Dynamics of postpartum endometrial cytology and bacteriology and their relationship to fertility in dairy cows

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ABSTRACT

Endometrial samples were obtained from 56 consecutively calving dairy cows examined for endometrial cytology and for aerobic and anaerobic bacterial growth. Changes over time, correlations between different cell types and between cell and bacterial populations and with fertility measures were calculated. The proportion of neutrophils in cytologic preparations decreased with time postpartum. Other cell types did not change significantly with time. The proportion of neutrophils early (day 0 and 7) postpartum was negatively correlated with neutrophil proportion at 5 or 7 weeks postpartum and positively correlated with fertility. Cows with high proportion of neutrophils at 7 days postpartum ($> 40\%$) were significantly more likely to become pregnant than those with lower proportions of neutrophils.

Escherichia coli were the bacteria most frequently isolated at 0 or 7 days postpartum but were uncommon after that. *Trueperella pyogenes* were most prevalent at 3 weeks postpartum, and were more likely to infect cows that had previously been infected with *E. coli*. Presence of *T. pyogenes* at 3 weeks postpartum increased the risk of concomitant or later infection with gram negative anaerobes. The presence of *T. pyogenes* at 3 weeks postpartum significantly reduced the risk of pregnancy at 150 days in milk (DIM). The presence of alpha-hemolytic *Streptococcus* spp. at 7 days postpartum was associated with improved reproductive performance. The proportion of neutrophils at 5 and 7 weeks postpartum was related to concomitant bacterial infection.

47 These findings suggest that rapid mobilization of neutrophils to the postpartum uterus is a
48 beneficial response for uterine health in dairy cows.

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50 Key words: Cytology; endometritis; infection; inflammation; uterus

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1. INTRODUCTION:

Prevalence of endometritis, as diagnosed by endometrial cytology, is high in dairy cows, persisting up to and beyond the end of the traditional postpartum voluntary waiting period. Several studies have confirmed a high prevalence of endometritis after 40 days postpartum and that the condition has a negative impact on subsequent reproductive performance [1-7]. In contrast, however, most publications maintain that the bovine uterus, although consistently contaminated or infected in the early postpartum period, is essentially sterile by about 28 days postpartum [8-11]. The distinction between proportion of infected cows and cows with subclinical endometritis is well documented by Sheldon, et al. [9]. Therefore, one aim of this study was to examine the relationship between bacterial infection and cytological evidence of endometritis and to determine if endometritis persisted after elimination of bacterial infection.

The relationship between persistent bacterial infection and inflammation is important in devising strategies for prevention and treatment of endometritis. For example, antibacterial treatments are unlikely to be effective if the inflammation occurs in the absence of bacterial infection, or persists long after the infection is resolved. This study was undertaken to further understanding of the relationship between uterine infection and evidence of endometrial inflammation (measured by endometrial cytology), and the relationships between uterine infection, endometritis, postpartum endometrial cytology and fertility in lactating dairy cows. We postulated that uterine bacterial populations would account for observed inflammation and that inflammation (and infection) would steadily decrease with time postpartum.

76 The relationship between bacterial infection and endometritis, which is often subclinical, could
77 also cast light on the pathogenesis of endometritis. For example, is endometritis largely a
78 reflection of a generalized inflammatory environment attributable to a form of metabolic
79 syndrome (extensive fat mobilization and insulin resistance) that is often recognized in
80 postpartum cows [12], or is it more simply a consequence of local bacterial infection (which
81 itself may follow more generalized immune impairment [13])?
82

83 Finally, characterization of the relationship between uterine infection and endometritis could cast
84 light on the mechanism of infertility in endometritis. Do bacteria play a direct role in altering the
85 uterine environment, rendering it hostile to spermatozoa or the developing zygote, or is infertility
86 mediated by more subtle changes brought about by inflammatory mediators at a uterine [14,15]
87 or ovarian [15-17] level?
88

89 While cross sectional studies of endometrial cytology abound, few have examined cows in a
90 longitudinal fashion and those that have, have concentrated on cows later in lactation, for
91 example at 35 and 56 days postpartum [2,18], or at 3, 5 and 7 weeks postpartum [4]. We
92 therefore examined endometrial cytology beginning immediately postpartum and continuing
93 until 49 days postpartum. Knowledge of the progression of endometrial cytology is important
94 for identifying periods in which clinically significant inflammation can be diagnosed and
95 distinguished from the physiological inflammation which occurs postpartum and is necessary for
96 the extensive uterine tissue remodeling required during uterine involution for return to fertility.
97

Our goals therefore were to characterize the progression of endometrial cytology in postpartum dairy cows, and to evaluate the relationship between uterine bacterial infection and endometrial cytology. The relationship between endometrial cytology or uterine bacterial infection at different postpartum periods and subsequent fertility was also investigated.

