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Short Communication



SHORT COMMUNICATION

# The effects of protein level on cytokines and chemokines in the uterine environment of beef heifers during development

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## Abstract

The development of replacement heifers is crucial for breeding success and herd efficiency. Nutritional management can affect not only reproductive development but also the inflammatory status of the uterine environment, which may impact reproductive functions such as pregnancy establishment and development. The study herein evaluated the concentration of cytokines and chemokines in the uterus of heifers supplemented with different levels of protein. Angus heifers ($n$ = 60) were blocked by body weight (**BW**) and randomly assigned to 1 of 3 treatments based on protein supplementation level: control of 10% crude protein (CON), 20% crude protein (P20), or 40% crude protein (P40). BW, body condition score, and blood samples were taken every 2 wk for 140 d to monitor development. Uterine flushes were performed monthly and concentrations of cytokines (IL-1α, IL-1β, TNF-α, IFN-γ, IL-10, VEGF-α, IL-17A, and IL-36RA) and chemokines (IL-8, MCP-1, MIP-1α, and MIP-1β) were quantified via ELISA multiplex. To test if there were mean differences in cytokines between the treatment groups or over time, PROC GLIMMIX (SAS v 9.4) was utilized. Concentrations of all cytokines and chemokines, except IL-1α, changed throughout heifer development ($P < 0.05$). Heifers in the P40 treatment group displayed reduced concentrations of MCP-1 ($P = 0.007$) and tended to have decreased concentrations of IFN-γ ($P = 0.06$). Cytokine IL-36RA tended ($P = 0.06$) to be affected by protein level, with the lowest concentrations observed in CON heifers. Most cytokines and chemokines increased following the initial month of supplementation ($P < 0.05$). The increase in concentrations after 1 mo may indicate an adaptive response in the uterus to diet change. Cytokines and chemokines fluctuated due to physiological changes occurring during development. Further research is needed to determine the influence of nutrition on uterine inflammation and long-term impacts on reproductive function.

**Key words:** cytokine, heifer development, inflammation, protein, uterine environment

**Abbreviations**

| | |
|---|---|
| BCS | body condition score |
| BW | body weight |
| CCL | C-C motif ligand |
| CON | control |
| CP | crude protein |
| CTLA8 | cytotoxic T-lymphocyte-associated antigen 8 |
| CXCL-8 | C-X-C motif ligand-8 |
| DDGs | dried distillers grains |
| DM | dry matter |
| IFN-γ | interferon-γ |
| IL | interleukin |
| MCP-1 | monocyte chemoattractant protein-1 |
| MIP | macrophage inflammatory protein |
| P20 | protein 20 treatment |
| $P_4$ | progesterone |
| P40 | protein 40 treatment |
| Th2 | T-helper 2 |
| TNF-α | tumor necrosis factor-α |
| ULF | uterine luminal fluid |
| VEGF-α | vascular endothelial growth factor-α |

## Introduction

The global population is expected to be ~10 billion people by 2050. The livestock industry must increase sustainable production to provide enough nutrient dense food even as land availability decreases (Elliot, 2013; Reynolds et al., 2015). The development of heifers for breeding is crucial toward achieving this goal through optimal reproductive efficiency in beef production. Heifer development is largely influenced by nutrition (Freetly et al., 2011; Perry, 2016), with the goal of achieving puberty early and meeting target body weight (**BW**) prior to the breeding season (Patterson et al., 1992; Perry, 2012). Reproductive functions suffer if adequate nutrition is not provided (Short et al., 1990); therefore, heifers often require nutrient supplementation to ensure development goals are achieved. Generally, heifer development occurs during seasons when forage quality is often lower, creating a need for protein supplementation for adequate growth and performance. However, feedstuffs utilized in different diets can influence systemic inflammation (Chandra, 1997; Zebeli et al., 2012). Additionally, nutritional status can influence immunological responses (Chandra, 1997), for example, cytokine expression systemically can decrease during nutritional deficiency (Kauffman et al., 1986). Specifically, nutrition may influence pro-inflammatory (i.e., Interlukin-1 family, TNF-α, and IFN-γ) and anti-inflammatory (i.e., IL-10 and TGF-β) cytokines and angiogenic cytokines (i.e., VEGF) that are crucial for development and reproductive processes. Therefore, increased inflammation caused by diet differences or nutrient deficiencies may impact the environment of the uterus and reproductive functions.

