REPRODUCTION
REVIEW

Genesis of clinical and subclinical endometritis in dairy cows

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Abstract

In brief: Clinical and subclinical endometritis are different manifestations of reproductive tract inflammatory disease in dairy cows. This review addresses the genesis of clinical and subclinical endometritis considering metabolic stress, innate immune dysfunction, and shifts in the composition of the uterine microbiota in the postpartum period.

Abstract: Up to half of dairy cows may develop one or more types of reproductive tract inflammatory disease within 5 weeks after calving. Clinical endometritis (CE) results from uterine bacterial dysbiosis with increased relative abundance of pathogenic bacteria associated with luminal epithelial damage. These bacteria cause endometrial stromal cell lysis, followed by massive polymorphonuclear neutrophil (PMN) migration, and pyogenesis. CE is defined as endometrial inflammation accompanied by purulent discharge. Purulent discharge is not always accompanied by uterine inflammation (being (rarely) vaginitis or (commonly) cervicitis), hence referred to as purulent vaginal discharge (PVD). Subclinical endometritis (SCE) is an asymptomatic uterine disease defined by a threshold of PMN on cytology that is associated with worse reproductive performance; it has not been linked with bacterial dysbiosis. Current evidence suggests that SCE is a result of metabolic and inflammatory dysfunction that impairs innate immune function and the ability of endometrial PMN to undergo apoptosis, necrosis, and ultimately achieve resolution of inflammation. CE and SCE are diagnosed between 3 and 5 weeks postpartum and commonly overlap, but they are considered distinct manifestations of reproductive tract inflammatory disease. This review addresses the genesis of CE and SCE in postpartum dairy cows considering metabolic stress, innate immune dysfunction, and shifts in the composition of the uterine microbiota.

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Background

Dairy cows are challenged by trauma of the birth canal, placental detachment, systemic inflammation and metabolic stress, immune suppression, and shifts in the uterine microbiota in the early postpartum period (Sheldon *et al.* 2008, Pascottini & LeBlanc 2020*a*). Inability to cope with one or more of these challenges may result in the development of different types of reproductive tract inflammatory disease, which affect up to 50% of postpartum dairy cows (LeBlanc 2014). Within 21 days postpartum (but more often between 3 and 10 days postpartum), dairy cows may develop metritis (Sheldon *et al.* 2019). Histologically, metritis encompasses inflammation of all uterine layers (endometrium, myometrium, and perimetrium) and is characterized clinically by fetid uterine discharge, which may be reddish-brown and watery or purulent and viscous, often with an enlarged, flaccid uterus (Sheldon *et al.* 2019). Healthy cows adapt to changes associated with the postpartum period and mount a robust immune and inflammatory response in their reproductive tract. The presence of purulent discharge in the vagina after 21 days postpartum, however, is associated with impaired reproductive performance. When this purulent discharge is diagnosed by vaginoscopy, a gloved hand, or the metricheck (McDougall *et al.* 2007, Pleticha *et al.* 2009) without performing endometrial cytology or biopsy, its origin is unknown (Dubuc *et al.* 2010*a*), and the condition is simply referred to as purulent vaginal discharge (PVD). This PVD may be caused by endometritis, cervicitis, or, rarely, vaginitis, or a combination of these (Dubuc *et al.* 2010*a*, Deguillaume *et al.* 2012).

The endometrium is the innermost lining of the uterus and is comprised of two layers. The *stratum*

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spongiosum (or *basalis*) is the deeper layer (rich in endometrial glands) and attaches to the underlying myometrium, and the *stratum compactum* (or *functionalis*) is the superficial layer that lines the uterine cavity. Histologically, endometritis refers to inflammation no deeper than the *stratum spongiosum* and involves damage of the luminal epithelium, vascular congestion, edema, and infiltration with inflammatory cells, notably macrophages and polymorphonuclear neutrophils (PMN) (Bondurant 1999). Endometritis is a physiological feature postpartum that is required for tissue repair and avoidance of dysbiosis. However, its persistence beyond 21 days postpartum is pathological. Endometritis may occur with or without purulent discharge. Clinical endometritis (CE) is PVD with endometrial inflammation diagnosed by cytology (typically > 5% PMN) (LeBlanc *et al*. 2002, Dubuc *et al*. 2010*a*). Subclinical endometritis (SCE) is not visible to the naked eye (by definition, there is no PVD), so SCE is diagnosed by endometrial cytology (typically > 5% PMN) (Dubuc *et al*. 2010*a*, Wagener *et al*. 2017).

