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Endometrial gene expression in response to lipopolysaccharide between estrous cycle phases and uterine horns in cattle

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Uterine bacterial community abundances shift throughout the estrous cycle, potentially altering the immunological environment of the uterus and impacting subsequent fertility. The objective of the current study was to evaluate the immunological impact of lipopolysaccharide (LPS), as a model for potentially pathogenic bacteria, throughout the uterine endometrium between the luteal and follicular phase of the estrous cycle. Bovine uterine tracts were harvested in mid-luteal ($n = 7$) or follicular ($n = 7$) phase. Explants were collected from the contralateral and ipsilateral horn relative to the dominant follicle or corpus luteum, then subjected to one of three treatments: uncultured control, cultured control, or cultured with LPS (1 $\mu\text{g}/\text{mL}$). Explants underwent RNA extraction and targeted RNA sequencing for expression analyses of 40 immune response related genes. Sequencing reads were mapped to *Bos taurus* genome in CLC Genomics Workbench. Resulting total read counts were normalized by housekeeping gene GAPDH and analyzed for overall expression profile by Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) and Variable Importance in Projection (VIP) analyses in Metaboanalyst. Individual gene expression differences were determined by GLIMMIX procedure in SAS with fixed effects of treatment, estrous phase, uterine horn, and their interaction, with random effect of individual uterus. Expression of 29 genes were affected among treatment groups, with seven genes increased in LPS treatment compared to other groups ($P < 0.05$). Multiple genes were affected by estrous phase and uterine horn, independent of treatment ($P < 0.05$). The OPLS-DA analyses indicated overall gene expression differences due to clustering by estrous cycle and treatment ($P < 0.001$), with no effect of uterine horn ($P > 0.10$). Similar clustering was observed between luteal and follicular phase explants of controls, but distinct separate clustering between phases with LPS treatment ($P = 0.001$). According to VIP analyses, mucins were identified as contributing the most to differences observed between phase and treatment. In conclusion,

estrous cycle phase resulted in differing overall endometrial gene expression profiles of immune response to LPS treatment. Therefore, altered immunological environment of the uterus in response to bacteria at different estrous cycle stages may lead to differences in reproductive success.

KEYWORDS

bovine, follicular, lipopolysaccharide, luteal, uterus, bacteria, gram-negative, cytokine

Introduction

Reproductive failures and pregnancy losses cost the beef industry millions of dollars annually (Bellows et al., 2002). In postpartum cows, bacterial infections in the uterus are a common cause of low rebreeding success (Bonnett et al., 1993; LeBlanc et al., 2002; Sheldon et al., 2009; Ribeiro et al., 2013). Through parturition and uterine involution, there is high risk for bacterial contamination and infection resulting in uterine diseases. Bacterial communities in healthy postpartum cattle have shown to influence rebreeding success (Ault et al., 2019a; Ault et al., 2019b). Cows that did not become pregnant had a greater abundance of uterine disease-associated bacterial genera in the uterus, although no presence of infection was observed (Ault et al., 2019a). Interestingly, many of these potentially pathogenic bacteria in postpartum cows with uterine disease have also been found in the healthy virgin heifer and pregnant uterus (Moore et al., 2017). Specific genera of potentially pathogenic bacteria associated with uterine diseases include *Acinetobacter*, *Fusobacteria*, *Proteus*, *Prevotella*, and *Peptostreptococcus*, and many are classified as Gram-negative bacteria. Gram-negative bacteria are defined by their cell wall composition with a thin peptidoglycan layer and outer membrane containing lipopolysaccharide (LPS), which is considered an endotoxin that can elicit a significant immune response in the host (Dong et al., 2017). Through toll like receptor signaling, LPS stimulates a pro-inflammatory reaction in various tissues, mainly through the production of cytokines and chemokines (Nagaraja et al., 1978; Takeuchi et al., 1999; Huemann and Roger, 2002; Elson et al., 2006; Lu et al., 2008). Cytokines and chemokines are commonly known for regulating inflammation and immune cell recruitment (Dimberg, 2010), but are also involved in reproductive processes such as corpus luteum (CL) function, embryo development, placental attachment, and preventing rejection of the fetus (Zolti et al., 1991; Simón et al., 1998; Penny et al., 1999; Ealy et al., 2021). The presence of cytokines and chemokines have shown to fluctuate across the estrous cycle and pregnancy, and throughout puberty attainment (Krakowski and Zdzisinska, 2007; Oliveira et al., 2013; Ault-Seay et al., 2021; