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—Original Article—

Persistence of uterine bacterial infection, and its associations with endometritis and ovarian function in postpartum dairy cows

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Abstract. We investigated the relationship between the persistence of uterine bacterial infections with cytologically determined endometritis and ovarian function in 65 postpartum Holstein cows. Vaginal mucus discharges were collected, and endometrial smear samples (n = 130) were collected for cytological and bacteriological examinations from the cows at weeks 5 and 7 postpartum (pp). Blood samples were collected at weeks 3, 5 and 7 pp to determine plasma progesterone concentrations to monitor ovarian activity. According to the bacteriological examination, cows were classified into four groups. The first group (n = 32; 49%) comprised cows negative for bacteria at weeks 5 and 7 pp. The second group (n = 11; 17%) comprised cows with bacterial infections at week 5 pp but that were clear of infection at week 7 pp. The third group (n = 12; 19%) comprised cows without bacteria at week 5 pp but that acquired an infection by week 7 pp. The fourth group (n = 10; 15%) comprised cows with bacterial infections at weeks 5 and 7 pp (persistence of infection). A positive correlation ($P < 0.001$) was noted between the severity of cytologically determined endometritis, purulent vaginal discharge and the persistence of infection. Cows with persistent infections had a significantly ($P < 0.01$) prolonged luteal phase compared with cows without infection. In conclusion, the prevalence of cytologically determined endometritis and prolonged luteal phase were significantly increased in cows with persistent infections.

Key words: Bacterial infection, Cows, Endometritis, Prolonged luteal phase

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Postpartum (pp) endometritis caused by persistent bacterial infection has a major impact on the fertility of dairy cattle [1–5]. The most substantial effects of the disease are an increase in the number of days to conception, increased numbers of services needed for conception and an increased risk of culling [6]. Because the effects of the disease are delayed and often only detectable with subsequent statistical analysis, the economic significance of this disease remains largely unknown, but it is speculated to exceed billions of dollars annually for the global dairy industry.

The pp involution of the uterus is considered a normally septic process; thus, more than 90% of cows have microorganisms in their uterus for the first 2 weeks following calving, 78% have them in their uterus between days 16 and 30, 50% have them in their uterus between days 31 and 45, and 9% have them in their uterus between days 45 and 60 [7]. Most of these bacteria are environmental contaminants and are cleared by the uterus without impairing fertility [8]. Bacteriological studies have identified *Arcanobacterium pyogenes* (recently reclassified as *Trueperella pyogenes*), *Bacteroides* spp., *Fusobacterium necrophorum*, *Escherichia coli*, *Streptococcus* spp., *Clostridia* spp., *Pseudomonas aeruginosa*, and *Staphylococcus* spp. as the bacteria most likely to be associated with bovine endometritis [9, 10]. *T. pyogenes*, *Prevotella* spp., *F. necrophorum* and *E. coli* are the major uterine pathogens [11–13]. Other bacteria, such as *Staphylococcus* spp., *Streptococcus* spp. or non-*E. coli* aerobic gram-negative rods have also been isolated as additional flora along with the major uterine pathogens [14, 15]. *T. pyogenes* cooperates with aerobic-facultative anaerobic bacteria and/or with gram-negative obligate anaerobes such as *F. necrophorum* and *Prevotella* spp. in formulating bacterial flora in the uterus [12–16]. However, severe endometrial lesions are mainly caused by *T. pyogenes*, which is the most prevalent bacterial type in the late pp period [13, 17, 18] and acts synergistically with anaerobic pathogens such as *F. necrophorum* [11, 19–21].

Sheldon *et al.* [3] defined clinical endometritis as the presence of a purulent uterine discharge detectable in the vagina 21 days or more pp or a mucopurulent discharge detectable 26 days pp and defined subclinical endometritis as inflammation limited to the endometrium at least 21 days pp with no detectable discharge from the vagina.

It is generally agreed that a high level of progesterone suppresses cervical mucus production, myometrial contractility, uterine gland secretion and the phagocytic activity of uterine neutrophils [18, 22] and is therefore permissive of at least short-term uterine infections

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[23]. Early ovulation and the elevation of circulating progesterone concentrations before the elimination of uterine bacterial contamination have been linked to the establishment of pyometra in pp cows [24].