Some pathophysiological events that drive reproductive tract inflammatory disease in dairy cattle have been described (Sheldon *et al*. 2009, Wagener *et al*. 2017, Pascottini & LeBlanc 2020*a*), but much remains to be unraveled about the nature and triggers of the different manifestations of disease. Until recently, the paradigm was that a healthy uterus is free of (pathogenic) bacteria, and that uterine disease is caused by infection with pathogenic bacteria within the uterine cavity. However, with the advent of culture-independent methods for bacteriology (16s rRNA gene and shotgun metagenomics sequencing), it was shown that potentially pathogenic bacteria are commonly present in the healthy uterus but kept in low relative abundance by 'beneficial bacteria' in combination with adequate immune system function (Jeon *et al*. 2015, Knudsen *et al*. 2016, Clemmons *et al*. 2017). Robust migration of PMN into the uterine lumen just after calving is required for uterine health (Prunner *et al*. 2014, Gilbert & Santos 2016, Pascottini *et al*. 2020*a*), although there is little information on the viability and function of these endometrial PMN. Other research has focused on the effects of mineral and energy status and the level of systemic inflammation on the immune status and health of the uterus (Dubuc *et al*. 2010*b*, Cheong *et al*. 2011, Martinez *et al*. 2012). Essentially, all high-yielding dairy cows experience a certain degree of hypocalcemia, negative energy balance, and systemic inflammation at the onset of lactation (Sordillo & Raphael 2013, Pascottini *et al*. 2022). However, some cows are more resilient, i.e., better able to adapt to these metabolic challenges and maintain uterine homeostasis (Sheldon *et al*. 2020). Thus, this review addresses immunologic, metabolic, and microbiologic drivers of different manifestations of reproductive tract inflammatory disease in the postpartum period and discusses the pathophysiology of CE or SCE in dairy cows.

Innate immune function in the uterus: quickly come, quickly go

Neutrophils are critical in the balance between physiological inflammation and repair (acute inflammation) and pathologic inflammation and tissue damage (chronic inflammation). There is recruitment of PMN into the uterus at parturition. Endometrial epithelial and stromal cells detect harmful alterations due to mechanical tissue injury (e.g. calving and placental detachment) or pathogen-induced damage (e.g., infection) (Sheldon *et al*. 2019). Pattern recognition receptors (PRR) recognize damage-associated molecular patterns (DAMP) and pathogen associated molecular patterns (PAMP). An important DAMP immunomodulatory signal in mammals are oxidized phospholipids, and the consequent cytokine cascade (notably interleukin (IL)-1α) is believed to trigger initial PMN taxis into the uterine cavity after calving (Healy *et al*. 2014, Zhivaki & Kagan 2022). We hypothesize that at an early stage of endometrial inflammation, PAMP may play no part since microbial dysbiosis (and infection) most commonly occurs between 3 and 10 days after parturition. The exact mechanisms of DAMP detection and signaling are uncertain (Zhivaki & Kagan 2022), but they result in the local production of pro-inflammatory cytokines that leak to the systemic circulation and act as PMN chemoattractants. Chemoattractants bind to surface receptors of circulating PMN allowing them to attach and roll on the vascular endothelium and migrate into the uterus (Pascottini & LeBlanc 2020*a*). Robust and rapid migration of PMN to the uterine cavity just after calving is associated with better uterine health (Gilbert & Santos 2016). As a result, within 1 week postpartum, PMN make up around 40% of nucleated cells present in the uterine lumen to control the (over)growth of potentially pathogenic bacteria and clear damaged and sloughed endometrium (Gilbert & Santos 2016). However, the quantity of uterine PMN should decrease after completing their task. Spent PMN undergo apoptosis and necrosis and abandon the uterine cavity via vaginal discharge. Apoptosis appears to be one signal for resolution of inflammation (Kolaczkowska & Kubes 2013). Persistent recruitment of PMN to the uterus by dysfunctional immune cells may induce endometrial cell damage and contribute to disease.

Common methods to study innate immune function in dairy cows are to measure various capabilities of PMN to kill bacteria *in vitro* such as phagocytosis, oxidative (or respiratory) burst, and, more recently, endocytosis and neutrophil extracellular trap formation (Pascottini *et al*. 2019, LeBlanc 2020, Xie *et al*. 2021). Adequate circulating PMN function is associated with

reduced uterine disease incidence (Cai *et al.* 1994, Kimura *et al.* 2002, Hammon *et al.* 2006). However, we found that the correlation between circulating and endometrial PMN function is weak (Lietaer *et al.* 2021*a*). Different genes and biological pathways are involved in the peripheral and local responses to endometrial inflammation (Raliou *et al.* 2019). The poor correlation of circulating and endometrial PMN transcripts and function suggests that the migration process of circulating PMN to the endometrium and the uterine environment itself, including cytokines and inflammatory mediators, might alter the viability or function of the activated PMN. Therefore, good function of PMN in circulation does not necessarily assure a strong innate immune response in the uterus. Moreover, in most of the studies in which the endometrial PMN proportions and function were measured, PMN viability (alive, apoptotic, or dead) was not assessed. Recent experiments in our laboratory (Lietaer *et al.* 2022, 2023*a*) revealed no major fluctuations in the percentage of viable endometrial PMN (around 30%) in healthy cows, but a decreasing percentage of apoptotic PMN and increasing proportion of necrotic PMN from 9 to 36 days postpartum. Thus, we speculate that PMN flux to the uterus that terminates with apoptosis reflects and contributes to the decreasing endometrial PMN% over time and resolution of uterine inflammation in healthy cows within 5 weeks after calving.