Prior to hemotrophic nutrient supply, the embryo must utilize substrates present in the uterine environment to grow and develop. To prevent rejection, the uterine environment must become immunotolerant to facilitate attachment and interaction with the embryo (Schjenken et al., 2012). The inflammatory status of the uterus is controlled by cytokines, which may work to create the immunotolerant environment needed for the establishment of pregnancy. Complex inflammatory cytokine networks demonstrate various roles in a wide range of reproductive and pregnancy-related processes such as ovulation (Espey et al., 2004), embryo development (Zolti et al., 1991), and implantation (Simón et al., 1998). Additionally, these cytokines can vary in different stages of the estrous cycle and pregnancy (Krakowski and Zdzisinska, 2007). Chemokines are a family of small cytokines, also known for their role in inflammation and immune surveillance (Dimberg, 2010). Chemokines have been found to be important in uterine remodeling for pregnancy establishment and recruitment of immune cells to the uterus during pregnancy. However, if the balance between cytokines or chemokines shifts to a pro-inflammatory dominated environment, the uterus may become unfavorable for pregnancy, which has been exhibited in humans and mice (Raghupathy, 2001). In the heifer endometrium, inflammation was a major pathway identified among differentially expressed genes between heifers of varying future reproductive success (Killeen et al., 2014). Therefore, identifying when inflammatory signals become present within the uterus of heifers could allow for alterations in management strategies to improve reproductive outcomes.

Inflammatory cytokines and chemokines also function to regulate the processes of angiogenesis, an important natural process used for healing by producing a precise balance of growth and inhibitory factors in healthy tissues. The physiological processes of the formation of new blood vessels and remodeling of existing vessels are likely to be induced by multiple angiogenic cytokines and growth factors, including vascular endothelial growth factor (**VEGF**). In the bovine uterus, the expression of VEGF, and its related ligands and receptors, increases during the implantation period (Hayashi et al., 2019). The increases in angiogenic factors during early pregnancy aids in vascular remodeling and maternal recognition of pregnancy to improve blood supply for pregnancy success (Hayashi et al., 2019). Other cytokines in the interleukin (**IL**)-17 and IL-36 families have multiple roles acting as both pro-inflammatory molecules and stimulants for the release of angiogenic factors.

Since nutrition can affect the overall inflammatory state of the animal and impact immune responses, it is vital to understand how the diet can affect the resident cytokines and chemokines in the uterine environment during heifer development. Therefore, we hypothesized cytokine and chemokine concentrations would be altered in the uterus of developing heifers consuming different levels of protein supplementation. These findings may be useful in developing nutritional strategies specific to optimizing cytokine production for uterine function and maximizing reproductive efficiency.

## Materials and Methods

All experimental procedures involving animals were approved by the University of Tennessee Institutional Animal Care and Use Committee.

### Experimental design and sample collection

Commercial Angus heifers ($n$ = 60, 235.5 ± 29.4 kg; 215 ± 24 d of age) were utilized to determine the effects of protein supplementation on cytokine concentrations in uterine luminal fluid (**ULF**). All animal procedures and diets have been previously described (Brandt et al., 2020). Briefly, all heifers had ad libitum access to native grass hay (95.62% dry matter [**DM**], 7.60% crude protein [**CP**], and 1.97% fat), trace mineral supplement, and water. Heifers were blocked by BW into 4 weight classes ($n$ = 15 heifers per class), and randomly assigned to 1 of 3 supplemental treatments: (1) control (**CON**) 10%

