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Immunology

Reproductive microbiome and cytokine profiles associated with fertility outcomes of postpartum beef cows

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Abstract

Shifts from commensal bacteria (for example, *Lactobacillus* in the phylum Firmicutes) within the reproductive tract have been associated with changes in local reproductive immune responses and decreased fertility in humans. The objective of this study was to characterize the microbiome and cytokine concentrations before artificial insemination (**AI**) in vaginal and uterine flushes from postpartum beef cows. Twenty *Bos indicus*-influenced beef cows (approximately 60 d postpartum and free of reproductive, health, or physical issues) were enrolled. The *B. indicus* prostaglandin (PG) 5-d + controlled intervaginal drug-releasing estrus synchronization protocol was initiated on day −8 of the study with timed AI on d0. Blood samples were collected on days −3, −1, and 28 via coccygeal venipuncture. Vaginal and uterine flushes were collected on days −3 and −1. Based on days 28 pregnancy status determined by transrectal ultrasonography, cows were identified as either Open (n = 13) or Pregnant (n = 7). Bacterial community analyses were conducted targeting the V4 hypervariable region of the 16S rRNA gene. Cytokine analyses were performed using the RayBiotech Quantibody Bovine Cytokine Array Q1 and MyBioSource ELISA kits per the manufacturer's instructions. Statistical analyses for bacteria relative abundance were conducted using PROC NPAR1WAY and for cytokine concentrations using PROC GLM in SAS 9.4. Uterine concentrations of interferon γ, interleukin (**IL**)1α, and IL21 were greater in Open than in Pregnant cows ($P < 0.05$). Regardless of pregnancy status, uterine IL13 increased from days −3 to −1 (9.76 vs. 39.48 ± 9.28 pg/mL, respectively; $P < 0.05$). Uterine relative abundance of the phylum Firmicutes decreased from days −3 to −1 in Open cows (60.4% ± 0.9% vs. 48.5% ± 3.2%; $P = 0.004$). In Open cows, the genus *Blautia* decreased in relative abundance within the uterus from days −3 to −1 (2.1% ± 0.2% vs. 0.9% ± 0.1%; $P = 0.002$). Uterine relative abundance of the phylum Tenericutes increased from days −3 to −1 in Pregnant cows (1.0% ± 0.1% vs. 7.6% ± 4.1%; $P = 0.002$). In Pregnant cows, the genus *Ureaplasma* tended to increase within the uterus from days −3 to −1 (0.08% ± 0.06% vs. 7.3% ± 4.1%; $P = 0.054$). These findings suggest a distinct difference in the reproductive microbiome and cytokine profiles before AI for resulting Open vs. Pregnant cows.

Lay Summary

Efficiently producing cattle to feed a growing population can come with many challenges. A few challenges occur soon after a cow has given birth, and subsequent reproductive performance can be impacted. Bacteria within the reproductive tract can trigger an immune response and together play a role in affecting fertility in cows. The objectives of this experiment were to distinguish the commensal vs. harmful bacteria that reside in the reproductive tract and to characterize the immune response in beef cattle via uterine and vaginal flushes. The results demonstrated that bacteria within the reproductive tract of beef cattle changes before breeding. The current study also suggests that changes in immune response before breeding can be associated with fertility outcomes. Additional research may be worthwhile to evaluate management tactics to positively shift bacteria within the reproductive tract and reduce inflammatory immune responses to improve fertility and increase reproductive efficiency. Future research is necessary to identify the causes of bacterial shifts and how it relates to pregnancy establishment.

Key words: beef cows, cytokine, fertility, microbiome, uterus, vagina

Abbreviations: AI, artificial insemination; BCS, body condition scores; CIDR, controlled intervaginal drug-releasing; CVs, coefficients of variation;CXCL, chemokine ligand; GnRH, gonadotropin-releasing hormone; IFN, interferon; IL, interleukin; LPS, lipopolysaccharides; MIG, monokine induced by gamma; MIP, macrophage inflammatory protein; P4, progesterone; PG, prostaglandin; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; RIA, radioimmunoassay; TAI, timed artificial insemination; TGFβ, transforming growth factor beta; TNF, tumor necrosis factor

Introduction

Reproductive efficiency is a significant economic determining factor for beef producers. In cattle, fertility issues can cost billions of dollars annually and can be attributed to factors such as nutrition, genetics, and stress (Richards et al., 1986; Bellows et al., 2002). Furthermore, these factors present challenging problems in the postpartum cow as they can affect the uterine involution process or the return of the uterus to the standard, nonpregnant size to become pregnant again (Sheldon et al., 2006). During this process, pathogenic bacteria may colonize the reproductive tract because the placenta or necrotic tissues are not fully expelled. Retention of these necrotic tissues may lead to bacterial infections, inflammation, or other uterine diseases, thus compromising fertility (Azawi, 2008; Potter et al., 2010). The immune system detects pathogenic bacteria in the reproductive tract and releases cytokines, which are small communication proteins that can initiate an inflammatory response through the communication of immune cells

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(Zhang and An, 2007). Cytokines can be included in two different categories, pro- and anti-inflammatory. Pro-inflammatory cytokines can be attributed to causing destruction to the host by producing feverish symptoms, tissue destruction, or other detrimental factors, with common examples including interferon (**IFN**)γ, interleukin (**IL**)1, IL6, and tumor necrosis factor alpha (**TNF**α; Dinarello, 2000). Cytokines that are categorized as anti-inflammatory have been seen to reduce inflammation and encourage healing with common examples including IL4, IL10, and IL13 (Dinarello, 2000). In addition, it is known that the immune system is integral in establishing and maintaining pregnancy, performing routine functions for reproduction, and clearing infections or diseases (Bazer et al., 2009).

The microbiome of the human reproductive tract has been relatively well-characterized. *Lactobacillus* dominates the healthy microbiome of the human reproductive tract, creating a low-pH environment (Koedooder et al., 2019). Commensal bacteria within the reproductive tract of cattle remain under-characterized. To date, the microbiome of the bovine reproductive tract is known to be more diverse compared with humans (Swartz et al., 2014). Ault et al. (2019a) demonstrated that relative bacterial abundances change during estrous synchronization. Greater bacterial abundances of specific phyla were seen in cows that have reduced concentrations of progesterone (P4), and in contrast, different phyla were observed in cows that had increased levels of P4 (Ault et al., 2019a). It was also noted that the diversity of bacterial communities would potentially shift as the estrous cycle progresses (Ault et al., 2019b). Specifically, a dramatic shift in bacterial communities' diversity was observed 2 d before artificial insemination (**AI**) with pregnant and non-pregnant cows each having distinct communities (Ault et al., 2019b).

To date, the relationship between reproductive microbiota and cytokines in beef cattle, as it pertains to fertility, remains unclear. Therefore, the first objective of this study was to determine the concentrations of certain pro- and anti-inflammatory cytokines in the uterus, vagina, and plasma and characterize relative bacterial abundance in the uterus and vagina of postpartum beef cows before AI (i.e., between 3 and 1 d before AI). The second objective was to identify associations between cytokine concentrations and relative abundances of bacteria related to fertility (e.g., pregnancy status). The hypothesis is that the relative abundances of commensal and pathogenic bacteria in the uterus and vagina of resulting Open and Pregnant cows would differ before timed AI. Additionally, it is hypothesized that the pro-inflammatory cytokine concentrations would be greater before AI in resulting Open cows compared to resulting Pregnant cows. Together, it is hypothesized that certain pathogenic bacteria will be correlated with pro-inflammatory cytokine levels in resulting Open cows.