In a recent longitudinal study in multiparous dairy cows, we collected endometrial cytobrush samples at 9, 21, and 36 postpartum to measure the viability (viable, apoptotic, and necrotic) and phagocytosis capacity of endometrial PMN (Lietaer *et al.* 2023*a*) by flow cytometry. At 36 days postpartum, cows were categorized as CE, SCE, or healthy. In cows with CE, endometrial PMN count, the percentage of viable, and the percentage of phagocytic endometrial PMN were greater at 36 days postpartum than in healthy cows (Lietaer *et al.* 2023*a*). This confirms CE to be associated with ongoing influx of new, viable PMN, rather than being an accumulation of apoptotic and necrotic cells. Therefore, CE represents persistent, active inflammation. Interestingly, the characteristics of endometrial PMN in cows with SCE seemed to be intermediate between those of CE and healthy cows. The viability of the endometrial PMN in cows with SCE was similar to CE cows at 36 days postpartum, but the percentage of phagocytosis was lesser than in CE cows and similar to healthy cows. Endometrial PMN in cows with SCE were still viable but less functional, supporting the hypothesis that the pathogenesis of SCE differs from CE (Fig. 1).

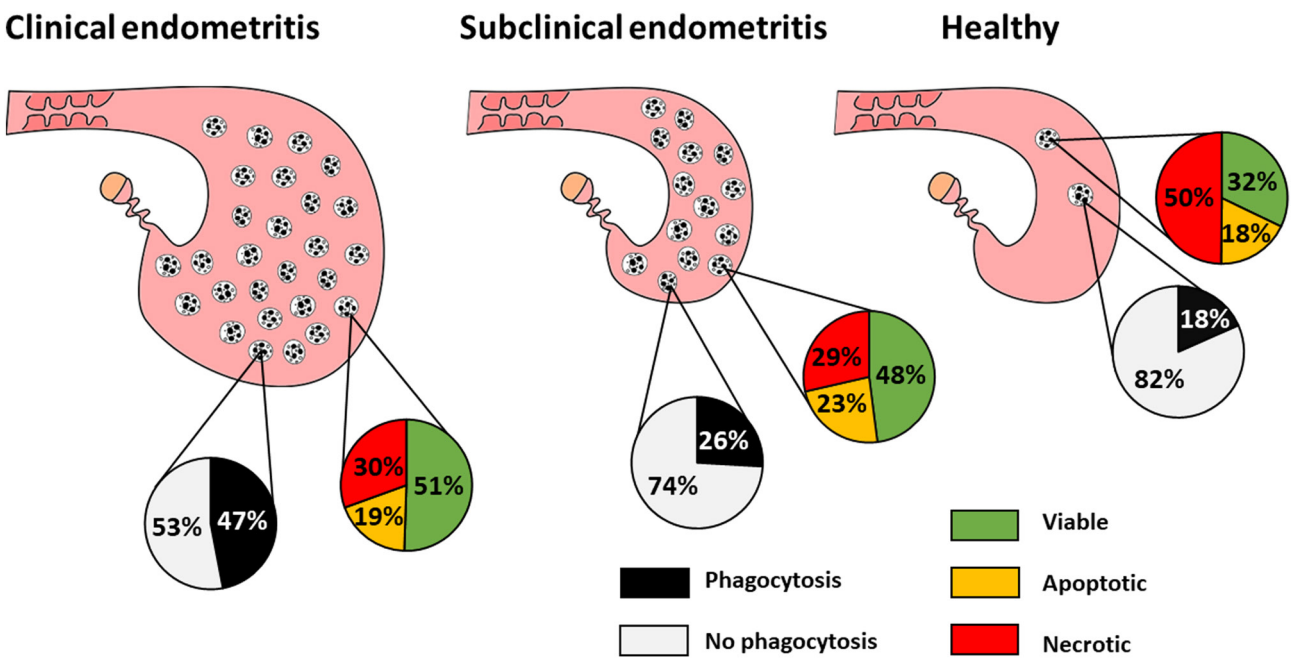

Figure 1 Differences in neutrophil viability and function in the uterus of postpartum dairy cows with differing uterine health status. Polymorphonuclear neutrophils in the uterus of healthy cows (n = 16) at 36 days postpartum had low viability and phagocytotic activity but in cows with clinical endometritis (n = 8) were highly viable and functional. Neutrophils from cows with subclinical endometritis (n = 8) represent an intermediate condition with high viability and moderate phagocytic activity. Neutrophil viability and phagocytosis were evaluated via flow cytometry. Pie charts are based on data from Lietaer *et al.* 2023*a*.