2. MATERIALS and METHODS:

2.1 Animals and animal procedures

All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee (protocol 2004-0078).

Consecutively calving cows ($n = 56$), calving over a 12-month period in 2004-05, in the College of Veterinary Medicine Dairy Herd were enrolled in this experiment. Endometrial samples were obtained by low volume uterine lavage on the day of calving and 7, 21, 35 and 49 days postpartum as previously described [6]. Briefly, a sterile plastic infusion pipette contained in a chemise was passed through the cervix under transrectal control and 20 ml sterile saline injected into the uterine lumen, gently agitated, and aspirated. Recovered fluid was used for aerobic and anaerobic bacterial culture by thoroughly wetting a Tran-Swab culturette (Fisher Scientific; Pittsburgh, PA) and a Port-a-cul® (Becton Dickinson, Franklin Lakes, NJ) swab and submitting them to the Animal Health Diagnostic Laboratory at Cornell University for aerobic and anaerobic bacterial culture, respectively. Remaining fluid was used for cytological evaluation of the uterus. An aliquot of fluid was transferred to a glass microscope slide using a cytocentrifuge, air dried and stained using a Romanowski staining technique (Diff Quik, VWR, Arlington

Heights, IL) [6]. Note that on the day of calving it was usually not necessary to inject fluid into the uterus before aspirating a sample. A pipette could simply be introduced and uterine content aspirated for further processing.

2.2 Bacterial culture

Bacterial samples were cultured at the Animal Health Diagnostic Laboratory as follows. For aerobic culture, the swabs were inoculated on to the following agar plates - Blood Agar (BP), Chocolate Agar (CHOC), Eosin Methylene Blue Agar (EMB) and Columbia Colistin Nalidixic Acid Agar (CNA) and struck for isolation of bacteria. The plates were incubated at 35°C with 6% carbon dioxide overnight before being examined. Individual bacterial colonies were identified using biochemical methods or Trek automated ID or MALDI-TOF biotyper.

The anaerobic cultures were performed in an anaerobic chamber on *Brucella* agar, Phenylethyl alcohol agar (PEA), *Bacteroides* bile esculin agar (BBE), and *Brucella* laked blood agar with kanamycin and vancomycin (LKV) at 35C. Individual bacterial colonies were identified using biochemical methods and/or MALDI-TOF biotyper.

2.3 Endometrial cytology

Endometrial cytology was evaluated by a single examiner blinded to the source of the sample. Nucleated cells (n = 200) were identified as endometrial epithelial cells, polymorphonuclear cells (presumptive neutrophils), large mononuclear cells (presumptive macrophages) and small mononuclear cells (presumptive lymphocytes). Examination was performed independently by both authors using bright field microscopy at 400 x magnification and the mean result used for

analysis [4,6,7,19-25]. Progression of cell population with days postpartum was evaluated by linear regression. The relationship of proportion of each cell type to subsequent reproductive performance (pregnancy to first artificial insemination, pregnancy by 150 days postpartum) was evaluated by calculating spearman correlation coefficients, because pregnancy is a dichotomous outcome. Where significant correlations were found, predictive values of selected parameters were chosen by Receiver Operating Characteristic curves. The predictor was then dichotomized at the predictive value with the highest sum of sensitivity and specificity, and used in Cox proportional hazards regression.

2.4 Statistical analysis

Demographic information and reproductive performance of cows were recovered from electronic herd records (DairyComp305, ValleyAg Software, Tulare, CA).

Results are presented as proportions or means (and standard errors). Associations between bacterial isolates at each time point and bacterial isolates at later time points, and contemporaneous and later cytology results, and the relationship between bacterial isolates and endometrial cytology results with pregnancy outcome were examined initially by calculating correlations, and then by multiple logistic regression (dichotomous outcome) including factors indicated by simple correlation coefficients and *P*-values. The effect of bacterial isolates on simultaneous endometrial cytology findings were first examined by univariate analysis. Bacterial species found to be related to any cytological outcome ($P < 0.2$) were included in a multivariate linear regression (continuous outcome) to explain variation in cell populations using manual backward exclusion of variables. Variables were retained in the final model if $P < 0.05$.

The relationship between bacterial species isolated at one time point with bacteria isolated later was examined initially by calculating spearman coefficients because presence or absence of a bacterial species was a dichotomous variable. Bacteria with significant relationships ($P < 0.2$) were included in multivariate logistic regression, with manual backward exclusion of variables. Variables were retained in the final model if $P < 0.05$. The relationship between bacterial species at different time points and cytological findings at each time point with reproductive outcomes was explored in a similar fashion. Pregnancy to first insemination and pregnancy at 150 days in milk were used as outcomes. When cytological parameters were found to be related to pregnancy outcomes, they were dichotomized using receiver operating characteristic (ROC) curve analysis to find the cutoff point with the highest sum of sensitivity and specificity. The dichotomized parameters were then used in a Cox regression model to calculate hazard of pregnancy. Cows declared “do not breed” were censored on the day that decision was made. Statistical significance was assumed at $P \leq 0.05$, with tendencies reported at $0.05 < P < 0.10$. All statistical calculations were performed using Stata IC 11.1 (StataCorp, College Station TX) or GraphPad Prism 5.04.