Materials and Methods

This study was conducted at the Texas A&M University Beef Cattle Systems in College Station, Texas, and all animal procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University (2020-0077).

Animals

Multiparous *B. indicus*-influenced beef cows (n = 20) from the Texas A&M University Beef Cattle Systems were used for data collection that occurred in May to June 2021. Each cow was approximately 60 d postpartum and free of any known reproductive, health, or physical ailments. Cows were subjected to the *B. indicus* prostaglandin (PG) 5-d + controlled intervaginal drug-releasing (**CIDR**) protocol (Beef Reproduction Task Force, 2021). The protocol is depicted in Figure 1. On day −8, ultrasonography was completed to identify cows with a corpus luteum, and prostaglandin $F_{2\alpha}$ (**$PGF_{2\alpha}$**; Lutalyse, 5 mL; 5 mg/mL; Zoetis Animal Health, Troy Hills, NJ) was administered, and CIDR devices (Eazi Breed CIDR; Zoetis Animal Health) were inserted. On days −3 and −1, ultrasonography was completed to identify cows that responded to the synchronization protocol (e.g., corpus luteum regression) and had a growing ovulatory follicle. On day −3, CIDRs were removed, $PGF_{2\alpha}$ was administered, and heat detection patches (Estrotect; Rockway, Inc., Spring Valley, WI) were applied. Cows were evaluated for patch scores with a scale of 0 to 4 (0, lost patch; 1, <25% activated; 2, 25% to 50% activated; 3, 50% to 75% activated; and 4, >75% activated) as described by Pohler et al. (2016a) on day −1 and day 0.

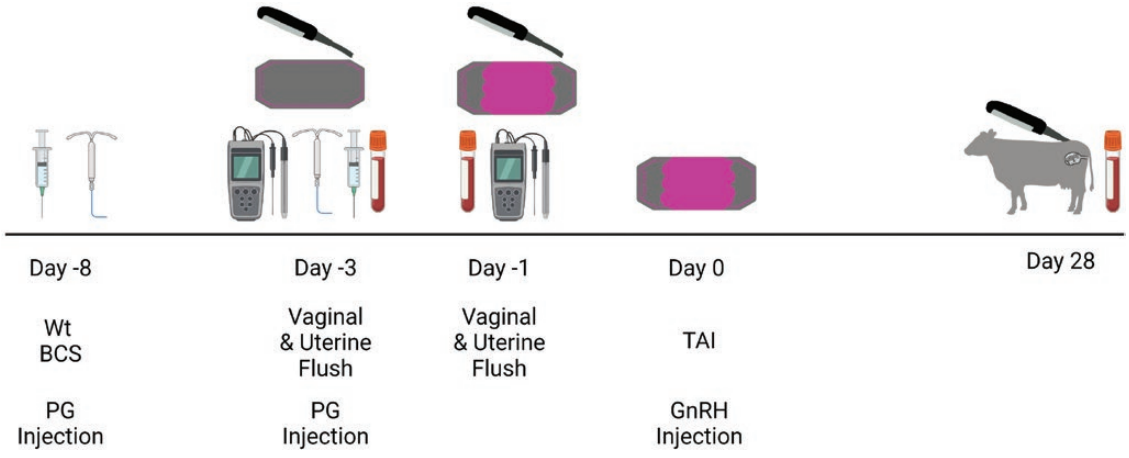

Figure 1. *Bos indicus* prostaglandin (PG) 5-d + CIDR protocol experimental timeline used for cows in May and June 2021 for estrus synchronization. Wt – weight; BCS – Body Condition Score; PG – Progesterone injection; TAI – Timed Artificial Insemination; GnRH – Gonadotropin-Releasing Hormone. Created with BioRender.com.

On day 0, a gonadotropin-releasing hormone (GnRH; Factrel, 100 μg; Zoetis Animal Health) injection was administered, and timed artificial insemination (**TAI**) occurred. Three bulls of known fertility and similar genetic composition were utilized. Two experienced AI technicians performed the inseminations. Experimental groups were assigned post hoc as determined by day 28 pregnancy status via transrectal ultrasonography resulting in Open (n = 13) and Pregnant (n = 7) cows.

Animal measurements and sample collections

Cattle body weight and body condition scores (**BCS**) were recorded on day −8 (at the initiation of the *B. indicus* PG 5-d + CIDR protocol). Vaginal and uterine flush samples were collected on days −3 and −1 for bacterial DNA extraction and sequencing and pH sampling as previously described (Ault et al., 2019a,b; Clemmons et al., 2017; Poole et al., 2023). For the flush samples, the perineal region was disinfected before sample collection to prevent the introduction of external bacteria into the reproductive tract. Sterile saline, 0.9% (Vetivex; Dechra Veterinary Products, Overland Park, KS) was drawn into a 60-mL syringe and infused into the vagina and recovered by vaginal lavage. Uterine flushes were collected by a process similar to the embryo transfer technique. A sterile Foley catheter was placed in the uterine body using a large speculum and a sterile chemise (WatanabeTecnologia Aplicada [WTA]; College Station, TX) to avoid vaginal contamination while placing the catheter into the uterus. Sterile saline was flushed through the catheter and into the uterus and collected by rectal massage. Two blank control flushes were collected through sterile Foley catheters, and these had minimum quality-filtered reads. An Orion Star portable pH meter (Thermo Fisher Scientific Inc., Waltham, MA) was used immediately after collection to measure the pH of flush samples. Vaginal and uterine flush samples were flash-frozen in liquid nitrogen and stored at −80 °C for later analysis.

Blood sampling and progesterone radioimmunoassay

Blood samples were collected on days −3, −1, and 28 via coccygeal venipuncture into 10 mL sterile vacutainer K_2 EDTA collection tubes (BD Vacutainer, Becton, Dickinson and Company, NJ). Samples were immediately placed on ice and centrifuged at 1,500 × g for 20 min at 4 °C . Plasma was transferred into 1.5 mL tubes and stored at −20 °C until P4 and cytokine analysis.

Progesterone concentrations were quantified per the manufacturer's instructions using a commercial double-antibody radioimmunoassay (**RIA**) kit (MP Biomedicals, Santa Ana, CA) as previously described (Pohler et al., 2016b). Concentrations were calculated using a standard curve, including high/low reference samples, for quality control. The intra- and inter-assay coefficients of variation (**CVs**) were 4.89% and 11.29%, respectively.

Cytokine assays

Cytokine analyses were conducted using the Multiplex Quantibody Bovine Cytokine Array Q1 kit (RayBiotech Life, Inc., Peachtree Corners, GA). Concentrations of IL13, IL1α, IL1F5, IL21, IFNα, IFNγ, TNFα, chemokine ligand (**CXCL**)9 or monokine induced by gamma (**MIG**), CXCL10 (interferon gamma-induced protein 10; interferon gamma-induced protein 10 [IP10]), and macrophage inflammatory protein (**MIP**)1b are measured in the Bovine Cytokine Array Q1 kit. Plasma samples were diluted 1:1 as suggested by the manufacturer and days −3 and −1 samples were assayed. Nondiluted uterine and vaginal flush samples were also analyzed. Assays were run per the manufacturer's instructions with slides being stored in a dark box at 4 °C before shipping to the manufacturer for laser scanning and data extraction. These assays have been previously validated by Poole et al. (2019) and (2020).