Uterine health and metabolic stress: the spark that lights the fire

Uterine inflammation is a necessary component of uterine involution (Pascottini & LeBlanc 2020*a*). However, adaptation to systemic metabolic stressors may be at least as important for the development of uterine disease as the local host interactions with bacteria and the concomitant inflammatory response in the reproductive tract. Prolonged or excessive hypocalcemia, reduced insulin sensitivity in peripheral tissue together with hypoglycemia, and upregulated lipolysis and sterile inflammation are all common phenomena that alone or synergistically might impair uterine involution (Pascottini *et al.* 2022).

Calcium availability in the 3 days after calving seems to be important for uterine health (Kimura *et al.* 2006, Martinez *et al.* 2012). Adequate amounts of ionized Ca are necessary to sustain immune function and myometrial contractions during and after calving to allow placental detachment and expulsion (Kimura *et al.* 2002). Dystocia and failure to expel the placenta within 24 h after calving are both risk factors for uterine disease (Kimura *et al.* 2002, Vieira-Neto *et al.* 2016). Lochia and necrotic placental tissue in the uterine lumen support growth of potentially pathogenic bacteria, especially anaerobes (Dohmen *et al.* 2000). Moreover, PMN rely on Ca for migration and for bactericidal activities like phagocytosis and oxidative killing (Martinez *et al.* 2014, Immler *et al.* 2018). Clinical hypocalcemia has been associated with an increased incidence and severity of PVD (Whiteford & Sheldon 2005). Subclinical hypocalcemia (<2.15 mmol/L serum total Ca within 72 h after calving) was a risk factor for metritis (Martinez *et al.* 2012) and cows with serum total Ca <1.75 mmol/L in the first week postpartum were at greater risk to be diagnosed with PVD in the fifth week postpartum (Ruiz-Garcia *et al.* 2022). We found lower Ca serum concentrations in the week before calving in cows that later had SCE, compared to healthy herd mates (Pascottini & LeBlanc 2020*b*). Larger studies with multiple measurements of blood Ca in the first week after calving are warranted to investigate the associations between subclinical hypocalcemia and SCE.

Glucose and glutamine are important energy substrates for endometrial cell metabolism (reviewed by Sheldon *et al.* 2018). In postpartum dairy cows, however, glucose demands are locally (in the uterus) and systemically exacerbated due to the substantial physiologic inflammation and lactogenesis, respectively. There is low systemic availability of glucose as homeorhetic changes make glucose primarily available to the mammary gland for milk production. Thus, intracellular glycogen stores are important for PMN function in postpartum dairy cows. Galvão *et al.* (2010) found lesser intracellular glycogen stores in circulating PMN from the first to the third week after calving in cows with SCE than in healthy cows, which could partly explain PMN dysfunction. Moreover, circulating PMN in transition cows are often exposed to high concentrations of lipid- and inflammation-derived metabolites that might influence in their function before they reach the uterine cavity (LeBlanc 2020).

Excessive negative energy balance and systemic inflammation in the early postpartum period are risk factors for CE or PVD, and SCE (Dubuc *et al.* 2010*b*, Cheong *et al.* 2011, Pascottini & LeBlanc 2020*b*). Dubuc *et al.* (2010*b*) found serum haptoglobin ≥ 0.8 g/L in the first week following parturition to be a risk factor for PVD and SCE. The same study demonstrated that cows were at greater risk for SCE but not PVD if serum β-hydroxybutyrate (BHB) was ≥ 1.1 mmol/L in the first week after calving. Similarly, other authors found associations of elevated non-esterified fatty acids (NEFA) and BHB in the postpartum period with SCE (Hammon *et al.* 2006, Galvão *et al.* 2010, Cheong *et al.* 2011). The link between these thresholds of metabolic markers and uterine disease are well characterized in observational studies, but their mechanistic pathways are complex and poorly understood. NEFAs may act as ligands for toll-like receptors (TLR; important mediators of inflammatory pathways (Hotamisligil & Erbay 2008)) and may affect PMN function in different directions. High NEFA concentrations can impair oxidative burst (Ster *et al.* 2012), limiting the capacity of PMN to kill bacteria. Conversely, high NEFA were also shown to increase PMN degranulation, apoptosis, and necrosis, which may contribute to the persistent endometrial cell damage in cows with SCE (Hammon *et al.* 2006, Scalia *et al.* 2006). *In vitro* studies have shown that BHB affected chemotaxis and respiratory burst capabilities (in combination with other ketone bodies) (Kremer *et al.* 1993, Suriyasathaporn *et al.* 1999). We found that serum haptoglobin concentrations were negatively associated with PMN degranulation, but positively associated with phagocytosis (Pascottini *et al.* 2021). *In vivo*, cows diagnosed with PVD or SCE had greater serum haptoglobin and BHB concentrations than healthy cows in the second week postpartum (i.e., weeks before diagnosis of the uterine disease), reflecting elevated metabolic stress and inflammation preceding uterine disease as observed in other studies (Huzzey *et al.* 2009, Dubuc *et al.* 2010*b*, Dervishi *et al.* 2016).