3. RESULTS

3.1. Demographics

Cows in this trial ranged in parity from 1 to 6 (median 2, interquartile range: 1-3). All cows calved spontaneously and had single, live calves, of which 57 % (32/56) were male. Mean days to first insemination were 60.9 (SD = 8.5 days; range 53 – 100 days). Pregnancy to first insemination was 31% (15/49; 7 cows were designated as “do not breed” for reasons unrelated to this trial) and 49 % of cows bred (24/49) were pregnant by 150 days postpartum.

3.2. Endometrial cytology

Figure 1 depicts the changes in cell populations in endometrial cytology preparations over time postpartum. Compared to the proportion of cells on the day of calving, the polymorphonuclear neutrophil (PMN) proportion was unchanged at 1 and at 3 weeks postpartum, but decreased by 5 and 7 weeks postpartum ($P < 0.001$; Fig. 1A). The proportion of macrophages ($P = 0.175$; Fig. 1B) and lymphocytes ($P = 0.384$; Fig. 1C) did not change significantly with time postpartum but the lymphocyte proportion at 7 weeks was greater than that at calving ($P = 0.013$). The proportion of neutrophils and lymphocytes tended to be negatively correlated with each other, significantly so at 7 d ($r = -0.45$, $P = 0.003$) and 21 d ($r = -0.51$, $P = 0.0002$) postpartum.

The cell populations most closely related to pregnancy outcomes were the proportions of PMN. Specifically, the PMN population at 7 d postpartum was positively correlated to pregnancy ($r = 0.41$; $P = 0.01$) and PMN proportion at 49 d postpartum was negatively correlated with pregnancy ($r = -0.38$; $P = 0.01$). The proportion of neutrophils at 7 d postpartum was negatively correlated with the proportion at 7 weeks postpartum ($r = -0.39$, $P = 0.02$). Since a positive effect of higher neutrophil proportion at 7 days postpartum has not previously been reported, it was explored further. For PMN at 7 days postpartum 40 % was chosen as a cutoff point based on receiver operating characteristic curves. This value provided the greatest sum of sensitivity and specificity with pregnancy at 150 days postpartum as the outcome. Using the dichotomized values for PMN proportion at 7 days postpartum as a variable in Cox's proportional hazards regression (with parity as covariate), cows with high PMN proportion were found to have

increased hazard of pregnancy (HR = 3.85; $P = 0.01$). Figure 2 depicts a Kaplan-Meier curve showing this result.

There was a tendency for a negative correlation between the proportion of macrophages at 7 d postpartum and pregnancy to first AI ($r = -0.27$, $P = 0.08$) and the proportion of lymphocytes at 7 d postpartum was positively correlated to risk of pregnancy by 150 d postpartum ($r = 0.32$, $P = 0.04$) despite the fact that the proportion of lymphocytes at 7 d postpartum was negatively correlated to the proportion of PMN at the same time ($r = -0.45$, $P = 0.003$). When these cell types were combined in Cox's proportional hazards analysis, the proportions of neutrophils and of lymphocytes remained significant, with both exercising a positive effect on hazard of pregnancy (Table 1).

Table 1. Coefficients from Cox's Proportional Hazards for pregnancy, depicting the influence of proportion of neutrophils and of neutrophils at 7 d postpartum.

Parameter	Hazard Ratio	95% CI	<i>P</i>
Neutrophils at 7 d	1.04	1.01 – 1.08	0.009
Lymphocytes at 7 d	1.08	1.006 – 1.17	0.034

No cytological parameter at 21 d postpartum was correlated with any fertility parameter (pregnancy to first AI, pregnancy by 150 DIM or pregnancy by 300 DIM).

At 35 and 49 d postpartum the proportion of neutrophils was negatively correlated to pregnancy at 150 DIM, consistent with numerous previous studies [6,7,26-30]. Given the well-known

nature of this relationship and the small size of the current study, the effect of elevated neutrophil proportion at 5 or 7 weeks postpartum was not further investigated.

3.3.Uterine bacteriology

The most common aerobic bacterial species isolated on the day of calving and at 7 d postpartum were alpha-hemolytic *Streptococcus* spp. and *Escherichia coli*. These species rapidly became less common. By 21 d postpartum *E. coli* was rare and *Trueperella pyogenes* was more common. Figure 3 depicts a prevalence of the most commonly isolated aerobic bacterial species.

Figure 4 shows similar results for anaerobic bacteria. Clostridial species (particularly *C. perfringens*) were most common in very early lactation. Other species were present at low prevalence throughout the study period.