Plasma and flush concentrations of transforming growth factor beta (**TGFβ**) and IL6 were determined in samples from days −3 to −1 utilizing commercially available Bovine TGFβ and IL6 ELISA kits (MyBioSource, San Diego, CA). Assays were previously validated in-house as described by Poole et al. (2021). The intra- and inter-assay CVs for TGFβ were 6.79% and 13.56%, respectively. The intra- and inter-assay CVs for IL6 were 5.60% and 14.64%, respectively.

DNA extraction and 16S rRNA gene amplicon sequencing

Microbiome analysis was performed on the vaginal and uterine flush samples from days −3 and −1. Samples were delivered to FERA Diagnostics and Biologicals Corp. (College Station, TX) for DNA extraction and 16S rRNA sequencing. Samples were transferred to 96-well plates, and DNA extraction was performed per manufacturer's instructions using the Mag-Bind Universal Pathogen 96 Kit (Omega Bio-Tek, Norcross, GA). The 16S amplicons were amplified by polymerase chain reaction (PCR) for individual metagenomic DNA samples as previously described (Bicalho et al., 2017). The V4 hypervariable region of the bacterial 16S rRNA bacterial genome was amplified with 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers using methods for the Illumina MiSeq platform (Caporaso et al., 2012). The generated 16S rRNA gene sequences were assembled using the standard MiSeq pipeline as previously described (Coil et al., 2015; Bringhenti et al., 2021; Tomazi et al., 2023). The representative sequences for each operational taxonomic unit were compared against the Greengenes database (https://greengenes2.ucsd.edu/) for taxonomy assignment, and only full-length, high-quality reads were used. The MiSeq reporter classification was based on the Greengenes database, and the output used from this workflow was a classification of reads at multiple taxonomic levels (kingdom, phylum, class, order, family, genus, and species). For the purposes of this study, the phylum and genus levels were used.

Statistical analysis

All variables were tested for normality and homogeneity of variance using the HOVTEST=LEVENE option in PROC GLM in SAS 9.4 (SAS Institute, Cary, NC). Bacterial abundances were non-normally distributed, and all other variables were normally distributed. Nonparametric ANOVA in SAS 9.4 (SAS Institute) was used for all bacterial abundance data with the independent variables of day or pregnancy status group. Significance was determined by Wilcoxon exact test for comparisons between pregnancy status groups and for comparisons among days (Weiss et al., 2017). Body weight, BCS, and patch score were analyzed using PROC GLM in SAS 9.4 by pregnancy status. Uterine and vaginal pH, progesterone, and cytokines were analyzed using PROC GLM in SAS 9.4 with fixed effects of day, status, and the interaction. For correlations between parametric data (progesterone and

cytokines), Pearson's correlations were analyzed using PROC CORR in SAS 9.4. For correlations using nonparametric data (bacterial abundances), Spearman's correlations were analyzed using PROC CORR in SAS 9.4. A statistical significance was reported at $P \leq 0.05$. A tendency was reported at $P > 0.05$ and ≤ 0.10.

Results

Body weight, BCS, progesterone, patch score, and pH

Weight ($P = 0.86$), BCS ($P = 0.67$), and patch scores ($P = 0.44$), did not differ by pregnancy status. Progesterone concentrations, uterine flush pH, and vaginal flush pH did not differ between Open and Pregnant cows ($P > 0.05$; Table 1). Progesterone concentrations differed by day decreasing from days –3 to –1 (0.89 vs. 0.08 ± 0.23 ng/mL, respectively; $P = 0.015$). Uterine flush pH (7.03 vs. 6.64 ± 0.04, respectively) and vaginal flush pH (7.12 vs. 6.64 ± 0.04, respectively) significantly decreased from days –3 to –1 ($P < 0.0001$).

Cytokine concentrations – plasma

Cytokine concentrations are listed in Tables 2, 3, and 4. No differences were found in plasma cytokine concentrations by day or status-by-day interaction (Table 2). Plasma concentrations of the cytokines were significantly greater in Open than Pregnant cows for the pro-inflammatory cytokine IFNα (389.6 ± 54.0 vs. 153.5 ± 73.6 pg/mL, respectively; $P = 0.014$), IL1F5 (846.7 ± 154.4 vs. 309.4 ± 210.4 pg/mL, respectively; $P = 0.047$), and the pro-inflammatory cytokine MIP1b (74.3 ± 11.3 vs. 27.9 ± 15.4 pg/mL, respectively; $P = 0.020$). Open cows tended to have greater plasma concentrations than Pregnant cows for the anti-inflammatory cytokine IL13 (1548.0 ± 320.1 vs. 467.0 ± 436.2 pg/mL; $P = 0.053$), the pro-inflammatory cytokine IL1α (277.5 ± 64.0 vs. 82.0 ± 87.1 pg/mL; $P = 0.079$), and the pro-inflammatory cytokine TNFα (2466.0 ± 538.1 vs. 788.3 ± 733.4 pg/mL; $P = 0.079$). Plasma concentrations of TGFβ, an anti-inflammatory cytokine, (549.8 ± 49.3 vs. 338.0 ± 36.2 pg/mL; $P = 0.001$) were greater in Pregnant than Open cows.

Cytokine concentrations – uterine flushes

The uterus had significantly increased levels of IL13 (39.5 ± 9.3 vs. 9.8 ± 9.3 pg/mL; $P = 0.030$), IL1α (7.5 ± 2.0 vs. 0.6 ± 2.0 pg/mL; $P = 0.019$), the anti-inflammatory cytokine IL1F5 (19.6 ± 2.7 vs. 6.1 ± 2.7 pg/mL; $P = 0.001$), the anti-inflammatory cytokine IL21 ($P = 0.026$), MIP1b (11.2 ± 2.0 vs. 1.3 ± 2.0 pg/mL; $P = 0.002$), and TNFα (37.9 ± 10.8 vs. 0.7 ± 10.8 pg/mL; $P = 0.020$) on day –1 when compared with day –3. Additionally, on day –1 compared with day –3 the uterus tended to have increased concentrations of the pro-inflammatory cytokine MIG (20.5 ± 6.7 vs. 2.1 ± 6.7 pg/mL; $P =$

Table 1. Summary statistics for progesterone (P4) concentrations and pH

	Open		Pregnant		P-value		
	Day –3	Day –1	Day –3	Day –1	Status	Day	Status*day
P4, ng/mL	0.48 ± 0.27^{ab}	0.09 ± 0.27^{b}	1.29 ± 0.36^{a}	0.06 ± 0.36^{b}	0.236	0.015*	0.195
Uterine, pH	6.99 ± 0.05^{a}	6.67 ± 0.05^{b}	7.08 ± 0.07^{a}	6.61 ± 0.07^{b}	0.741	<0.0001*	0.175
Vaginal, pH	7.11 ± 0.05^{a}	6.67 ± 0.05^{b}	7.13 ± 0.06^{a}	6.61 ± 0.06^{b}	0.734	<0.0001*	0.435

^{abc}Between day in each row for each pregnancy status separately indicates $P \leq 0.05$.
*Indicates main effect was determined significant at $P \leq 0.05$.