The identification of inflammatory and energetic biomarkers as risk factors for reproductive tract inflammatory disease in the early postpartum period strengthens the hypothesis that adaptation to metabolic demands associated with milk production is essential for the avoidance of disease. Metabolic stress however may be triggered in the prepartum period, and some cows may not adapt to this challenge (Pascottini *et al.* 2020*c*). A recent study identified thresholds of

serum NEFA concentrations in the prepartum period associated with the risk of developing postpartum diseases. Nicola *et al.* (2022) collected blood samples 1 to 14 days before calving but found no relationship between prepartum serum NEFA concentrations and subsequent development of PVD, CE, or SCE. The hypothesis for the lack of association was that the half-life of circulating PMN is short (less than 12 h) (Paape *et al.* 2002), so PMN exposed to metabolic stress prepartum may be associated with early postpartum diseases such as retained placenta and metritis, but not directly with reproductive tract inflammatory disease after 21 days postpartum.

As reviewed by LeBlanc (2010), the second week postpartum appears to be a critical time for the pathogenesis of uterine disease due to divergent systemic and uterine inflammatory status in healthy versus affected cows. We explored this hypothesis further with new analyses of our data on metabolic markers in the weeks before diagnosis of PVD or SCE (Pascottini & LeBlanc 2020*b*). We fitted nonmetric multidimensional scaling plots (Fig. 2) from data resulting from blood sampling at 7, 15, and 21 days postpartum for total Ca, total protein, albumin, globulin, cholesterol, urea, glucose, gamma-glutamyl transferase, aspartate aminotransferase, glutamate dehydrogenase, BHB, NEFA, haptoglobin, and insulin-like growth factor-1. At 35 days postpartum, cows were categorized as PVD, SCE, or healthy. Although the differences were not consistent over time, separation (clustering) of the SCE group at 15 days postpartum may support the hypothesis that SCE reflects maladaptation to a metabolic challenge and (dys)regulation of inflammation (Fig. 2).

Uterine microbiome: all about balance

During the last decades, comprehensive research revealed that only a small percentage of microorganisms are purely pathogenic, while most are neutral or vital for other (higher) organisms, or ecosystems (Berg *et al.* 2020). Commensal microorganisms can live on or within a host's organs without causing any harm. The collective community of living microorganisms (pathogens or parasites, commensals, and mutualists) in the same ecological niche is called 'microbiota' (Berg *et al.* 2020). The microbiome can be defined as the combination of microbiota and their 'theater of activity', including microbial proteins, lipids, polysaccharides, nucleic acids, metabolites, and signal molecules (Berg *et al.* 2020). The uterine cavity of healthy postpartum dairy cows is a crowded 'theater' with a high diversity of bacteria in an intricate network. Hence, there is yet no consensus on the nature of a 'healthy' uterine microbiota and how this microbiota interacts with the immune system of the host to achieve symbiosis. However, it seems that the healthy uterine microbiome consists of a blend of beneficial and potentially pathogenic bacteria which continuously train and develop the immune system of the host while the immune system coordinates tolerance and maintenance of crucial properties of host-microbe symbiosis (Zheng *et al.* 2020).

To illustrate the interaction between pathogenic bacteria and the immune system of the host in the endometrium of dairy cows, TLR-4 is the most studied PRR while lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria (notably *Escherichia coli*) is the most explored PAMP (reviewed