The effects of individual bacterial species and major categories (aerobic and anaerobic) at different intervals postpartum on subsequent pregnancy were explored. Interestingly, the presence of *Streptococcus* spp. (alpha-hemolytic Streptococci) at 1 week postpartum was positively associated with pregnancy (Cox HR = 3.4, P = 0.02). There was a positive correlation between the presence of streptococci at 7 d postpartum and proportion of PMN at the same time. Cows with *Streptococcus* spp. infection at 7 d postpartum had 59.5 ± 4 % neutrophils in endometrial cytology and those without the presence of this bacterium had 34.8 ± 4 % ($P = 0.04$).

The presence of *Trueperella pyogenes* at 3 weeks postpartum was associated with reduced risk of pregnancy at 150 days postpartum (OR = 0.102, 95% CI = 0.022 – 0.466; P = 0.003). The number of anaerobic isolates was small after 3 weeks postpartum. Isolation of *Clostridium perfringens* early postpartum did not influence subsequent reproduction, or subsequent bacterial populations. It had no direct effect on endometrial cytology. The presence of anaerobes at 5 weeks postpartum (predominantly *Fusobacterium necrophorum* and *Prevotella melaninogenica*) tended to reduce the hazard of pregnancy (HR = 0.157, 95% CI = 0.019 – 1.26; P = 0.081). In this study we found no influence of *Mycoplasma* or *Ureaplasma* species on reproductive performance, although numbers were severely limiting.

Cows with *E. coli* infection at 7 d postpartum had increased risk of having *T. pyogenes* at 21 d postpartum (OR = 4.8, 95% CI: 1.3 – 17.4; P = 0.02) and also of being infected with the gram negative anaerobes *F. necrophorum* and *P. melaninogenica* (OR = 4.5; 95 % CI 1.06 – 19.4; P = 0.042). In turn, cows with *T. pyogenes* at 21 d postpartum were more likely to have concomitant infection with the gram negative anaerobes (OR = 18.4; 95 % CI 3.0 – 114; P = 0.002) and tended to have greater risk of infection with these pathogens at 35 d postpartum (OR = 9.7; 95 % CI 0.89 – 105; P = 0.06).

3.4.Bacterial isolates and cytology

The relationship between bacterial isolates and endometrial cytology was explored. In particular, the bacterial species associated with the PMN proportion at 1 week and 5 or 7 weeks postpartum were investigated, since they had positive or negative effects on subsequent reproduction.

The presence of aerobic bacteria (all species combined) on the day of calving was associated with a lower contemporaneous proportion of PMN. Cows with infection had 43 ± 5 % PMN compared to 60 ± 5 % PMN for those uninfected ($P = 0.04$). A similar observation was made for anaerobic bacteria. Cows infected with anaerobes had 40 ± 9 % PMN while those free of anaerobic infection had 61 ± 4 % PMN ($P = 0.03$).

By 1 week postpartum cows with streptococci isolated from the uterus had higher PMN proportions in endometrial cytology than those free of streptococci (60 ± 5 % and 35 ± 4 %, respectively; $P = 0.37$). This has been alluded to above. Other individual bacterial species had no significant effect in very early lactation.

There was no correlation between cytological parameters at 3 weeks postpartum and any reproductive parameter (pregnancy to first AI, pregnancy by 150 days, or pregnancy by 300 days). However, the presence of bacteria in the uterus at 3 weeks postpartum was associated with higher PMN proportion at this time. Cows with no isolated bacteria had 38 ± 5 % PMN and those with bacterial isolates of any species had 57 ± 3 % PMN ($P = 0.002$). Considering only aerobic bacteria, the PMN proportions were 40 ± 5 % and 57 ± 3 %, respectively ($P = 0.01$), and for anaerobes alone 42 ± 4 % and 59 ± 4 %, respectively ($P = 0.015$). When bacterial species were considered individually, the only significant effect observed was due to the presence of *T. pyogenes*. Cows from which *T. pyogenes* was isolated had 62 ± 3 % PMN while those free of the bacterium had 42 ± 4 % PMN ($P = 0.003$). The presence of *T. pyogenes* and the concurrent presence of gram negative anaerobes (either *Prevotella melaninogenica* or

Fusobacterium necrophorum or both) at 3 weeks postpartum was correlated ($r = 0.58$, $P = 0.0003$).

The proportion of PMN in endometrial cytology samples at 35 d postpartum was influenced by the presence of *T. pyogenes* at 21 and at 35 d postpartum and by the presence of the gram negative anaerobes at 35 d postpartum. Anaerobic pathogens were combined because of small numbers in each species. The final regression model for predicting percentage of PMN at 35 d postpartum included only *T. pyogenes* at 3 weeks and anaerobes at 5 weeks postpartum. It is summarized in Table 2.