Table 2. Cytokine and chemokine concentrations (pg/mL) in plasma samples from resulting Open and Pregnant cows on days –3 and –1

Plasma	Open		Pregnant		P-value		
	Day –3	Day –1	Day –3	Day –1	Status	Day	Status*day
IFNα	331.7 ± 76.4^{ab}	447.6 ± 76.4^{a}	191.5 ± 104.1^{ab}	115.5 ± 104.1^{b}	0.014*	0.828	0.300
IFNγ	769.1 ± 252.4	782.0 ± 252.4	650.4 ± 344.0	675.6 ± 344.0	0.711	0.950	0.984
IL13	1506.5 ± 452.6	1589.4 ± 452.6	451.1 ± 616.8	482.9 ± 616.8	0.053†	0.916	0.963
IL1α	284.4 ± 90.4	270.6 ± 90.4	97.7 ± 123.2	66.3 ± 123.2	0.079†	0.835	0.936
IL1F5	821.1 ± 218.3	872.3 ± 218.3	299.5 ± 297.6	319.3 ± 297.6	0.047*	0.893	0.952
IL21	15165 ± 3947	8730 ± 3947	7691 ± 5379	3235 ± 5379	0.178	0.256	0.835
IP10	1007.6 ± 264.3	1076.1 ± 264.3	610.3 ± 360.2	791.8 ± 360.2	0.288	0.695	0.859
MIG	3388 ± 1351	3751 ± 1351	1819 ± 1841	2331 ± 1841	0.361	0.788	0.963
MIP1b	58.8 ± 16.0^{ab}	89.7 ± 16.0^{a}	33.5 ± 21.8^{b}	22.2 ± 21.8^{b}	0.020*	0.612	0.277
TNFα	2788.5 ± 777.6	2143.5 ± 777.6	955.9 ± 1059.8	620.7 ± 1059.8	0.079†	0.601	0.869
IL6	39.7 ± 6.3	37.9 ± 6.3	35.7 ± 8.5	30.6 ± 8.5	0.454	0.647	0.830
TGFβ	342.7 ± 51.2^{a}	333.4 ± 51.2^{a}	539.3 ± 69.7^{b}	560.3 ± 69.7^{b}	0.001*	0.924	0.805

^{abc}Between day in each row for each pregnancy status separately indicates $P \leq 0.05$.
*Indicates main effect was determined significant at $P \leq 0.05$.
†Indicates main effect was determined as a tendency at $P > 0.05$ and ≤ 0.10.

Table 3. Cytokine and chemokine concentrations (pg/mL) in uterine flush samples from resulting Open and Pregnant cows on days –3 and –1

Uterine	Open		Pregnant		P-value		
	Day –3	Day –1	Day –3	Day –1	Status	Day	Status*day
IFNα	17.1 ± 12.9	49.3 ± 12.9	34.4 ± 17.5	14.3 ± 17.5	0.568	0.695	0.098†
IFNγ	7.1 ± 5.0 ^{ab}	20.9 ± 5.0 ^{a}	0.4 ± 6.8 ^{b}	3.2 ± 6.8 ^{b}	0.049*	0.173	0.367
IL13	13.2 ± 11.0 ^{b}	46.6 ± 11.0 ^{a}	6.3 ± 15.0 ^{b}	32.4 ± 15.0 ^{ab}	0.426	0.030*	0.783
IL1α	1.1 ± 2.3 ^{b}	13.3 ± 2.3 ^{a}	0.1 ± 3.2 ^{b}	1.7 ± 3.2 ^{b}	0.030*	0.019*	0.067†
IL1F5	6.6 ± 3.2 ^{b}	18.5 ± 3.2 ^{a}	5.5 ± 4.4 ^{b}	20.8 ± 44 ^{a}	0.873	0.001*	0.664
IL21	35.8 ± 60.1 ^{b}	300.4 ± 60.1 ^{a}	0.0 ± 81.9 ^{b}	68.7 ± 81.9 ^{b}	0.071†	0.026*	0.181
IP10	30.4 ± 117.1	342.5 ± 117.1	29.1 ± 159.6	143.0 ± 159.6	0.478	0.137	0.484
MIG	2.4 ± 7.9 ^{b}	33.4 ± 7.9 ^{a}	1.8 ± 10.8 ^{b}	7.5 ± 10.8 ^{ab}	0.172	0.061†	0.189
MIP1b	2.3 ± 2.4^{b}	13.4 ± 2.4 ^{a}	0.0 ± 3.3^{b}	8.9 ± 3.3^{ab}	0.233	0.002*	0.754
TNFα	1.4 ± 12.8 ^{ac}	21.0 ± 12.8 ^{bc}	0.0 ± 17.4 ^{ac}	54.7 ± 17.4 ^{b}	0.299	0.020*	0.259
IL6	0.0 ± 4.5	5.69 ± 4.5	0.0 ± 6.2	12.9 ± 6.2	0.522	0.089†	0.522
TGFβ	14.6 ± 30.9	47.3 ± 30.69	33.5 ± 42.1	108.1 ± 42.1	0.288	0.156	0.574

^{abc}Between day in each row for each pregnancy status separately indicates $P \leq 0.05$.
*Indicates main effect was determined significant at $P \leq 0.05$.
†Indicates main effect or interaction was determined as a tendency at $P > 0.05$ and ≤ 0.10.

Table 4. Cytokine and chemokine concentrations (pg/mL) in vaginal flush samples from resulting Open and Pregnant cows on days –3 and –1

Vaginal	Open		Pregnant		P-value		
	Day –3	Day –1	Day –3	Day –1	Status	Day	Status*day
IFNα	21.2 ± 12.4 ^{b}	83.8 ± 12.4 ^{a}	27.8 ± 16.8 ^{b}	24.9 ± 16.8 ^{b}	0.085†	0.050*	0.033*
IFNγ	6.6 ± 5.1 ^{b}	27.9 ± 5.1 ^{a}	11.2 ± 7.0 ^{ab}	3.3 ± 7.0 ^{b}	0.110	0.281	0.022*
IL13	25.4 ± 14.6	60.3 ± 14.6	11.9 ± 19.9	33.8 ± 19.9	0.260	0.113	0.713
IL1α	16.2 ± 7.5 ^{ab}	7.9 ± 7.5 ^{a}	37.6 ± 10.3 ^{b}	4.3 ± 10.3 ^{a}	0.330	0.027*	0.175
IL1F5	21.0 ± 7.0	36.5 ± 7.0	24.5 ± 9.6	41.6 ± 9.6	0.611	0.059†	0.925
IL21	104.1 ± 137.0	478.3 ± 137.0	212.7 ± 186.7	240.1 ± 186.7	0.695	0.228	0.296
IP10	866.5 ± 328.2^{a}	10.6 ± 328.2^{a}	2373.7 ± 447.2^{b}	108.8 ± 447.2^{a}	0.048*	0.0003*	0.081†
MIG	49.4 ± 27.7	37.0 ± 27.7	80.6 ± 37.7	10.4 ± 37.7	0.945	0.220	0.388
MIP1b	8.3 ± 6.9	22.3 ± 6.9	4.3 ± 9.3	9.7 ± 9.3	0.320	0.247	0.605
TNFα	17.0 ± 30.1	66.4 ± 30.1	0.0 ± 41.1	0.0 ± 41.1	0.255	0.498	0.498
IL6	12.1 ± 5.0 ^{a}	0.0 ± 5.0 ^{a}	42.5 ± 6.8 ^{b}	0.0 ± 6.8 ^{a}	0.016*	<0.0001*	0.016*
TGFβ	31.4 ± 56.3 ^{a}	11.3 ± 56.3 ^{a}	250.2 ± 76.7 ^{b}	12.7 ± 76.7 ^{a}	0.110	0.064†	0.115

^{abc}Between day in each row for each pregnancy status separately indicates $P \leq 0.05$.
*Indicates main effect or interaction was determined significant at $P \leq 0.05$.
†Indicates main effect or interaction was determined as a tendency at $P > 0.05$ and ≤ 0.10.