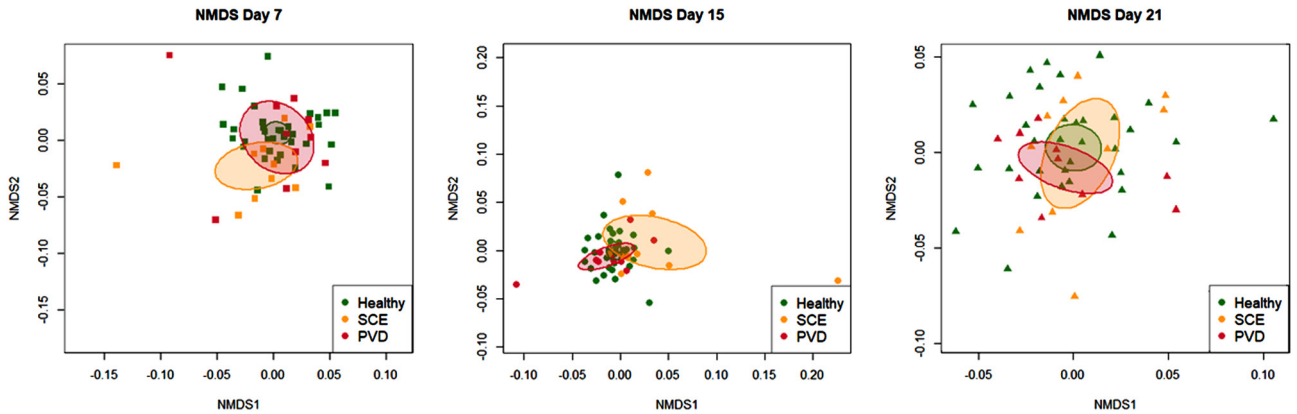

Figure 2 Nonmetric multidimensional scaling (NMDS) plots representing the mineral, metabolic, and inflammatory state of 58 Holstein cows from which blood samples were collected at 7, 15, and 21 days postpartum. Cows were diagnosed healthy (n = 38), with purulent vaginal discharge (PVD; n = 10) or subclinical endometritis (SCE; n = 10), at 35 days postpartum. Metabolites and markers measured in serum were total Ca, total protein, albumin, globulin, cholesterol, urea, glucose, γ-glutamyl transferase, aspartate aminotransferase, glutamate dehydrogenase, β-hydroxybutyrate, non-esterified fatty acids, haptoglobin, and insulin-like growth factor-1. Each shape (squares, circles, and triangles at days 7, 15, and 21 postpartum, respectively) coordinates represent variations in serum concentrations of metabolites and markers from each cow. Cows with SCE (orange circles) are clustered at 15 days postpartum. This suggests that the metabolic profile of cows with SCE differs from that of healthy cows and cows with CE at this time point. The plots were constructed from data in Pascottini & LeBlanc 2020*b*.

by Carneiro *et al.* 2016). In bacteria, LPS-binding protein and CD14 operate in tandem to release LPS molecules from their cell envelopes. In endometrial and stromal epithelial cells, LPS is detected by TLR-4 and this interaction activates inflammatory gene expression through nuclear factor-κB and mitogen-activated protein kinase signaling. Consequently, inflammation is stimulated via the secretion of pro-inflammatory cytokines including tumor necrotic factor-α, IL-1α, IL-1β, and IL-6 (Herath *et al.* 2006, Ghasemi *et al.* 2012). However, the microbial biomass in the postpartum uterus is immense and the mechanisms of PAMP activation may differ among bacteria and types of host cell.

Metritis, PVD, and CE are strongly correlated with the identification of (pathogenic) microorganisms (Jeon *et al.* 2015, Galvão *et al.* 2019, Pascottini *et al.* 2020*b*). The most prevalent bacteria cultured from uterine samples of dairy cows with postpartum uterine disease are *Trueperella pyogenes*, *Escherichia coli*, *Prevotella melaninogenica*, and *Fusobacterium necrophorum* (Williams *et al.* 2005, Carneiro *et al.* 2016, Gilbert & Santos 2016). These bacteria have long been regarded as 'classic uterine pathogens'; however, the role of *Escherichia coli* in uterine disease has been disputed (Bonnett *et al.* 1993, Dohmen *et al.* 1995). Recent studies using culture-independent techniques associated *Bacteroides*, *Fusobacterium*, *Helcoccus*, *Filifactor*, and *Porphyromonas* with metritis (Jeon *et al.* 2015) and *Bacteroides*, *Ureaplasma*, *Helcoccus*, *Fusobacterium*, *Trueperella*, *Prevotella*, and *Porphyromonas* with PVD and CE (Machado *et al.* 2012, Miranda-CasoLuengo *et al.* 2019, Pascottini *et al.* 2020*b*). *Trueperella pyogenes* is the main pyogenic agent. Damaged endometrial epithelia caused by excessive or persistent trauma after calving (e.g., dystocia and retained placenta) or other pathogens (e.g., *Fusobacterium* and *Prevotella*) are the entrance gateways. *Trueperella pyogenes* are commensal bacteria in the uterus of healthy cows in low relative abundance while they proliferate once the epithelium is breached and the endometrial stroma is exposed (Amos *et al.* 2014, Ormsby *et al.* 2022). This is because endometrial stromal cells are exceptionally vulnerable to the pore-forming toxin pyolysin produced by *Trueperella pyogenes* while endometrial epithelial cells are more resistant (Amos *et al.* 2014, Ormsby *et al.* 2022). Thus, *Trueperella pyogenes* and other pathogenic bacteria, often accompanied by trauma or a dysfunctional immune function, act synergistically in the development of uterine disease in dairy cows. However, this is only one example of the multiple interactions among bacteria and between bacteria and the host that occur in the postpartum uterus. The role of viruses and other microorganisms is less explored, although bovine herpesvirus-4 has been found to possess a tropism for endometrial cells (*in vitro*) and has been associated with uterine disease (Donofrio *et al.* 2007, Yang *et al.* 2019).

