben des Endometriums positiv für *Histophilus* (*H.*) *somni* bei der SCE-Gruppe, die ein Placebo erhielt, im Vergleich zu der gesamten Gruppe (H und SCE), die *L. buchneri* DSM 32407 erhielt.

Schlussfolgernd zeigte die erste Studie die stärkste Korrelation von endometrialen pro-inflammatorischen mRNA-Gehalten zum uterinen Gesundheitsstatus (H, SCE und CE) an den Tagen 45-51 pp. Dies weist darauf hin, dass die mRNA-Expressionsanalyse besonders zu diesem Zeitpunkt des Puerperiums eine aussagekräftige Technik zur Bewertung der Stärke endometrialer Entzündung darstellen könnte.

Die zweite Studie zeigte, dass die intrauterine Verabreichung von *L. buchneri* DSM 32407 zunächst einen immunstimulierenden Effekt hatte, mit einer anschließenden Herunterregulierung des endometrialen Immunsystems an den Tagen 45-51 pp. Überdies war die intrauterine Verabreichung von *L. buchneri* DSM 32407 vorteilhaft für die nachfolgende Reproduktionsleistung von klinisch gesunden Kühen (H und SCE). Zusammenfassend gibt dies Hinweise darauf, dass eine intrauterine Verabreichung von *L. buchneri* DSM 32407 an den Tagen 24-30 pp ein uterines Milieu unterstützt, welches mit der Gesundheit des Uterus an den Tagen 45-51 pp assoziiert wird und eine nachfolgende Verbesserung der Reproduktionsleistung herbeiführt.

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10. Previous publications of the presented data

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- **Peter S**, Gärtner MA, Michel G, Ibrahim M, Klopfleisch R, Lübke-Becker A, Jung M, Einspanier R, Gabler C (2018):
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  Detection of lactic acid bacteria in the bovine uterus and vagina by real-time PCR approaches.
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  Gdańsk, Poland – 27.02.-01.03.2013.
  In: Reproductive Biology; **13** (Suppl. 2), S. 53–54

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14. Selbstständigkeitserklärung

Hiermit bestätige ich, Sarah Peter, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 24.06.2019 Sarah Peter

Detaillierte Einschätzung der Beteiligung von Sarah Peter an den Publikationen, die im Rahmen dieser kumulativen Dissertation präsentiert wurden:

Beteiligung	1. Publikation (Bovine uterine health status and endometrial inflammation)	2. Publikation (Intrauterine influence of *Lactobacillus buchneri*)
Studiendesign	30%	30%
Datensammlung	85%	85%
Datenanalyse	80%	80%
Schreiben des Manuskriptes	85%	85%
Editieren des Manuskriptes	55%	55%

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