0.061) and IL6, a pleiotropic cytokine, (9.4 ± 3.8 vs. 0.0 ± 3.8 pg/mL; $P = 0.089$). Uterine concentrations of the pro-inflammatory cytokine IFNγ (1.78 ± 4.8 vs. 14.0 ± 3.5 pg/mL; $P = 0.049$) and IL1α (0.9 ± 2.3 vs. 7.2 ± 1.7 pg/mL; $P = 0.030$) were decreased in Pregnant than in Open cows regardless of the day. Furthermore, there was a tendency for IL21 (34.4 ± 57.9 vs. 168.1 ± 42.5 pg/mL; $P = 0.071$) to have decreased concentrations in Pregnant cows in relation to Open within the uterine flush samples. There was a tendency for a status-by-day interaction of IFNα ($P = 0.098$; Table 3). Moreover, a tendency for IL1α ($P = 0.067$; Table 3) to have a status-by-day interaction.

Cytokine concentrations – vaginal flushes

Concentrations of IFNα (24.5 ± 10.4 vs. 54.4 ± 10.4 pg/mL; $P = 0.050$), IL1α (26.9 ± 6.4 vs. 6.1 ± 6.4 pg/mL; $P = 0.026$), the pleiotropic cytokine IP10 (1620.1 ± 277.4 vs. 59.7 ± 277.4 pg/mL; $P < 0.001$) and IL6 (27.3 ± 4.2 vs. 0.00 ± 4.2 pg/mL; $P < 0.001$) differed significantly between days –3 to –1 within the vagina. There were greater concentrations of IL1α, IP10, and IL6 on day –3 as well as greater concentrations of IFNα on day –1. There was a tendency for IL1F5 (22.7 ± 5.9 vs. 39.1 ± 5.9 pg/mL; $P = 0.059$) to have an increased concentration on day –1 and a tendency for TGFβ (140.8 ± 47.6 vs. 12.0 ± 47.6 pg/mL; $P = 0.064$) to have increased concentrations on day –3 in the vagina. Comparing vaginal cytokine concentrations in Open and Pregnant cows, a tendency for IFNα (52.5 ± 8.7 vs. 26.4 ± 11.9 pg/mL; $P = 0.085$) to have lesser concentrations and a significant difference in IP10 (438.6 ± 232.0 vs. 1241.3 ± 316.2 pg/mL; $P = 0.048$) and IL6 (6.0 ± 3.5 vs. 21.2 ± 4.8 pg/mL; $P = 0.016$) to have greater concentrations in Pregnant cows. A status-by-day

interaction of IFNα (P = 0.033), IFNγ (P = 0.022), and IL6 (P = 0.016) occurred, while a tendency was seen in IP10 (P = 0.081) concentrations (Table 4).

Relative abundance – phylum

An average of 26,146 ± 3,444 sequences were present per sample after quality control, with the number of sequences ranging from 571 to 127,719 among samples. Among all samples, a total of 30 phyla were detected, of which Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Tenericutes were the most abundant (greater than 1% relative abundance, Figure 2). Differences for phyla less than 1% relative abundance for both pregnancy status group and day are listed in Supplementary Tables 1 and 2.

In uterine samples, Firmicutes were greater in Open cows than in Pregnant cows on day −3 (60.4% ± 0.9% vs. 55.7% ± 2.1%; P = 0.046). In vaginal samples, Actinobacteria tended to be more abundant in Open cows than in Pregnant cows on day −3 (1.73% ± 0.32% vs. 1.66% ± 1.09%; P = 0.097). No differences were detected on day −1 between pregnancy status for phyla greater than 1% relative abundance in the uterus or vagina.

Differences in phyla greater than 1% relative abundance were detected between days in both vaginal and uterine samples. In vaginal samples from Open cows, Proteobacteria tended to decrease from days −3 to −1 (10.1% ± 2.95% vs. 9.6% ± 3.04%; P = 0.072). In contrast, no differences in phyla greater than 1% relative abundance were detected in vaginal samples by day from Pregnant cows. In uterine samples from Open cows, a significant decrease in Firmicutes was observed from days −3 to −1 (60.4% ± 0.9% vs. 48.5% ± 3.2%; P = 0.004). In Pregnant cow uterine samples, there was a significant increase from days −3 to −1 in Tenericutes (1.00% ± 0.10% vs. 7.58% ± 4.14%; P = 0.002). For the phylum Euryarchaeota there was a significant decrease by day for both Open cows (2.9% ± 0.2% vs. 1.3% ± 0.3%; P < 0.001) and Pregnant cows (2.87% ± 0.24% vs. 1.49% ± 0.30%; P = 0.007).

Relative abundance – genus

At the genus level, a total of 757 genera were detected among all samples. Differences for genera less than 1% relative abundance are listed in Supplementary Table S3.

Differences in genera greater than 1% relative abundance were detected between days in both vaginal and uterine samples (Figure 3). From the vaginal samples of Open cows, there were significant decreases in the relative abundance of the genera *Olsenella* (1.19% ± 0.11% vs. 0.70% ± 0.15%; P = 0.022) and *Thalassospira* (1.05% ± 0.09% vs. 0.60% ± 0.11%; P = 0.009) from days −3 to −1. Within the vaginal samples of Open cows there was a tendency for the genus *Porphyromonas* (1.45% ± 0.07% vs. 1.54% ± 0.16%; P = 0.072) to increase and a tendency for the genera *Methanobrevibacter* (2.06% ± 0.23% vs. 1.41% ± 0.35%; P = 0.091) and *Rhodothermus* (1.60% ± 0.08% vs. 1.30% ± 0.16%; P = 0.057) to decrease from days −3 to −1. In vaginal samples