Using culture-dependent methods, several studies failed to find associations between the presence of 'pathogenic' bacteria and SCE (Sens & Heuwieser 2013, Madoz *et al.* 2014, Prunner *et al.* 2014). A newer study using sequencing of bacterial16S rRNA of uterine flushings at 30 days postpartum concluded that SCE was not associated with the uterine pathogens found in cows with CE in the same study (Wang *et al.* 2018). In a longitudinal study, we collected endometrial cytobrush samples from dairy cows at 10, 21, and 35 days postpartum, on which we performed 16S rRNA gene sequencing (Pascottini *et al.* 2020*b*). At 35 days postpartum, cows were retrospectively categorized as CE, SCE, or healthy. Neither alpha or beta diversity, nor individual bacteria phyla or genera abundances differed between healthy cows and those with SCE. The distribution of the top 15 bacterial genera among CE, SCE, and healthy cows in the fifth week postpartum is shown in Fig. 3. We also performed aerobic and anaerobic bacterial culture on the endometrial samples collected at 35 days postpartum. Overall, bacteria that grew in culture were often present within the most abundant bacteria found in the16S rRNA gene sequencing. These results support that culture-dependent studies that failed to find an association between SCE and pathogenic bacteria were not biased by the limitations of lack of growth in (anaerobic) culture media and support the hypothesis that SCE is associated with maladaptation

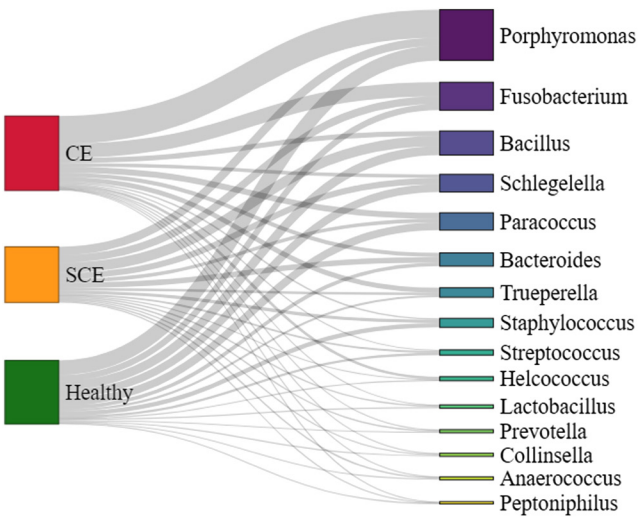

Figure 3 Alluvial plot illustrating the relative abundance of the 15 most abundant uterine bacteria genera in cows diagnosed as healthy ($n = 8$), with clinical endometritis (CE; $n = 5$), or subclinical endometritis (SCE; $n = 8$) at 35 days postpartum. The plot was constructed based on endometrial cytobrush 16S rRNA gene sequencing data derived from Pascottini *et al.* 2020*b*. Potentially pathogenic bacteria are present in cows diagnosed healthy, CE, and SCE. Higher relative abundance (thicker lines) of *Porphyromonas* and *Fusobacterium* can, however, be seen in cows diagnosed with CE than in SCE or healthy, but the relative abundances of bacteria genera were similar between healthy and SCE cows.