CP supplement consisting of 100% corn, (2) 20% CP supplement (**P20**) consisting of 25% corn and 75% dried distillers grains (**DDGs**), and (3) 40% CP supplement (**P40**) consisting of 25% DDGs and 75% soybean meal. This resulted in diets of ~11%, 15%, and 19% overall CP for CON, P20 and P40 heifers, respectively. Heifers were housed by weight class in ~1 ha lots on native grass with 5 heifers each, resulting in a total of 4 pens per treatment with 1 pen for each weight class. Protein supplements were provided 4 times weekly for 140 d. BW, body condition score (**BCS**), and plasma were taken every 14 d. ULF was collected every 28 d for cytokine quantification by insertion of a Foley catheter through the cervix into the uterine body. Sterile saline (20 mL) was administered through the catheter, manipulated within the uterus, and collected via syringe. All samples were stored at –80 °C until cytokine and hormone analyses could be performed. Cytokine concentrations of IL-1α, IL-1β, TNF-α, IFN-γ, IL-10, IL-17A/CTLA8, IL-36RA/IL-1F5, IL-8/CXCL-8, MCP-1/CCL-2, MIP-1α/CCL-3, MIP-1β/CCL-4, and VEGF-α were quantified within ULF using the MILLIPLEX MAP Bovine Cytokine/Chemokine Magnetic Bead Panel (MilliporeSigma, Burlington, MA) according to manufacturer protocol, and analyzed on the Luminex 200 system (Luminex, Austin, TX) at the University of Tennessee Institute of Agriculture Genomics Hub. Interassay coefficient of variation averaged 22.5% across all cytokines. Progesterone ($\mathbf{P_4}$) concentrations were quantified in plasma using the ImmuChem $P_4$ double antibody radioimmunoassay kit (MP Biomedicals, Costa Mesa, CA) according to manufacturer protocol, and validated by Pohler et al. (2016). Onset of puberty was determined by circulating $P_4 \geq 1$ ng/mL across 2 consecutive samplings representative of a normal estrous cycle pattern (Polat et al., 2009). Five heifers were determined to be pubertal at the initiation of protein supplementation, and 31 heifers were pubertal by the end of study. Progesterone concentrations by treatment and over time, as well as general puberty analyses, are previously reported by Brandt et al. (2020).

## Statistical analyses

A randomized complete block design with sampling and repeated measures was implemented for all statistical analyses in SAS 9.4 (SAS Institute, Cary, NC) blocking on weight class with pen as the experimental unit and heifer as the sampling unit. The GLIMMIX procedure was performed with fixed effects of treatment, sampling time, and the interaction. Random effects included block and heifer nested within the block by treatment. The repeated measure of sampling time was included in the model. Progesterone concentration, BCS, and BW were included as covariates in the model but were removed if nonsignificant ($P > 0.05$). Normality of cytokine concentrations was determined by Shapiro–Wilk statistic > 0.8. Cytokine concentrations that were not normally distributed were log transformed to achieve normality for analyses. Raw means are reported in the following results to express biological relevance.

## Results

The covariate, $P_4$, had a negative relationship with VEGF-α ($P = 0.05$) and MIP-1β ($P = 0.02$), and a positive relationship with IL-36RA ($P < 0.001$) concentrations in ULF during heifer development. Pubertal status, however, was not significant among all cytokines ($P > 0.10$). BW was a significant covariate to IL-1β ($P = 0.02$) with a positive relationship. There were no significant interactions ($P > 0.10$) between sampling day and protein supplement for any of the cytokines and chemokines. Additionally, no effect of sampling day or protein supplementation treatment was detected for IL-1α ($P > 0.10$). Monocyte chemoattractant protein-1 (MCP-1) was affected by protein supplement, with lower concentrations of MCP-1 in the P40 group than the control or P20 groups (Figure 1A; $P = 0.007$). Concentrations of IFN-γ within the uterine lumen tended to