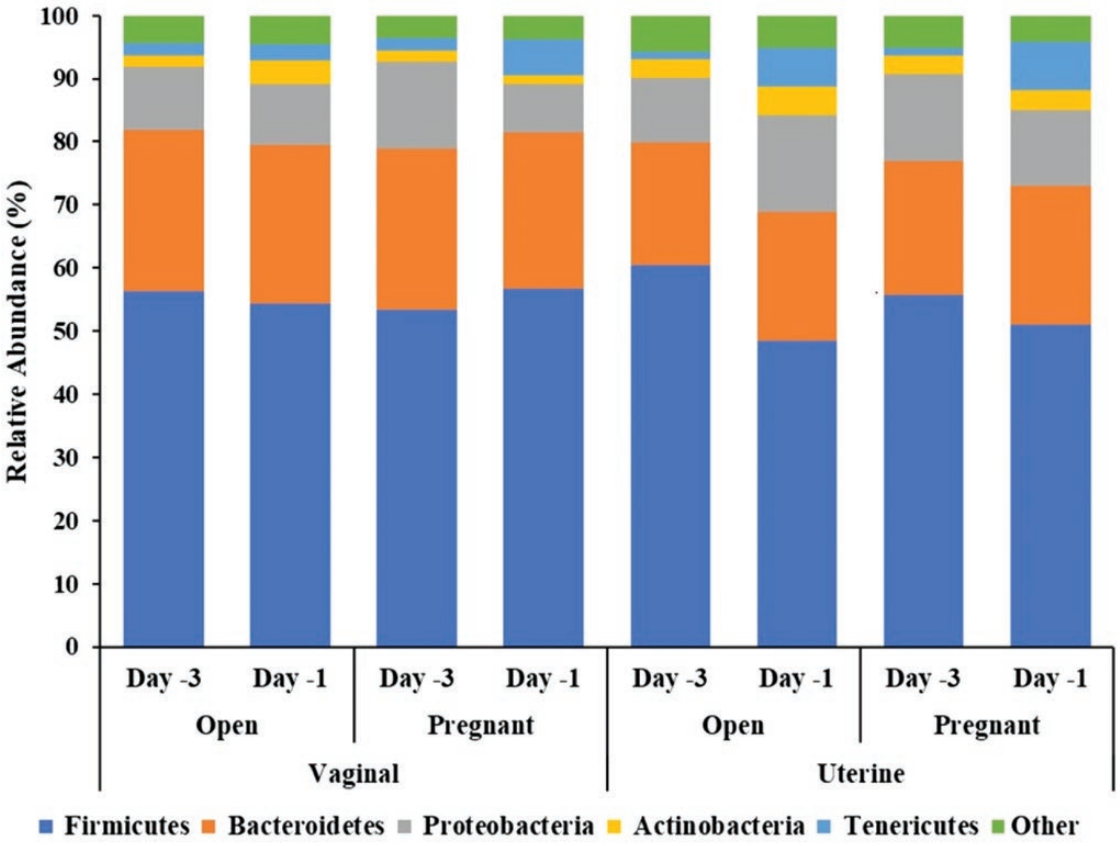

Figure 2. Relative abundance of phyla greater than 1% relative abundance. A total of 30 phyla were detected in all samples. Samples were collected on days −3 and −1 from the uterus and vagina in resulting Open and Pregnant post-partum beef cows.

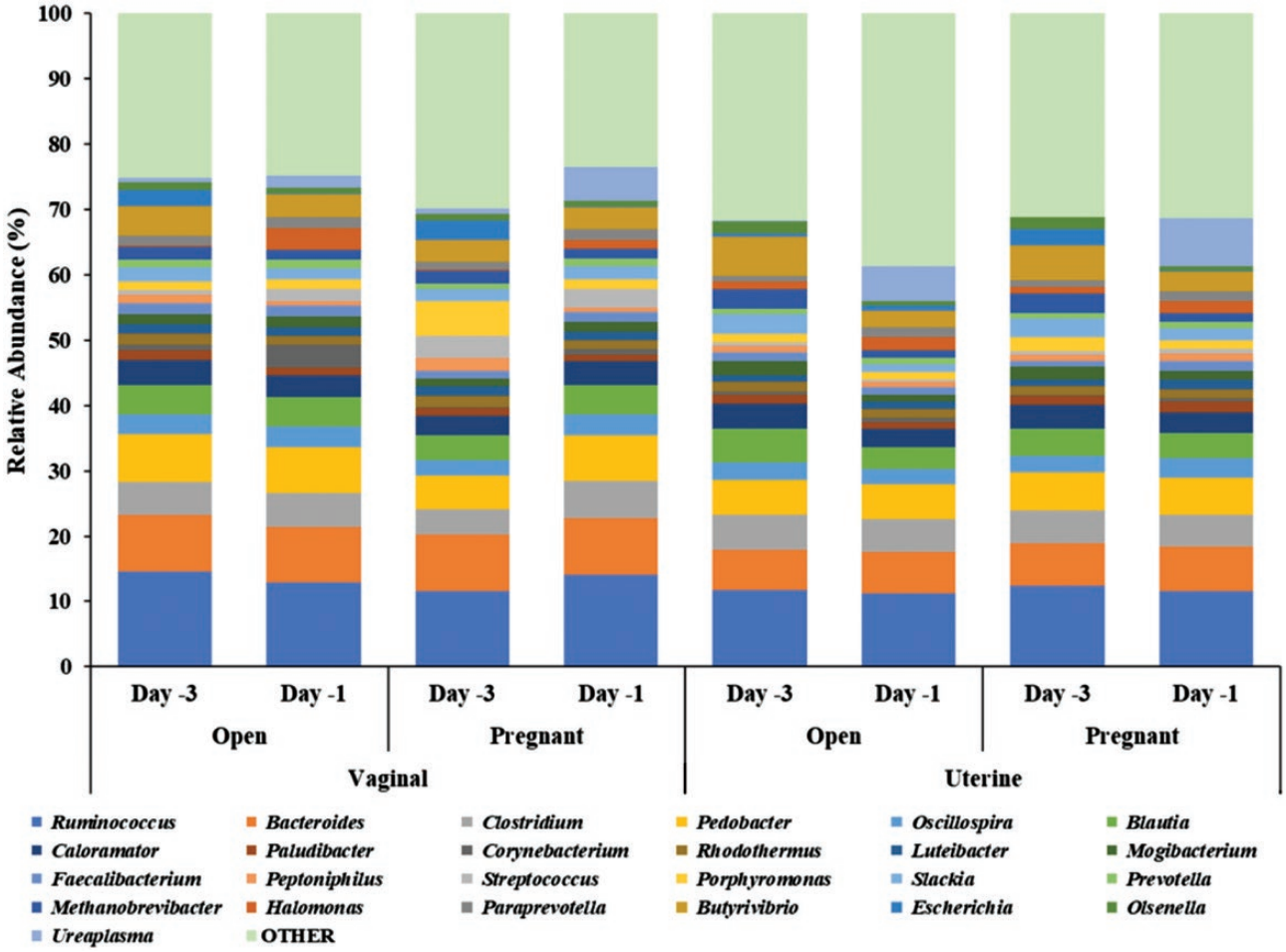

Figure 3. Relative abundance of genera greater than 1% relative abundance. A total of 757 genera were detected in all samples. Samples were collected on days −3 and −1 from the uterus and vagina in resulting Open and Pregnant post-partum beef cows.

from Pregnant cows, *Clostridium* (3.89% ± 0.72% vs. 5.68% ± 0.47%; $P = 0.097$) tended to increase from days −3 to −1. *Halomonas* (0.23% ± 0.18% vs. 1.29% ± 0.91%; $P = 0.026$) exhibited a significant increase in relative abundance. From the vaginal samples of Open (1.31% ± 0.14% vs. 0.66% ± 0.09%; $P = 0.0004$) and Pregnant (1.99% ± 0.98% vs. 0.53% ± 0.09%; $P = 0.018$) cows, there were significant decreases in the relative abundance of the genera *Peptoniphilus* from days −3 to −1.