Table 2. Linear regression model for effect of uterine bacteria on the proportion of neutrophils at D 35 postpartum. (Overall $r^2 = 0.36$; $P = 0.0005$)

Variable	Coefficient	95 % CI	<i>P</i>
<i>T. pyogenes</i> at 21 d	15.34	1.12 – 29.57	0.035
Gram negative anaerobes at 35 d	17.31	11.56 – 57.85	0.004
Constant	11.07	3.88 – 18.25	0.004

The proportion of PMN in endometrial cytology samples at 49 d postpartum was affected by the contemporaneous presence of *T. pyogenes* or *Prevotella melaninogenica*. Bacteria present before 7 weeks did not significantly affect PMN at 7 weeks. The effect of these two organisms was evaluated using multiple linear regression, the results of which are summarized in Table 3.

Table 3. Linear regression model for effect of uterine bacteria on the proportion of neutrophils at D 49 postpartum. (Overall $r^2 = 0.43$; $P < 0.0001$)

Variable	Coefficient	95 % CI	<i>P</i>
<i>T. pyogenes</i> at 49 d	12.67	0.046 – 25.30	0.049
<i>P. melaninogenica</i> at 49 d	20.03	5.70 – 34.36	0.008
Constant	7.19	3.42 – 10.95	0.001

4. DISCUSSION

Perhaps the most unexpected finding of this study was the observation that high proportions of neutrophils in endometrial cytology samples obtained soon after parturition are beneficial to uterine health and subsequent pregnancy. The fact that bacterial infection with any species of bacterium was less likely in cows with high proportions of uterine neutrophils early postpartum is consistent with this finding, as is the observation that PMN proportion at 7 d postpartum is negatively associated with PMN at 7 weeks postpartum. High uterine PMN population in the later postpartum period is well known to be associated with poor reproductive outcome [4-6,22,26,27,31]. It seems that cows that are capable of recruiting large numbers of neutrophils rapidly to the uterus in the immediate postpartum period are likely to have the healthiest postpartum course. This is also consistent with the well-established finding that retention of the fetal membranes in cows is associated with failure to recruit adequate numbers of neutrophils to the placental interface [32]. Furthermore, it has been clearly shown that negative energy balance in the periparturient period is a major risk factor for postpartum endometritis [13,22,23,28] and that cows with improved periparturient energy balance had higher proportions of neutrophils in

cytological preparations at 7 d postpartum [24]. Additionally, it appears that diminished expression of inflammatory cytokines in the periparturient period may increase risk of postpartum uterine disease [33]. Therapeutic interventions to increase mobilization of neutrophils to the early postpartum uterus may be beneficial to uterine health and reproduction, for example by local application of chemokines such as CXCL8 (formerly IL-8), which is involved in neutrophil recruitment to the placental interface at parturition [32]. Conversely, this finding might explain why therapeutic interventions with the potential to inhibit neutrophil recruitment to the uterus, such as non-steroidal inflammatory treatment in the periparturient period, have been found to be detrimental to health and fertility of cows in some studies [34-36].

We found that the proportion of neutrophils in postpartum endometrial cytology remained high for the first three weeks postpartum, declining thereafter. This finding suggests that neutrophils at this time are a reflection of physiological inflammatory processes associated with uterine remodeling and that it is not appropriate to diagnose pathological inflammation before 21 days postpartum.

Another novel finding of this study is that infection with alpha-hemolytic streptococci, generally regarded as non-pathogenic, opportunist contaminants of the bovine uterus [37], had a positive association with subsequent pregnancy as well as being associated with the recruitment of large numbers of neutrophils to the uterus in the early postpartum period. Bonnett et al. previously reported that alpha-hemolytic streptococci isolated at 26 and 40 d postpartum were associated with reduced endometrial inflammation in endometrial biopsies obtained at the same time [38], but our observation is the first to associate very early presence of alpha hemolytic streptococci with

beneficial effects on subsequent inflammation and on fertility, occurring much later in lactation. This raises the possibility of successful probiotic treatment of early postpartum cows with alpha-hemolytic streptococci to mediate recruitment of uterine neutrophils with a potentially beneficial effect on subsequent uterine health and reproductive performance. A similar approach has yielded success in colonizing the nasopharynx of infants with a beneficial flora [39].

A major goal of this project was to determine whether endometritis, as indicated by endometrial cytology, persisted after resolution of bacterial infection. Our results indicate that PMN proportion in cytological preparations was related to contemporaneous or immediately preceding bacterial infection from 3 weeks postpartum onwards. Specifically, the presence of *T. pyogenes* in the uterus at 3 weeks postpartum mediated the increase in uterine PMN at that time and was also a risk factor for concurrent and subsequent infection with the gram negative anaerobes *P. melaninogenica* and *F. necrophorum* as was also reported by Bonnett [38]. This triad of bacteria accounted for most of the variance in PMN proportion observed at 5 and 7 weeks postpartum. High proportion of uterine PMN at this stage postpartum has consistently been related to poor reproductive performance. We therefore conclude that, at least for the period studied here, endometrial cytological evidence of endometritis reflects concurrent bacterial infection. This also suggests that uterine infection with these pathogens persists longer than has previously been recognized. Earlier workers regarded the uterus as essentially sterile after 4 weeks postpartum [40-42].