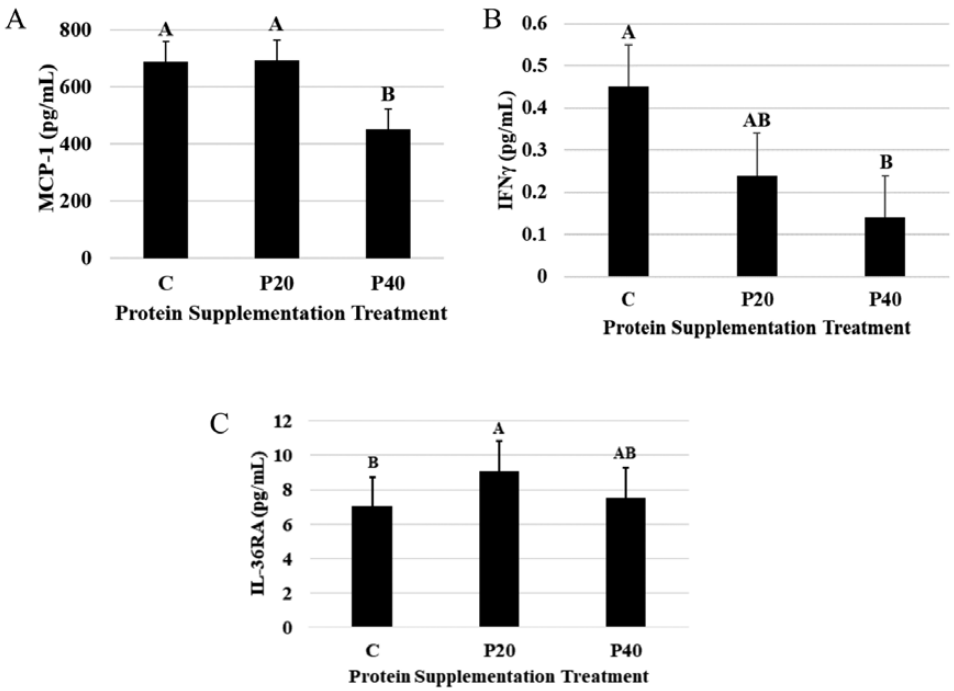


**Figure 1.** Uterine luminal concentrations of MCP-1 (A), IFN-γ (B), and IL-36RA (C) were affected by protein supplementation. [ABCD] Bars that do not share a letter denotes differences at $P \leq 0.05$.

decrease with increased protein supplementation (Figure 1B; $P = 0.06$). Protein supplementation also tended (Figure 1C; $P = 0.06$) to affect IL-36RA, with the highest concentrations in P20 heifers and lowest in CON heifers. All other cytokines and chemokines were not affected by the protein supplementation treatment ($P > 0.10$).

Uterine luminal levels of IL-1β, TNF-α, IL-10, IFN-γ, IL-8, MCP-1, VEGF-α, MIP-1α, MIP-1β, IL-36RA, and IL-17A differed by sampling day regardless of protein supplementation type (Table 1; $P < 0.05$). Interestingly, IL-1β, TNF-α, IL-10, IFN-γ, VEGF-α, IL-17A, IL-36RA, and MCP-1 concentrations significantly increased by the first 28 d of supplementation following the initiation of treatments ($P < 0.05$). Of these cytokines, IL-1β, IFN-γ, VEGF-α, IL-36RA, and MCP-1 concentrations were the highest at day 28; the lowest values for TNF-α, IL-10, IL-8, and MCP-1 were observed on day 0 (Table 1; $P < 0.05$).

The concentrations of inflammatory cytokines IL-1β, TNF-α, IL-10, IFN- γ, and IL-36RA fluctuated in the uterine lumen throughout development with no apparent overall trend (Table 1). Concentrations of IL-1β peaked after day 28 of treatments, with lowest values observed at day 56 ($P < 0.001$). Pro-inflammatory cytokine TNF-α ($P < 0.001$) and anti-inflammatory cytokine IL-10 ($P = 0.002$) followed similar patterns to IL-1β throughout development with lowest values at day 0 and increasing to the highest values between days 56 and 84, then decreasing to intermediary levels at day 140. All other days had significantly lower concentrations, with the lowest observed value on day 112. The highest value for IFN-γ was detected on day 28 with similar values on day 84 ($P = 0.002$). Anti-inflammatory cytokine IL-36RA followed a decreasing trend through development (Table 1; $P < 0.001$), with the highest values on day 28 and lowest values on day 140.

Angiogenic cytokine IL-17A (Table 1; $P = 0.03$) followed an overall decreasing trend throughout the supplementation period, with the highest values on day 28 and lowest values on day 140. When evaluating VEGF-α, elevated levels were maintained throughout development; the concentrations were the highest after 28 d of supplementation, decreased at day 84, and then returned to levels similar to concentrations at day 0 (Table 1; $P = 0.003$).

Chemokines MIP-1α, MIP-1β, and IL-8 increased during heifer development, albeit with varying patterns (Table 1; $P < 0.05$). Macrophage inflammatory protein-1β concentrations were greatest at the end of development on day 140 but lowest on day 112 ($P < 0.001$). Similar to MIP-1β, MIP-1α concentrations were the highest at the end of development on day 140 ($P < 0.001$). IL-8 had the lowest concentrations at the initiation of the study on day 0, with steady increases each month until the highest concentrations at day 140 (Table 1; $P < 0.001$). Monocyte chemoattractant protein-1 had the lowest concentrations at day 0, increased to its highest concentrations at day 28, and remained at comparable levels during remaining period (Table 1; $P = 0.007$).