Within the uterus of Open cows, there was a decrease in relative abundance of the genera *Blautia* (5.05% ± 0.26% vs. 3.46% ± 0.37%; $P = 0.002$), *Mogibacterium* (2.08% ± 0.24% vs. 0.99% ± 0.14%; $P = 0.001$), *Natronincola* (1.41% ± 0.22% vs. 0.73% ± 0.12%; $P = 0.006$), and *Olsenella* (1.87% ± 0.23% vs. 0.72% ± 0.13%; $P = 0.001$) from days −3 to −1. In contrast, *Caloramator* (3.97% ± 0.24% vs. 2.76% ± 0.46%; $P = 0.091$) tended to increase from days −3 to −1 in the uterus of Open cows. Additionally, there was an increase in *Paraprevotella* (1.04% ± 0.15% vs. 1.59% ± 0.16%; $P = 0.037$) in Pregnant cows from days −3 to −1. There was also a tendency for *Ureaplasma* (0.08 ± 0.06% vs. 7.28 ± 4.86%; $P = 0.055$) to increase from days −3 to −1. From days −3 to −1, there was a significant decrease in the relative abundance of the genera *Butryvibrio* in Open cows (5.96% ± 0.54% vs. 2.40% ± 0.36%; $P < 0.001$) and Pregnant cows (5.33% ± 0.69% vs. 3.02% ± 0.51%; $P = 0.018$), for *Slackia* in Open cows (3.06% ± 0.19% vs. 1.15% ± 0.21%; $P < 0.001$) and Pregnant cows (2.85% ± 0.32% vs. 1.86% ± 0.19%; $P = 0.018$) and for *Methanobrevibacter* in Open cows (2.88% ± 0.21% vs. 1.13% ± 0.25%; $P < 0.001$) and Pregnant cows (2.92% ± 0.26% vs. 1.43% ± 0.35%; $P = 0.007$).

Correlations

There were no significant correlations between plasma P4 and plasma cytokines ($P > 0.05$). Plasma P4 was positively correlated with uterine IFNα concentrations regardless of pregnancy status or day ($r = 0.41$; $P = 0.01$). In addition, plasma P4 was negatively correlated with vaginal IL1F5 concentrations regardless of pregnancy status or day ($r = -0.32$; $P = 0.04$). Regardless of pregnancy status or day, plasma P4 was positively correlated with relative abundance of *Methanobrevibacter* in both the uterus ($r = 0.36$; $P = 0.02$) and vagina ($r = 0.37$; $P = 0.02$). Plasma P4 also positively correlated with vaginal relative abundance of *Streptococcus* ($r = 0.47$; $P = 0.002$).

Within the uterus of Open cows, there was a negative correlation between *Blautia* and the cytokines IL1F5 ($r = -0.46$; $P = 0.01$), IL21 ($r = -0.52$; $P = 0.01$), and MIG ($r = -0.44$; $P = 0.02$). Within the uterus of Open cows, a negative correlation was seen between *Butyrivibrio* and IL1α ($r = -0.44$; $P = 0.02$), IL1F5 ($r = -0.39$; $P = 0.04$), IL21 ($r = -0.46$; $P =$

0.02), and MIG (r = –0.44; P = 0.02). Within the uterus of Open cows, a negative correlation was seen between *Mogibacterium* and IL1α (r = –0.39; P = 0.04) and MIP1b (r = –0.46; P = 0.01). Furthermore, in the uterus of Open cows, there was a negative correlation between *Slackia* and IL1α (r = –0.39; P = 0.04), IL1F5 (r = –0.43; P = 0.03), IL21 (r = –0.47; P = 0.01), MIG (r = –0.47; P = 0.01) and MIP1b (r = –0.40; P = 0.04). Finally, within the uterus of Open cows, there was a negative correlation between MIP1b and *Methanobrevibacter* (r = –0.60; P < 0.01). In uterine flush samples of Pregnant cows, there was a negative correlation between *Methanobrevibacter* and the cytokines IFNγ (r = –0.63; P = 0.01), IL13 (r = –0.55; P < 0.01), MIP1b (r = –0.60; P < 0.02), and IL1α (r = –0.75; P < 0.01). The vagina of Open cows had a significant negative correlation between IFNγ and the genera of *Peptoniphilus* (r = –0.39; P = 0.04) and *Olsenella* (r = –0.41; P = 0.03). In the vagina of Pregnant cows, there was a negative correlation seen between MIG and the genera *Clostridium* (r = –0.74; P < 0.01), while a positive correlation was observed with *Peptoniphilus* (r = 0.80; P < 0.01). Additionally, there was a positive correlation observed between *Peptoniphilus* and the cytokines IL1α (r = 0.82; P < 0.01) and IP10 (r = 0.83; P < 0.01). A negative correlation was observed between *Clostridium* and IL1α (r = –0.53; P < 0.04) in the vagina of Pregnant cows.

Discussion

The reproductive microbiome of livestock animals has not been as well characterized compared with humans; however, previous research has found that the microbial composition in the reproductive tract of cattle differs from that in humans (Ravel et al., 2011). Swartz et al. (2014) noted that the abundance of *Lactobacillus,* which is commonly found to dominate the human vaginal microbiome, is found in much less abundance in the cow and ewe (less than 1% relative abundance). The aim of this study was to determine concentrations of certain pro- and anti-inflammatory cytokines in the uterus, vagina, and plasma and characterize relative bacterial abundance in the uterus and vagina of postpartum beef cows before AI. Additionally, fluctuations in bacteria communities are closely tied to and controlled by the immune system (Hooper et al., 2012; Belkaid and Hand, 2014). Therefore, an additional objective was to identify associations between cytokine concentrations and relative abundances of bacteria related to fertility in beef cattle.