This study confirmed the well-established observations that the presence *E. coli* immediately postpartum or during the first postpartum week predisposes cows to *T. pyogenes* infection at 3

weeks postpartum [43-46] and that *T. pyogenes* after 21 days postpartum is associated with concomitant or subsequent infection with gram negative anaerobes *F. necrophorum* and *P. melaninogenica*, with increased risk of endometritis, and with reduced risk of pregnancy [37,38,45,47,48]. Modern studies have employed metagenomic methods to define the microbiome of the postpartum uterus. Not surprisingly, these have suggested that the gram-negative anaerobes are much more common than indicated by conventional culture methods [45,47], but the importance of these organisms and of *T. pyogenes* in mediating uterine disease and infertility in our study is consistent with more comprehensive modern knowledge.

5. CONCLUSIONS

Rapid recruitment of neutrophils to the uterine lumen, reflected in a high neutrophil proportion at calving and 7 d postpartum was associated with improved fertility. Persistent high neutrophil proportions at 35 and 49 d postpartum were detrimental to fertility. *Trueperella pyogenes* at and after 21 d postpartum and gram negative anaerobes (*F. necrophorum* and *P. melaninogenica*) at and after 21 d postpartum mediated increased neutrophil proportion at 35 and 49 d postpartum and reduced fertility. Presence of alpha-hemolytic streptococci in the early postpartum period was beneficial to subsequent fertility.

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7. REFERENCES:

- [1] Cheong SH, Nydam DV, Galvão KN, Crosier BM, Gilbert RO. Cow-level and herd-level risk factors for subclinical endometritis in lactating Holstein cows. *J Dairy Sci* 2011;94: 762-70.
- [2] Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. Definitions and diagnosis of postpartum endometritis in dairy cows. *J Dairy Sci* 2010;93: 5225-33.
- [3] Dubuc J, Duffield TF, Leslie KE, Walton JS, Leblanc SJ. Effects of postpartum uterine diseases on milk production and culling in dairy cows. *J Dairy Sci* 2011;94: 1339-46.
- [4] Galvão KN, Frajblat M, Brittin SB, Butler WR, Guard CL, Gilbert RO. Effect of prostaglandin F-2 alpha on subclinical endometritis and fertility in dairy cows. *Journal of Dairy Science* 2009;92: 4906-13.
- [5] Vieira-Neto A, Gilbert RO, Butler WR, Santos JEP, Ribeiro ES, Vercouteren MM, Bruno RG, Bittar JHJ, Galvao KN. Individual and combined effects of anovulation and cytological endometritis on reproductive performance of dairy cows. *Journal of Dairy Science* 2014;97: 1-11.
- [6] Gilbert RO, Shin ST, Guard CL, Erb HN, Frajblat M. Prevalence of endometritis and its effects on reproductive performance of dairy cows. *Theriogenology* 2005;64: 1879-88.
- [7] Sheldon IM, Lewis GS, LeBlanc S, Gilbert RO. Defining postpartum uterine disease in cattle. *Theriogenology* 2006;65: 1516-30.
- [8] Sheldon IM, Dobson H. Postpartum uterine health in cattle. *Animal Reproduction Science* 2004;82-83: 295-306.
- [9] Sheldon IM, Williams EJ, Miller AN, Nash DM, Herath S. Uterine diseases in cattle after parturition. *Vet J* 2008;176: 115-21.
- [10] Sheldon IM. The postpartum uterus. *The Veterinary clinics of North AmericaFood animal practice* 2004;20: 569-91.
- [11] Sheldon IM, Rycroft AN, Zhou C. Association between postpartum pyrexia and uterine bacterial infection in dairy cattle. *The Veterinary record* 2004;154: 289-93.
- [12] Sordillo LM, Contreras GA, Aitken SL. Metabolic factors affecting the inflammatory response of periparturient dairy cows. *Anim Health Res Rev* 2009;10: 53-63.
- [13] Galvão KN, Flaminio MJ, Brittin SB, Sper R, Fraga M, Caixeta L, Ricci A, Guard CL, Butler WR, Gilbert RO. Association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. *J Dairy Sci* 2010;93: 2926-37.
- [14] Hill J, Gilbert R. Reduced quality of bovine embryos cultured in media conditioned by exposure to an inflamed endometrium. *Aust Vet J* 2008;86: 312-6.