## Discussion

The use of various levels of supplementary protein had not been previously evaluated regarding inflammation in the uterus. Most research has focused on the diets control of inflammation in the digestive system as the gastrointestinal tract is a major reservoir for immune cells and regulator of immune responses. The successful development of replacement heifers is highly dependent on the nutrition provided throughout puberty, conception, and into the first gestation (Funston et al., 2012; D'Occhio et al., 2019). Diet has been shown to impact systemic inflammatory responses (reviewed in Galland, 2010), therefore, the presence of cytokines and chemokines in the uterus may also be impacted and potentially alter early reproductive success. A major objective of the current study was to determine the effect of protein supplementation on cytokines and chemokines in the uterus; however, many cytokines and chemokines were not impacted by the amount of protein supplementation. Different levels of CP have previously been shown to alter local and systemic inflammatory responses in mice (Tak et al., 2017) and swine (Opapeju, 2010). In mice, increased CP consumption was associated with increases in pro-inflammatory expression (Tak et al., 2017). In swine, decreased CP consumption following a challenge of *Escherichia coli* led to a decreased inflammatory response systemically (Opapeju, 2010). Only intrauterine levels of MCP-1 were determined to be significantly impacted by protein level in the current study, while IFN-γ and IL-36RA tended to be affected by protein supplementation. This could be due to the protein treatment only being a supplement and not a major portion of the diet resulting in smaller difference in protein amongst treatment groups.

**Table 1.** The effects of protein supplementation length on ULF cytokine concentrations in beef heifers during development

| | Day of supplementation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Cytokine[1] | 0 | 28 | 56 | 84 | 112 | 140 | SEM[2] | P-value |
| IFNγ | 0.04$^{b}$ | 0.10$^{a}$ | 0.06$^{ab}$ | 0.12$^{a}$ | 0.03$^{b}$ | 0.03$^{b}$ | 0.02 | 0.001 |
| IL-1β[3] | 0.39$^{bc}$ | 1.27$^{a}$ | 0.36$^{c}$ | 0.91$^{ab}$ | 0.13$^{c}$ | 0.33$^{c}$ | 0.23 | <0.001 |
| IL-8[4] | 339$^{c}$ | 545$^{bc}$ | 388$^{bc}$ | 541$^{bc}$ | 606$^{b}$ | 909$^{a}$ | 135 | <0.001 |
| IL-10 | 0.28$^{c}$ | 1.22$^{ab}$ | 0.99$^{ab}$ | 1.84$^{a}$ | 0.63$^{bc}$ | 0.89$^{ab}$ | 0.45 | <0.001 |
| IL-17A | 0.08$^{b}$ | 0.25$^{a}$ | 0.15$^{ab}$ | 0.13$^{abc}$ | 0.06$^{bc}$ | 0.04$^{c}$ | 0.05 | 0.030 |
| IL-36RA[5] | 1.14$^{c}$ | 4.74$^{a}$ | 3.06$^{ab}$ | 2.42$^{b}$ | 0.47$^{d}$ | 2.66$^{b}$ | 0.83 | <0.001 |
| MCP-1[4] | 460$^{c}$ | 803$^{a}$ | 645$^{abc}$ | 474$^{bc}$ | 625$^{abc}$ | 669$^{ab}$ | 84 | 0.007 |
| MIP-1α | 0.88$^{b}$ | 9.73$^{a}$ | 3.17$^{a}$ | 8.09$^{a}$ | 3.19$^{a}$ | 6.55$^{a}$ | 3.58 | <0.001 |
| MIP-1β[5] | 2.04$^{c}$ | 9.17$^{ab}$ | 6.12$^{b}$ | 5.50$^{bc}$ | 7.81$^{b}$ | 20.40$^{a}$ | 4.16 | <0.001 |
| TNFα | 0.41$^{c}$ | 10.44$^{a}$ | 1.06$^{bc}$ | 7.28$^{a}$ | 0.67$^{c}$ | 2.52$^{b}$ | 1.50 | <0.001 |
| VEGFα[4,5] | 508$^{bcd}$ | 662$^{a}$ | 454$^{cd}$ | 457$^{d}$ | 627$^{ab}$ | 588$^{abc}$ | 70 | 0.003 |

[1]Data were log transformed to achieve normality unless otherwise noted for analyses but are reported as backtransformed means and SEM.
[2]SEM was pooled with each cytokine.
[3]BW was found to be a significant covariate.
[4]Data were normally distributed and thus were not log transformed during analyses.
[5]Progesterone was found to be a significant covariate.