Cytokines and chemokines (cytokines with chemotactic activity such as MIG and MIP1b) influence inflammatory responses and can upregulate pro- or anti-inflammatory immune responses (Zhang and An, 2007). Known pro-inflammatory cytokines, such as IFNα, TNFα, and IL1α, were more abundant in circulation before AI for resulting Open cows than in Pregnant cows in the current study. Though changes in these pro-inflammatory cytokines were not always observed in the flush samples in the current study. Interestingly, IL6 was not greater in resulting Open cows. Ishikawa et al. (2004) observed that "healthy" cows had declining concentrations of IL6 following the postpartum period. Similar results were also seen by Poole et al. (2021) where greater concentrations of IL6 were seen 21 d before AI or approximately day 60 postpartum. Additionally, the presence of the IL1 family is correlated with decreased fertility (Herath et al., 2009) and corresponds with the circulating and uterine IL1α concentrations in the current study. In addition, MIP1b is a chemokine that is stimulated either by bacterial endotoxin such as lipopolysaccharides (**LPS**), or pro-inflammatory cytokines such as IL1 (Maurer and von Stebut, 2004). Similar to elevated concentrations of IL1α, plasma concentrations of MIP1b was also increased in resulting Open cows in the current study. Pleiotropic cytokines, such as IL21, had greater uterine concentrations in Open cows compared with Pregnant cows on days –1. This particular cytokine, IL21, has been shown to have multiple roles in both pro- and anti-inflammatory responses, the immunosuppressive actions of IL21 suggest that it is a "double-edged" sword (Spolski and Leonard, 2014). Stimulation of IL21 could lead to either suppression or induction of a proper immune response within certain locations of the reproductive tract (Leonard and Wan, 2016). Typically described as a "IL-4-like" molecule, IL13 has been categorized as an anti-inflammatory cytokine (Zourbas et al., 2001). Various studies have exhibited that IL13 is an inhibitor of autophagy, or the process where the cell breaks down old and damaged cell parts (Ma et al., 2013; Dickinson et al., 2014). ?>Hickey et al. (2013) observed that concentrations of IL13 were increased in uterine lavages of murine models compared with vaginal lavages. They also noted that there were greater concentrations during proestrus and estrus in comparison to diestrus (Hickey et al., 2013). This potentially could align with the current study with uterine concentrations of IL13 increasing from days –3 to –1 as cattle were expressing estrus before TAI on day 0. However, further investigation of the relationship between IL13 and estrus expression in cattle is needed. It cannot be discounted that the use of a CIDR could have influenced the vaginal cytokine concentrations, with some researchers observing incidences of vaginitis when using a CIDR in dairy cattle (Chenault et al, 2003; Fischer-Tenhagen et al, 2012). In beef cattle, it has been reported that vaginitis caused by CIDR use does not influence pregnancy rates (Dias et al., 2019). Moreover, in the current study, no inflammation or infection was detected. Results from the current study indicate that Pregnant cows had increased circulating TGFβ leading up to TAI. These results are similar to Poole et al. (2021) in which uterine TGFβ concentrations were greater in resulting Pregnant than Open cows. Though it is important to note that TGFβ did not differ in flush samples in the current study. Because of TGFβ being an anti-inflammatory cytokine and some cytokines of the IL family (e.g., IL1α) being characterized as pro-inflammatory, the observed differences between resulting Open and Pregnant cows in the current study suggest that some circulating cytokine concentrations just before breeding may be associated with fertility outcomes.

Bacterial communities of the uterus and vagina shift over time with hormonal changes, potentially to aid the uterus in preparing for pregnancy (Ault et al., 2019a,b; Poole et al., 2023). Changes in the taxonomic composition of bacterial communities could potentially affect fertility. Across multiple studies, various phyla such as Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Tenericutes have been investigated in the reproductive tract of cattle (Swartz et al., 2014; Clemmons et al., 2017; Messman et al., 2020; Pickett et al., 2022). In the current study, it was identified that in the uterus of Open cows, the phylum Firmicutes was more abundant than in Pregnant cows and decreased in abundance from days –3 to –1. Santos and Bicalho (2012) found that most cattle containing a greater relative abundance of Firmicutes

developed endometritis, supporting the adverse effects that result in decreased fertility. Within the phylum of Firmicutes, various genera such as *Peptoniphilus* in the vagina and *Blautia*, *Mogibacterium*, and *Natronincola* in the uterus were more abundant in Open cows and decreased from days −3 to −1 in the current study. *Blautia* has been seen as a common isolate within fecal matter and of the gut microbiome ?>(Koskey et al., 2014). It is a Gram-positive staining bacterium that has been associated with antibiotic resistance associated with clinical infections in humans (Liu et al., 2008; Murphy and Fick, 2013). *Mogibacterium* has been seen to be isolated from the oral pockets of adult humans (Nakazawa et al., 2000). Liu et al. (2016) also noted that *Mogibacterium* has been associated with ammonia assimilation in the rumen of dairy cattle. Interestingly, *Butyrvibrio* was decreased in both Pregnant and Open females from days −3 to −1. The genus *Butyrvibrio* has been found to be one of the most common bacteria found within the rumen (Pavelich et al., 2017; Rodríguez Hernáez et al., 2018). Previous studies have associated *Butyrvibrio* with the uterus of nonpregnant cattle (Clemmons et al. 2017; Ault et al., 2019a). Prior research indicated that *Butyrvibrio* and *Mogibacterium* were associated with the pro-inflammatory cytokine, IL6, in the uterus of nonpregnant cattle (Poole et al., 2021). Interestingly, in the current study, these bacteria were negatively correlated with certain cytokines such as IL1α, IL1F5, and IL21 in the uterus of Open cows. These bacteria (*Butyrvibrio*, *Mogibacterium*) are classified as Gram-positive bacteria, meaning they do not contain LPS in their cell wall; however, do have other cell wall constituents that induce inflammation (Moreillon and Majcherczyk, 2003). Recently, it has been shown that LPS stimulates gene expression of certain cytokines, such as IL1α and IL1F5 in bovine endometrial explants (Ault-Seay et al., 2022). Therefore, this could explain the negative correlation between these Gram-positive bacteria and cytokines such as IL1α and IL1F5 in the uterus of Open cows. Together with prior research and the current findings, it suggests that *Butyrvibrio* and *Mogibacterium* could be negatively impacting fertility and are associated with immune responses in cattle, though the exact mechanisms require further investigation. In the current study, it was identified that the relative abundance of phylum Tenericutes and the genus *Ureaplasma* in the uterus of Pregnant cows increased from days −3 to −1. Multiple studies have shown that the presence of *Ureaplasma* is associated with a healthy uterine environment and positive fertility outcomes (Jeon et al., 2015; Ault et al., 2019a; Poole et al., 2021). However, Crane and Hughes (2018) found that *Ureaplasma* was associated with pregnancy losses in cattle. Future studies are needed to determine how these various bacteria, specifically at the species level, relate to reproductive health in beef cattle.

There is a distinct difference in the relative abundance of the genus *Lactobacillus* and vaginal pH between humans and other mammals. The median vaginal pH of women is 4.5, due to *Lactobacillus* producing lactic acid, while other mammals averaged a pH of 6.8 (Miller et al., 2016). Uterine and vaginal flush pH decreased by day in the current study. A decreased pH has been previously shown to allow for increased sperm lifespan within the female reproductive tract (Perry and Perry, 2008). It has been suggested that estradiol may influence pH; however, there were no differences observed in estrus expression in the current study. Interestingly, the relative abundance of *Methanobrevibacter* in both the uterus and vagina was positively correlated with plasma progesterone. It has been previously shown in mice that supplementation of progesterone increases the fecal abundance of *Methanobrevibacter* compared with placebo mice (Nuriel-Ohayon et al., 2019). In addition, few studies have shown that bacteria such as anaerobic bacteria (e.g., *Blautia*, *Methanobrevibacter*) can influence environmental pH such as reproductive tract pH (Ozenc et al, 2010: Roper, 2014). Therefore, shifting bacterial populations or progesterone concentrations by day could possibly explain the pH results of the flush samples.

In conclusion, the relative abundance of various bacteria found in the uterus and vagina of cattle shifted throughout the estrus synchronization protocol before AI. Future research should focus on the specific causes of bacterial shifts that prevent the establishment and maintenance of pregnancy. The current study also suggests that immunological factors throughout estrus synchronization can be associated with fertility outcomes in beef cattle. In particular, a pro-inflammatory environment appears to impede pregnancy establishment and maintenance. Additional research may be worthwhile to evaluate management tactics to maintain a healthy reproductive tract environment to reduce fertility problems and improve herd reproductive efficiency.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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