- [15] Gilbert RO. The effects of endometritis on the establishment of pregnancy in cattle. *Reprod Fertil Dev* 2011;24: 252-7.
- [16] Herath S, Williams EJ, Lilly ST, Gilbert RO, Dobson H, Bryant CE, Sheldon IM. Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. *Reproduction* 2007;134: 683-93.
- [17] Sheldon IM, Price SB, Cronin J, Gilbert RO, Gadsby JE. Mechanisms of Infertility Associated with Clinical and Subclinical Endometritis in High Producing Dairy Cattle. *Reproduction in Domestic Animals* 2009;44: 1-9.
- [18] Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. Risk factors for postpartum uterine diseases in dairy cows. *J Dairy Sci* 2010;93: 5764-71.
- [19] Gilbert RO, Shin ST, Guard CL, Erb HN. Incidence of endometritis and effects on reproductive performance of dairy cows. *Theriogenology* 1998;49: 251-.
- [20] Santos NR, Lamb GC, Roman HB, Gilbert RO. Postpartum endometrial cytology in beef cows. *Theriogenology* 2005;64: 796-.
- [21] Galvão KN, Brittin SB, Frajblat M, Gilbert RO. Defining cutoff points for subclinical endometritis at different stages of lactation. *Journal of Dairy Science* 2007;90: 13-.
- [22] Cheong SH, Nydam DV, Galvão KN, Crossier BM, Gilbert RO. Cow-level and herd-level risk factors for subclinical endometritis in lactating Holstein cows. *Journal of Dairy Science* 2011;94: 762-70.
- [23] Yasui T, McCann K, Gilbert RO, Nydam DV, Overton TR. Associations of cytological endometritis with energy metabolism and inflammation during the periparturient period and early lactation in dairy cows. *J Dairy Sci* 2014;97: 2763-70.
- [24] Yasui T, McArt JA, Ryan CM, Gilbert RO, Nydam DV, Valdez F, Griswold KE, Overton TR. Effects of chromium propionate supplementation during the periparturient period and early lactation on metabolism, performance, and cytological endometritis in dairy cows. *J Dairy Sci* 2014;97: 6400-10.
- [25] de Boer MW, LeBlanc SJ, Dubuc J, Meier S, Heuwieser W, Arlt S, Gilbert RO, McDougall S. Invited review: Systematic review of diagnostic tests for reproductive-tract infection and inflammation in dairy cows. *J Dairy Sci* 2014;97: 3983-99.
- [26] Kasimanickam R, Duffield TF, Foster RA, Gartley CJ, Leslie KE, Walton JS, Johnson WH. Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. *Theriogenology* 2004;62: 9-23.
- [27] Barlund CS, Carruthers TD, Waldner CL, Palmer CW. A comparison of diagnostic techniques for postpartum endometritis in dairy cattle. *Theriogenology* 2008;69: 714-23.
- [28] Hammon DS, Evjen IM, Dhiman TR, Goff JP, Walters JL. Neutrophil function and energy status in Holstein cows with uterine health disorders. *Veterinary immunology and immunopathology; Veterinary immunology and immunopathology* 2006;113: 21-9.
- [29] Lincke A, Drillich M, Heuwieser W. Subclinical endometritis in dairy cattle and its effect on reproductive performance--a review on recent publications. *Berliner und Munchener tierarztliche Wochenschrift* 2007;120: 245-50.
- [30] de Boer MW, LeBlanc SJ, Dubuc J, Meier S, Heuwieser W, Arlt S, Gilbert RO, McDougall S. Invited review: Systematic review of diagnostic tests for reproductive-tract infection and inflammation in dairy cows. *J Dairy Sci* 2014.
- [31] Dubuc J, Duffield TF, Leslie KE, Walton JS, Leblanc SJ. Randomized clinical trial of antibiotic and prostaglandin treatments for uterine health and reproductive performance in dairy cows. *J Dairy Sci* 2011;94: 1325-38.