Limited data are available on the persistence of *T. pyogenes* and anaerobic bacteria in the uterus of cows and their association with cytologically determined endometritis and purulent vaginal discharge under field conditions. The aim of this study was to examine the prevalence and persistence of bacterial infections, specifically *T. pyogenes* and anaerobic bacteria isolated at early pp stages using cytobrush techniques, in relation to the percentage of polymorphonuclear neutrophils (PMN%), conditions of vaginal mucus discharges and ovarian function in dairy cows.

Materials and Methods

Animals

This study was conducted at three commercial dairy farms in Iwate Prefecture, Japan. We used 65 cows, and the average parity of the cows was 2.4 ± 0.2. On the farms, cows were fed a total mixed ration based on corn silage, grass silage and concentrates.

Evaluation of vaginal mucus discharges

Cows were examined at postpartum weeks 5 (34.9 ± 0.2 days) and 7 (48.6 ± 0.2 days) using a sterilized Metricheck (Simcro Tech, Hamilton, New Zealand). The vaginal mucus discharge score was used to classify its characteristics [25]. Thus, clear or translucent mucus scored 0; a discharge score of 1 described mucus containing flecks of white or off-white pus; score of 2 described a discharge containing less than 50% white or off-white mucopurulent material; score of 3 described a discharge composed of more than 50% white or yellow pus; and a score of 4 described a discharge composed of more than 50% white or yellow pus with a fetid odor.

Uterine swab collection

For each animal, uterine endometrial samples (n = 130) were collected at pp weeks 5 and 7 using a cytobrush (Puritan Medical Products Company L.L.C., Guilford, ME, USA) adapted for use in cattle [4, 26]. Briefly, the cytobrush was used to collect endometrial samples by insertion via the cervix into the uterine body. To protect the cytobrush from contamination, the catheter was covered with a disposable plastic sleeve. The sleeve was retracted, and the cytobrush was pushed gently forward and rolled along the uterine wall. Thereafter, the cytobrush was retracted into the catheter to prevent contamination during passage through the outer genital tract.

Bacterial culture technique

Sterilized swabs were rolled against the cytobrush and collected in transport medium (Seed Swab No. 2; Eiken Chemical, Tokyo, Japan) on the farms, and they were then transported to the laboratory. Aliquots were then plated on a blood agar plate, a chocolate agar plate and a deoxycholate-hydrogen sulfide-lactose agar plate and incubated at 35.0 ± 1.0 C under aerobic conditions. Another aliquot was placed on a brain–heart infusion agar plate and incubated at 35.0 ± 1.0 C under anaerobic condition. Colonies grown for 24 and 48 h under aerobic conditions or for 48 h under anaerobic conditions were harvested. If no colony was observed in any of the plates by 48 h, the samples were placed on Gifu Anaerobic Medium (GAM) semisolid for enrichment culture, incubated at 35.0 ± 1.0 C under aerobic conditions and checked for bacterial growth for 24, 48 and 72 h. This medium allows bacteria to grow under both aerobic (on the surface of the medium) and anaerobic (within the medium) conditions. If bacterial growth was observed, a drop of the GAM was placed on each of the above plate types and cultured under aerobic and/or anaerobic conditions. Bacterial species were identified by standard laboratory procedures [27]. Isolates were classified as either aerobic bacteria that require oxygen as a terminal electron acceptor and will not grow in the absence of oxygen or anaerobic bacteria that do not use oxygen for growth and metabolism but obtain their energy from fermentation reactions. Facultative anaerobes that can grow either using oxygen or anaerobically using fermentation reactions to obtain energy were classified as aerobes [28].

Diagnosis of cytological endometritis

Cytology samples were prepared by rolling the cytobrush onto sterile glass microscope slides at the farms and fixed immediately using a cytofixative (Cytokeep II, Alfresa Pharma Corporation, Osaka, Japan). Slides were then brought to the laboratory within 3 h and stained with Diff-Quik (Sysmex, Kobe, Japan) for 20 sec. Cytological assessment was used to determine the PMN% by counting 200 leukocytes under a microscope (× 400 magnification). The threshold values for the PMN% indicating cytologically determined endometritis were ≥ 6% at week 5 and ≥ 4% at week 7 [29]. Cows having cytologically determined endometritis but not having mucopurulent or purulent discharge were considered to have had subclinical endometritis [3].