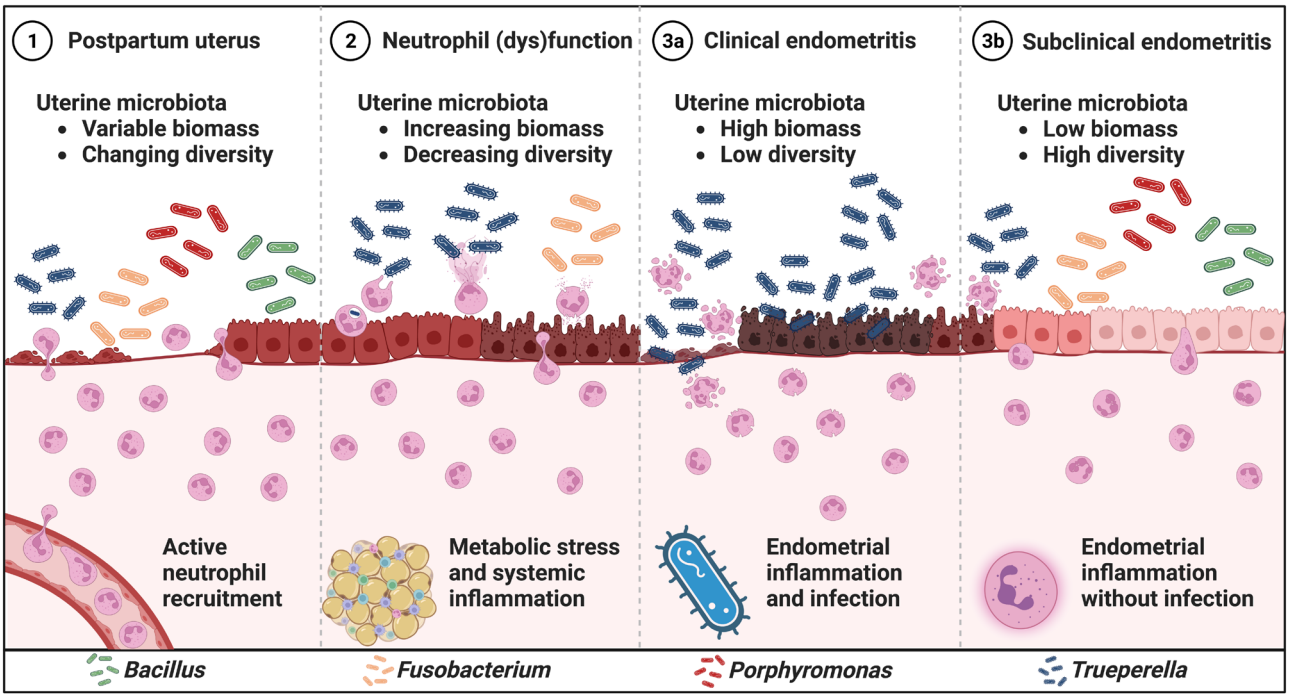

Figure 4 Postpartum events associated with the development of clinical or subclinical endometritis in dairy cows. (1) Robust and rapid migration of neutrophils to the uterine cavity just after calving is necessary to allow for tissue repair and control of bacterial dysbiosis. (2) Exacerbated negative energy balance and systemic inflammation are associated with neutrophil dysfunction. (3a) Clinical endometritis results from bacterial dysbiosis and infection, which are often a consequence of excessive trauma and inadequate immune response. (3b) Subclinical endometritis is the result of transition period maladaptation including excessive metabolic stress, which results in immune dysfunction in the absence of bacterial dysbiosis.

to negative energy balance and dysregulation of inflammation rather than uterine infection.

The number of studies exploring the composition of the bovine uterine microbiota using culture-independent methods increased greatly in the last 5–10 years. A pitfall of these studies, however, is (cross-) contamination during sampling as well as during DNA extraction (assuming that the identified bacteria are viable, which is not always the case) (Eisenhofer *et al.* 2019). Most bovine studies used a transcervical approach for endometrial sampling, increasing the risk of contamination with vaginal and cervical microbiota (Knudsen *et al.* 2016, Ault *et al.* 2019). Furthermore, during DNA extraction, the existence of 'reagent microbiota' has been described (Salter *et al.* 2014, de Goffau *et al.* 2018), also known as the 'kitome.' Thus, as explored in two recent studies by our research group (Lietaer *et al.* 2021*b*, 2023*b*), it is important to include environmental and reagent control samples and to use bioinformatic methods to 'decontaminate' collected data to achieve consistent results.

Conclusions

In Fig. 4, we summarize the events occurring from calving to the establishment of CE or SCE in dairy cows. Clinical endometritis is characterized by uterine dysbiosis and bacterial infection that is often a consequence of excessive trauma and inadequate immune response. However, it is unclear why some cows experience PVD without endometrial inflammation (i.e. apparently cervicitis alone). SCE is not associated with uterine dysbiosis. The weight of current data supports the hypothesis that SCE is the result of transition period maladaptation including excessive metabolic stress, which results in immune dysfunction. Hence, calving at an optimal body condition, proper feeding management (timely delivery, correct composition, and adequate space at the feed bunk), calving pen hygiene, clean and skillful obstetrical interventions, and prevention of retained placenta are key strategies to prevent reproductive tract inflammatory diseases. In the future, targeted modulation of the innate immune response around calving would help to control or treat uterine disease. Ultimately, understanding the microbial dynamics that cause infections in the uterus will allow the development of novel alternatives to antibiotics to prevent and treat infections.

Declaration of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data availability

The data presented in Figs. 1, 2, and 3 are available from the corresponding author upon request.

Author contribution statement

OBP Conceptualization, data analysis, figure preparation, writing of the original draft. SL, GG, JLMRL, GO review and editing. All authors have read and agreed to the published version of the manuscript.

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