Monocyte chemoattractant protein-1 regulates cell migration and infiltration into tissues. In the reproductive tract, MCP-1 has been shown to be important in uterine macrophage accumulation during pregnancy within mice (Wood et al., 1999) as well as during ovine embryonic attachment and placentation (Asselin et al., 2001). The inflammatory macrophages that are recruited are essential for the establishment of early pregnancy since they influence uterine epithelial cell remodeling to increase receptivity and secrete inflammatory cytokines to facilitate implantation and restructuring of the uterus (Care, 2011). The promoted synthesis of cytokines to modulate this inflammatory response within the uterus is due to the active components within seminal fluid that cooperate with the endometrial epithelial cells to facilitate embryo tolerance and the expansion and implantation in mammals for successful pregnancy (Robertson, 2005). Whether the decreases in MCP-1 herein coincide with increasing CP are indicative of a negative impact of protein supplementation needs to be explored further to understand this interaction.

The current study indicated IFN-γ tended to decrease in the uterus as CP supplementation level increased. Interferon-γ is associated with the pro-inflammatory response and recruiting immune cells to fight pathogens. In mice and humans, the regulation of IFN-γ concentrations have shown to be vital regarding pregnancy success or failure. The presence of IFN-γ is necessary for remodeling and vascularization of the endometrium during early pregnancy prior to implantation (Wu et al., 2006). Natural killer cells that produce IFN-γ have also been found in the bovine endometrium around the time of maternal recognition of pregnancy, potentially to assist in placental attachment and angiogenesis (Oliveira et al., 2013; Vasudevan et al., 2017). However, excessive elevation in IFN-γ concentrations may disrupt the balance between pro- and anti-inflammatory cytokines resulting in complications such as infertility, pregnancy loss, or preterm birth (Raghupathy, 2001; Wilczyński, 2005). Gene expression of IFN-γ was higher in the endometrium of heifers that had nonviable embryos recovered on day 7, compared with heifers with viable embryos (Beltman, 2013). Failure to balance IFN-γ with other inflammatory cytokines may lead to a dysfunctional uterine environment. Therefore, the decrease observed in IFN-y with increasing CP may benefit the uterine environment to maintain an immune-tolerant state. Further studies are needed to evaluate breeding success from different inflammatory environments to determine the benefits for developing an optimal uterine environment in heifers.

The concentrations of inflammatory and angiogenic cytokines increased after 1 mo of protein supplementation treatments. Cytokines often act synergistically and perform similar biological functions to activate and recruit other immune cells and stimulate acute inflammation, both locally and systemically (Dinarello, 1989). In the bovine uterus, the occurrence of these pro-inflammatory cytokines together is typically associated with the postpartum period and the development of disease such as endometritis. Elevation of pro-inflammatory cytokines signals to the immune system the presence of pathogens and requirement for clearance of the infected tissue (Koh et al., 2018). When comparing weeks following parturition, cows that developed endometritis had a reduced expression of pro-inflammatory cytokines earlier in the postpartum period than cows that did not develop endometritis (Galvão et al., 2011). Therefore, animals with a reduced expression of pro-inflammatory cytokines in the uterus may fail to properly respond to the presence of bacteria and be more likely to develop postpartum uterine diseases. The cause of reduced pro-inflammatory cytokine expression in the uterus is unknown, however, changes in the feedstuff provided to cattle have been shown to disrupt rumen bacterial communities and upregulate systemic pro-inflammatory cytokines (Zebeli and Metzler-Zebeli, 2012; Zebeli et al., 2012). The increase in systemic inflammation due to ruminal acidosis has been detected in the uterus, leading to increases in endometrial pro-inflammatory cytokine expression (Bilal et al., 2016). Throughout development, heifers did not exhibit any clinical signs of disease; therefore, the interpretation of our results supports functions of inflammatory cytokines outside of a diseased state. The increased concentration of inflammatory and angiogenic cytokines following the first month of supplementation may reflect effects due to dietary protein supplementation not detected by individual cytokine analyses.