Cervical diameter

The cervical diameter (in cm) was determined by transrectal palpation. The operator held the cervix with the fingers (thumb and pointer) and measured the cervical diameter with the two fingers and a ruler in millimeter increments immediately after palpation in all cows at weeks 5 and 7.

Determination of plasma progesterone and resumption of ovarian cyclicity

Blood samples were collected at weeks 3, 5 and 7 pp from the coccygeal vessels into vacuum heparinized tubes and transported on ice to the laboratory. Plasma was separated by centrifugation at 2,000 × *g* for 15 min, harvested and stored at −20 C until assayed for progesterone. Plasma progesterone (P_4) concentrations were measured in duplicate using time-resolved fluorescence immunoassay kits (DELFIA Progesterone Reagents, Wallac Oy, Turku, Finland) according to the manufacturer's protocol with the modification described previously [30]. Briefly, 25 µl of a series of standard solutions (0, 0.33, 1.33, 4.0, and 12.0 ng/ml, prepared by the addition of a known amount of P_4 into charcoal-treated progesterone-free bovine serum) and the plasma samples were dispensed into a 96-well assay microplate (precoated with anti-rabbit IgG as a secondary antibody), followed by the addition of 100 µl anti-progesterone antibody and europium-labeled tracer to each well. The plate was then incubated for 2.5 h at 22 C, washed with wash solution three times, supplemented with 200 µl enhancement solution, and shaken gently for 5 min at 22 C. Fluorescence was detected using a Wallac 1420 multilabel

Table 1. Distribution of different types of bacteria isolated from the uterus of postpartum cows at weeks 5 or 7

Bacterial findings	Cows positive for bacterial infection at week 5 and not at week 7[1]	Cows positive for bacterial infection at week 7 and not at week 5[2]	Cows positive for bacterial infection at weeks 5 and 7[3]	
	Week 5	Week 7	Week 5	Week 7
T. pyogenes and anaerobes	2	0	10	9
α-streptococcus	4	3		
T. pyogenes and aerobes	1	0		1
Gram negative bacilli	1	3		
Gram positive bacilli	1			
Fusobacterium spp.	1			
α-streptococcus and anaerobes	1			
α-streptococcus and Corynebacterium pyogenes		2		
Corynebacterium pyogenes		1		
T. pyogenes		1		
α-streptococcus and E. coli		2		
Total	11	12	10	10

[1] Cows with bacterial infections at week 5 but were spontaneously clear of the infection without intervention at week 7. [2] Cows with no bacterial load in the uterus at week 5 but that acquired the infection at week 7. [3] Cows with persistence of uterine bacterial pathogens at weeks 5 and 7.

counter (Arvo MX; Elmer Perkin Life and Analytical Sciences, Wallac Oy, Turku, Finland). The assay sensitivity was 0.22 ng/ml, and the median effective dose value of the standard curve was 1.4 ng/ml. The intra- and interassay coefficients of variation were 5.2% and 10.4%, respectively. Cows with a plasma P_4 level of ≥1 ng/ml were considered to exhibit luteal activity [31, 32]. Ovulation was considered to have taken place 5 days before the first rise of P_4 to 1 ng/ml or higher [33–35]. Normal or abnormal resumption of pp ovarian cyclicity was defined as follow: in normal resumption of ovarian cyclicity, ovulation occurred ≤ 45 days after calving followed by regular ovarian cycles, while in delayed resumption of ovarian cyclicity, ovulation followed by regular ovarian cycles did not occur until > 45 days after calving [35]. Cows with one or more ovarian cycles with luteal activity of > 20 days were diagnosed as having a prolonged luteal phase (PLP) [35].

Statistical analysis

The PMN% and vaginal mucus score for the cows with different statuses of bacterial infections at weeks 5 and 7 were analyzed using the nonparametric Kruskal–Wallis test. Further comparisons among groups were made using Dunn's multiple comparison test. The prevalence of cytologically determined endometritis and purulent vaginal discharge, and the prevalence of delayed ovarian activity at week 3 pp and PLP in cows with and without bacterial infection at weeks 5 and 7 pp were analyzed by Fisher's exact test. Correlations between cows with bacterial infections in relation to the PMN% and vaginal mucus score at weeks 5 and 7 pp were analyzed by Spearman's test (GraphPad Prism Version 5.01, GraphPad Software, San Diego, CA, USA). Differences were considered significant if $P < 0.05$.