- [32] Kimura K, Goff JP, Kehrli ME, Jr., Reinhardt TA. Decreased neutrophil function as a cause of retained placenta in dairy cattle. *Journal of Dairy Science* 2002;85: 544-50.
- [33] Galvao KN, Felipe MJ, Brittin SB, Sper R, Fraga M, Galvao JS, Caixeta L, Guard CL, Ricci A, Gilbert RO. Evaluation of cytokine expression by blood monocytes of lactating Holstein cows with or without postpartum uterine disease. *Theriogenology* 2012;77: 356-72.
- [34] Shwartz G, Hill KL, VanBaale MJ, Baumgard LH. Effects of flunixin meglumine on pyrexia and bioenergetic variables in postparturient dairy cows. *J Dairy Sci* 2009;92: 1963-70.
- [35] Stahringer RC, Neuendorff DA, Randel RD. The effect of aspirin administration and parity on plasma salicylate concentrations and postpartum reproductive parameters in Brahman cows. *Prostaglandins Other Lipid Mediat* 1999;58: 125-38.
- [36] Duffield TF, Putnam-Dingwell H, Weary DM, Skidmore AK, Neuder LM, Raphael W, Millman S, Newby N, Leslie KE. Effect of flunixin meglumine treatment following parturition on cow health and milk production. *Journal of Dairy Science* 2009;92: 118.
- [37] Williams EJ, Fischer DP, Pfeiffer DU, England GC, Noakes DE, Dobson H, Sheldon IM. Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. *Theriogenology* 2005;63: 102-17.
- [38] Bonnett BN, Martin SW, Gannon VP, Miller RB, Etherington WG. Endometrial biopsy in Holstein-Friesian dairy cows. III. Bacteriological analysis and correlations with histological findings. *Can J Vet Res* 1991;55: 168-73.
- [39] Sprunt K, Leidy G. The use of bacterial interference to prevent infection. *Can J Microbiol* 1988;34: 332-8.
- [40] Griffin JF, Hartigan PJ, Nunn WR. Non-specific uterine infection and bovine fertility. I. Infection patterns and endometritis during the first seven weeks post-partum. *Theriogenology* 1974;1: 91-106.
- [41] Elliott L, McMahon KJ, Gier HT, Marion GB. Uterus of the cow after parturition: bacterial content. *Am J Vet Res* 1968;29: 77-81.
- [42] Bekana M, Jonsson P, Kindahl H. Intrauterine bacterial findings and hormonal profiles in post-partum cows with normal puerperium. *Acta Vet Scand* 1996;37: 251-63.
- [43] Bicalho RC, Machado VS, Bicalho ML, Gilbert RO, Teixeira AG, Caixeta LS, Pereira RV. Molecular and epidemiological characterization of bovine intrauterine *Escherichia coli*. *J Dairy Sci* 2010;93: 5818-30.
- [44] Bicalho ML, Machado VS, Oikonomou G, Gilbert RO, Bicalho RC. Association between virulence factors of *Escherichia coli*, *Fusobacterium necrophorum*, and *Arcanobacterium pyogenes* and uterine diseases of dairy cows. *Vet Microbiol* 2012;157: 125-31.
- [45] Machado VS, Oikonomou G, Bicalho ML, Knauer WA, Gilbert R, Bicalho RC. Investigation of postpartum dairy cows' uterine microbial diversity using metagenomic pyrosequencing of the 16S rRNA gene. *Vet Microbiol* 2012;159: 460-9.
- [46] Sheldon IM, Rycroft AN, Dogan B, Craven M, Bromfield JJ, Chandler A, Roberts MH, Price SB, Gilbert RO, Simpson KW. Specific strains of *Escherichia coli* are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. *PLoS One* 2010;5: e9192.
- [47] Santos TMA, Gilbert RO, Bicalho RC. Metagenomic analysis of the uterine bacterial microbiota in healthy and metritic postpartum dairy cows. *Journal of Dairy Science* 2011;94: 291-302.

537 [48] Machado VS, Bicalho ML, Meira Junior EB, Rossi R, Ribeiro BL, Lima S, Santos T,
538 Kussler A, Foditsch C, Ganda EK, Oikonomou G, Cheong SH, Gilbert RO, Bicalho RC.
539 Subcutaneous Immunization with Inactivated Bacterial Components and Purified Protein
540 of *Escherichia coli*, *Fusobacterium necrophorum* and *Trueperella pyogenes* Prevents
541 Puerperal Metritis in Holstein Dairy Cows. PLoS One 2014;9: e91734.
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Postpartum progression of PMN proportion

Progression of macrophage proportion

Progression of lymphocyte proportion

545 figure 1. Proportion of leukocytes in endometrial cytology preparations by week postpartum.
546 Columns not sharing a common lower case superscript differ ($P < 0.05$) A. Neutrophil
547 proportion remained high until 21 days postpartum and declined thereafter. At 35 and 49 days
548 postpartum proportion of neutrophils was significantly lower than on Days 0, 7 and 21, which
549 did not differ from each other. B. Macrophage proportion did not change significantly as days
550 postpartum increased. C. Lymphocyte proportion did not change significantly as days
551 postpartum increased. However, lymphocyte proportion on day 49 was higher than on Day 0,
552 but not different from other days.
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556 Figure 2. Proportion of PMN at 7 days postpartum was related to subsequent reproductive
557 performance. Cows with PMN proportion > 40 % had higher likelihood of pregnancy ($P = 0.01$).

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559

560 Figure 3. The presence of alpha-hemolytic streptococci during the first 49 d postpartum was
 561 beneficial to future reproductive performance.

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Figure 4. Prevalence of common bacteria isolated from the uterus of postpartum cows, and total proportion of cows from which aerobes or anaerobic organisms were isolated.