Although there were minimal effects of protein supplementation, all cytokines and chemokines, except IL-1α, were found to significantly shift throughout development. Previous studies have indicated that systemic cytokines may fluctuate by stage of the estrous cycle (Krakowski and Zdzisinska, 2007; Oliveira et al, 2013). The fluctuations observed in the current dataset may reflect changes during puberty attainment or even differences in estrous cycle stage based on animals initiating cyclicity at different times. However, to maintain focus on the objective of this study which was to elucidate the impacts of protein supplementation on uterine environment during the natural onset of puberty this variation was expected and unavoidable. The progesterone and onset of puberty data has been reported previously (Brandt, 2020). Within the endometrium of heifers, the gene expression of IL-1β and IFN-γ, but not IL-1α, IL-6, and IL-10, was affected by stage of the estrous cycle (Oliveira et al., 2013). The current study indicated cytokines IL-1β, TNF-α, IL-10, and IFN-γ fluctuated with no apparent pattern throughout development which may suggest that some effects on cytokines may have been lost due to estrous cycle variation. IL-10 followed a similar pattern to pro-inflammatory cytokine TNF-α in the current study which may indicate an attempt at maintenance between pro- and anti-inflammatory cytokines and could be indicative of increased reproductive success. The amount of IL-10 released in response to a uterine infection may impact future reproductive success by changing the functional focus of IL-10. During pregnancy, IL-10 expression by endometrial immune cells was increased compared with cyclic heifers (Vasudevan et al., 2017) and goats (Imakawa et al., 2005). Postpartum cows that were considered infertile by failure to rebreed had a higher ratio of IL-1α and IL-1β expression to IL-10 expression than those that successfully rebred (Herath et al., 2009). The concentration of IL-1α was relatively stable in the current study; therefore, the impact on rebreeding may be driven by IL-1β concentration. By the end of the current study, 59% of heifers had reached puberty (Brandt et al., 2020). Therefore, the fluctuations observed in uterine cytokine concentrations across development from weaning until breeding may be due to various physiological changes as heifers achieved puberty.

The uterus and other female reproductive organs such as the ovary and placenta exhibit regular intervals of rapid growth with high vascularity and blood flow (Reynolds et al., 2002). During this study, $P_4$ influenced IL-36RA as well as VEGF-α which supports findings that $P_4$ appears to be a primary regulator of uterine vascular function such as angiogenesis in all mammalian species (Murray and Wynn, 2011). This is in contrast with previous research that activated monocytes that produce inflammatory cytokines may contribute to the inhibition of $P_4$ synthesis (Al-Gubory et al., 2012;

Quirk et al., 2013). Additionally, our results demonstrated VEGF-α spiked once early in development and remained elevated through the end of treatments to satisfy the blood flow dependency for development. However, anti-inflammatory cytokine IL-36RA and the angiogenic cytokine IL-17A, both had a negative trend over time which may indicate sufficient blood supply to support physiological function after the establishment of puberty. Another known physiological function of VEGF is related to bone formation and estrogen production, where both bone formation and estrogen secretion increased during early stages of puberty in humans (Emons et al., 2010). Studies on rats showed that in uterus and bone tissue, VEGF expression is upregulated by estrogens (Hyder et al., 1996; Mekraldi et al., 2003). This correspondence of VEGF and estrogen could potentially be relevant within the ULF of bovine to promote puberty.

In conclusion, cytokines and chemokines fluctuate in the uterine lumen throughout heifer development, potentially in response to hormonal variations as heifers achieve pubertal status and physiological changes during growth. The lack of protein correlation could have an association with the numerous processes that must occur in order for cytokines to be expressed in the reproductive tract compared with the gastrointestinal tracts production of cytokines due to the diet. An effect on IFN-γ concentrations may be suggestive of a benefit in the uterine environment for pregnancy establishment. The observed increases in intrauterine cytokines immediately following the beginning of protein supplementation could indicate an initial adaptive response to a diet change. Anti-inflammatory cytokines, such as IL-10, maintained the balance between inflammatory cytokines by following similar concentration patterns throughout development as pro-inflammatory cytokine, TNF-α. Overall, days of development influenced immunological responses through cytokine profiles produced in the uterus of developing heifers. Further studies are needed to fully understand the long-term impacts of dietary manipulations on the uterine environment for replacement heifer reproductive success.

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## Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

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