Results

Bacteriological findings at weeks 5 and 7

According to the bacteriological examinations at weeks 5 and 7, the cows were classified into four groups. The first group (n = 32; 49%) comprised cows with negative bacterial growth at both weeks 5 and 7. The second group (n = 11; 17%) comprised cows with bacterial infections at week 5 but that were spontaneously clear of the infection without intervention at week 7. The third group (n = 12; 19%) was cows with no bacterial load in the uterus at week 5 but that acquired an infection at week 7. The fourth group (n = 10; 15%) comprised cows with persistence of uterine bacterial pathogens at weeks 5 and 7. In the animals of the fourth group, *T. pyogenes* and anaerobes were isolated from all 10 cows (100%) at week 5 and nine cows (90%) at week 7. In cows with bacterial infection at week 5 and not at week 7, only two out of 11 cows (18.2%) were infected with *T. pyogenes* and anaerobes at week 5 (Table 1).

PMN% and vaginal mucus score for the different cows with bacterial infections at both weeks 5 and 7

There were significant differences in PMN% ($P < 0.0001$) and the severity of vaginal mucus discharge ($P < 0.001$) between cows with persistence of bacterial infections at both weeks 5 and 7 compared with those without bacterial infection at those times (Table 2).

Prevalence of cytologically determined endometritis and purulent vaginal discharge in cows with absence or persistence of uterine bacterial infection at both weeks 5 and 7

Cows with persistence of bacterial infection at both weeks showed a significantly higher prevalence ($P < 0.001$) of cytologically determined endometritis and purulent vaginal discharge compared with those without bacterial infections at weeks 5 and 7 (Table 3 and 4).

Persistence of bacterial infections in relation to cytological and clinical endometritis

In cows with persistent bacterial infections, the percentages of both clinical and cytologically determined endometritis were 40% at week 5 and 60% at week 7 (Table 5). The percentage of cytologically

Table 2. Parity, PMN%, vaginal mucus score and cervical diameter among cows with different bacterial infections at weeks 5 or 7

Parameters	No.	Parity	PMN%		Vaginal mucus score		Cervical diameter (cm)	
			Week 5	Week 7	Week 5	Week 7	Week 5	Week 7
Cows negative for bacterial infection at weeks 5 and 7	32	2.3 ± 0.3	1.7 ± 0.7[a]	3.0 ± 0.6[a]	0.8 ± 0.1[a]	1.0 ± 0.2[a]	3.7 ± 0.2[a]	3.8 ± 0.1
Cows positive for bacterial infection at week 5 and not at week 7	11	1.8 ± 0.3	10.1 ± 3.8[ab]	6.5 ± 3.8[a]	1.5 ± 0.4[ab]	1.3 ± 0.3[ab]	3.6 ± 0.2[a]	3.5 ± 0.2
Cows positive for bacterial infection at week 7 and not at week 5	12	2.7 ± 0.5	5.5 ± 2.6[a]	1.9 ± 1.0[a]	1.2 ± 0.2[ab]	1.3 ± 0.2[ab]	4.2 ± 0.2[ab]	4.4 ± 0.3
Cows positive for bacterial infection at weeks 5 and 7	10	3.1 ± 0.3	18.8 ± 3.8[b]	21.8 ± 4.6[b]	2.6 ± 0.4[b]	2.6 ± 0.4[b]	4.6 ± 0.1[b]	4.1 ± 0.2
P value			0.0003	0.0001	0.001	0.007	0.01	0.057

Table 3. Prevalence of cytological endometritis in cows with and without bacterial infection at weeks 5 and 7

Cows	Week 5					Week 7				
	PMN[†]		Total	Cytological endometritis (%)	P	PMN[†]		Total	Cytological endometritis (%)	P
	≤ 6%	≥ 6%				≤ 4%	≥ 4%			
Cows negative for bacterial infection at both weeks	29	3	32	9.4	0.0001	24	8	32	25	0.0004
Cows positive for bacterial infection at both weeks	1	9	10	90		1	9	10	90	
Total	30	12	42			25	17	42		

[†] The diagnostic criteria for cytological endometritis were ≥ 6% and ≥ 4% PMNs at weeks 5 and 7, respectively.

Table 4. Prevalence of purulent discharge in cows with and without bacterial infection at weeks 5 or 7

Cows	Week 5					Week 7				
	Vaginal mucus score		Total	(%)	P	Vaginal mucus score		Total	(%)	P
	1–2	3–4				1–2	3–4			
Cows negative for bacterial infection at both weeks	32	0	32	0	0.0003	30	2	32	6.3	0.0009
Cows positive for bacterial infection at both weeks	5	5	10	50		4	6	10	60	
Total	37	5	42			34	8	42		

determined endometritis declined from 50% at week 5 to 30% at week 7 when it was not associated with clinical endometritis (a purulent vaginal discharge).

Correlation between bacterial infection in relation to PMN% and vaginal mucus score at both weeks 5 and 7

There were positive correlations between the persistence of bacteria (*T. pyogenes* and anaerobes) in the uterus of cows and the PMN% at weeks 5 ($P < 0.0001$) and 7 ($P < 0.0001$) as well as the scores of vaginal mucus discharge at weeks 5 ($P = 0.001$) and 7 ($P = 0.001$). Conversely, the absence of bacterial infections in the uterus at both weeks was negatively associated with PMN% and vaginal mucus discharge scores (Table 6).

Relationship between the absence or persistence of bacteria at weeks 5 or 7 and postpartum resumption of ovarian activity and PLP

Resumption of ovarian cyclicity at week 3 pp showed no significant difference ($P = 0.7$) in cows without uterine bacterial infections compared with that those showing persistence of infection. However, cows with persistent uterine bacterial infections showed a significant PLP ($P = 0.005$) compared with those without bacterial infections at both weeks (Table 7).

Discussion

Establishment of microbial infections in the reproductive tract can have negative consequences for reproduction in pp cows. Most periparturient cows experience bacterial contamination of the uterus after parturition, but only a fraction of them develop subclinical or clinical diseases. It is not well understood how one female can resolve uterine infections after parturition while another develops a disease. Perhaps those that develop metritis or endometritis are exposed to a greater bacterial load at parturition than those that successfully restore the uterus to a healthy condition [36]. This study is one of a few reported trials that have conducted follow-up examinations of single cows with pp bacterial infections to determine the ability or inability of the uterus to clear itself. The previous studies examined

Table 5. Distribution of cows with a high PMN% and vaginal mucus score (3 or 4) together or alone at weeks 5 or 7

Cows	No.	Cows with both a high PMN% and high vaginal mucus score‡		Cows with a high PMN%#		Cows with a high vaginal mucus score§	
		W5	W7	W5	W7	W5	W7
Cows negative for bacterial infection at weeks 5 and 7	32	0	0	3 (9.4%)	8 (25%)	0	2 (6.3%)
Cows positive for bacterial infection at week 5 and not at week 7	11	2 (18.2%)	0	3 (27.3%)	4 (36.4%)	2 (18.2%)	1 (9.1%)
Cows positive for bacterial infection at week 7 and not at week 5	12	1 (8.3%)	0	2 (16.7%)	1 (8.3%)	0	0
Cows positive for bacterial infection at weeks 5 and 7	10	4 (40%)	6 (60%)	5 (50%)	3 (30%)	1 (10%)	0

‡ Cows had cytological and clinical endometritis (mucus discharge scored 3 or 4). # Cows had cytological endometritis with mucus discharge scored 1 or 2. § Cows with mucus discharge scored 3 or 4 without cytological endometritis.

Table 6. Comparison of correlation coefficients (r) among cows with different statuses of bacterial infection in relation to the PMN% and vaginal mucus score at weeks 5 or 7

Parameters	PMN% at W5		PMN% at W7		Vaginal mucus score at W5		Vaginal mucus score at W7	
	R	P	r	P	r	P	r	P
Cows negative for bacterial infection at weeks 5 and 7[1]	–0.39	< 0.01	–0.13	0.32	–0.39	< 0.01	–0.30	0.02
Cows positive for bacterial infection at week 5 and not at week 7[2]	0.08	0.51	–0.05	0.68	0.1	0.44	–0.01	0.97
Cows positive for bacterial infection at week 7 and not at week 5[3]	–0.04	0.74	–0.29	0.02	0.01	0.97	0.01	0.94
Cows positive for bacterial infection at weeks 5 and 7[4]	0.49	< 0.01	0.54	< 0.01	0.43	< 0.01	0.41	< 0.01

[1] Cows with negative bacterial growth at both weeks 5 and 7. [2] Cows with bacterial infections at week 5 but that were spontaneously clear of the infection without intervention at week 7. [3] Cows with no bacterial load in the uterus at week 5 but that acquired the infection at week 7. [4] Cows with persistence of uterine bacterial pathogens at weeks 5 and 7.

Table 7. Percentage of cows with delayed resumption of ovarian activity at week 3 postpartum (delayed) and a prolonged luteal phase (PLP) in cows with and without bacterial infection at weeks 5 and 7

Cows	Total	Delayed (%)	PLP (%)
Cows negative for bacterial infection at weeks 5 and 7	32	25 (78.1)	4 (12.5)[a]
Cows positive for bacterial infection at weeks 5 and 7	10	9 (90.0)	6 (60.0)[b]
Total	42	34	10

[ab] P = 0.005.

postpartum cows by cytology only one time, but in the present study, each cow was followed up by endometrial samples two times at weeks 5 and 7 to detect the type of bacteria that persisted in the uterus and deteriorated the uterine condition through the PMN count and the ability of the cow to eliminate the infection. One of the main objectives was to investigate the persistence of different bacterial species in the uterus of individual pp cows in relation to the prevalence of cytologically determined endometritis, purulent vaginal discharge and ovarian function.

The range of bacterial species isolated from intrauterine samples agreed with those in previous reports [13, 21]. However, in this study, *T. pyogenes* was not isolated alone except in one cow at week 7. *T. pyogenes* was associated mainly with aerobic-facultative anaerobic bacteria such as *F. necrophorum* and *Prevotella* spp. [12, 16]. This highlights the importance of these pathogens in inducing endometritis. *F. necrophorum* produces a leukotoxin, *Prevotella melaninogenicus* produces a substance that inhibits phagocytosis, and *T. pyogenes* produces a growth factor for *F. necrophorum* [5]. *T. pyogenes* is the most frequently isolated uterine pathogen [21, 37]. It is rarely isolated alone in pp animals [18, 38]. Although *E. coli* plays a key role in the metritis–endometritis syndrome complex [13, 17, 39], the number of *E. coli*-positive samples was surprisingly low in the present study. *E. coli* was identified in association with α- *Streptococcus* in two cows at week 7. These results indicate that the prevalence of *E. coli* infection decreased in the late pp period, while the prevalence of *T. pyogenes* and anaerobes increased. In the first few days after calving, *E. coli* dominates the uterus, and *T. pyogenes* is found later in animals with severe clinical endometritis [8, 40].

Uterine cytology has been established to distinguish healthy cows from those with subclinical or cytological endometritis based on elevated proportion of PMNs in endometrial samples [4, 26, 41]. To our knowledge, there is only limited information available about the relationship of bacterial findings and the PMN%. Therefore, this study analyzed the relationships between intrauterine bacteriological isolates, vaginal mucus discharge and cytological findings indicative of cytologically determined endometritis. *T. pyogenes* and anaerobes were the most prevalent bacteria persisting in the uterus of cows and were positively associated with a purulent vaginal discharge, as reported earlier [13, 17, 40]. Such a correlation did not exist in cows infected with bacteria at week 5 alone or week 7 alone, and

these animals did not show persistence of infection. Westermann *et al.* [42] demonstrated a significant correlation between the proportion of PMNs and the presence of *T. pyogenes*. Correlations were not significant between the PMN% and the presence of other bacteria isolated from uterine samples. Westermann *et al.* [42] indicated that *T. pyogenes* had a more pronounced effect on cellular uterine response than other bacteria. This information is valuable for the interpretation of intrauterine findings of cows with clinical and subclinical endometritis, and it is in accordance with reports that *T. pyogenes* causes more severe endometrial lesions than *E. coli* [43, 44].

In the present study, some individual cows were identified as positive for some bacterial species at week 5, but not at week 7. This might be due to the ability of the uterus to eliminate the infection. Some other cows were negative for bacterial infections at week 5 but acquired an infection at week 7. This might have arisen from a lowered immune response by the uterus. In the same aspect, some cows were diagnosed as negative for bacterial isolates but showed a high PMN%. This was in agreement with a study by Huszenicza *et al.* [40], who found that uterine pathogens could also be isolated from the uteri of cows that did not show clinical signs of endometritis. It has been previously reported that most cows that are negative for subclinical endometritis at pp week 4 are also negative at week 8. Similarly, a higher proportion of cows that have subclinical endometritis at pp week 4 are also positive at week 8 [45].

Interestingly, the number of samples positive for *T. pyogenes* and anaerobes remained high at week 7, similar to the findings at week 5 in cows with persistence of bacterial infection at both weeks. Moreover, they had both cytological and clinical endometritis that increased from week 5 to week 7. In some other cows infected only at week 5 or week 7, the incidence of both cytologically determined endometritis and clinical endometritis combined was low and disappeared later at week 7. Some cows had only cytologically determined endometritis without clinical endometritis, and some cows had clinical endometritis without cytologically determined endometritis. The assumption that purulent vaginal discharge is linked with the presence of endometrial inflammation has never been validated [29]. It is also possible that the term "clinical endometritis" might not be appropriate because most affected cows did not have endometrial inflammation. Cytological endometritis might reflect the presence of uterine inflammation, leading sometimes but not always to drainage of purulent material into the vagina. It is also unclear whether a purulent vaginal discharge can lead to cytological endometritis [29].

In the present study, there were no significant differences between cows with negative bacterial growth and cows with persistence of infection at both weeks in terms of early resumption of ovarian cyclicity at week 3 or the percentage of anovulatory cows. However, cows with persistence of bacteria showed a PLP ($P = 0.005$) compared with those without infection at both weeks. It was clear that *T. pyogenes* and anaerobes could prolong the luteal phase and diminish ovarian activity, leading to persistence of infections, a purulent vaginal discharge and cytological endometritis. Sheldon *et al*. [5] integrated the mechanisms of infection and immunity in the female reproductive tract of cattle that ultimately regulate reproductive efficiency. Postpartum cows that have uterine infections are less likely to ovulate because growth of the dominant follicle is slower and there are lower concentrations of plasma estradiol. If cows do ovulate, then endometrial cytokines might alter steroidogenesis by luteal cells and this could contribute to a lower secretion of progesterone or increase the PGE2/PGF2 ratio, which might extend the luteal phase. Delayed onset of ovarian activity in cows with endometritis as compared with healthy cows has been reported [46, 47]. However, Dohmen *et al.* [17] did not find a relationship between bacteriological findings and ovarian activity. Moreover, Subandrio *et al.* [48] failed to find any consistent relationship between stage of the estrous cycle and uterine neutrophil function.

It is difficult to know whether the presence of *T. pyogenes* and anaerobes retards the resumption of ovarian cyclicity or whether the delayed pp ovarian activity enhances infection and persistence by *T. pyogenes* and anaerobes. When ovulation occurs before the uterus has expelled all the exudates and debris, a heavy growth of bacteria such as *T. pyogenes* occurs, and the corpus luteum (CL) is retained for a prolonged interval [49]. Furthermore, the first CL to develop in cows with uterine disease secretes less progesterone than in normal animals [50]. Kaneko and Kawakami [51] reported that an infusion of *T. pyogenes* into the uterus caused luteal regression; consequently, first-wave dominant follicles, which normally become atretic, ovulated in half of the infused cows. However, the CL did not regress in the remaining cows, and the mechanism determining the fate of the CL remained unclear [52]. The serum concentrations of P_4 in cows with PLP were higher than in those with normal ovarian activity, indicating that the occurrence of PLP in the clinically healthy high-producing dairy cows was not associated with uterine infection [53].

In conclusion, the persistence of uterine infections in dairy cows was associated with the presence of *T. pyogenes* and anaerobes in the uterus of pp dairy cows. The prevalence rates of cytological endometritis, purulent vaginal discharge and prolonged luteal phase were increased in cows with persistence of infections in utero at weeks 5 and 7. Although the mechanism by which pathogenic bacteria affect ovarian function remains to be determined, effective prevention and treatment for *T. pyogenes* and anaerobic bacteria infections should be implemented to mitigate the persistence of uterine infections and inflammation, and thereby normalize ovarian function in pp dairy cows.

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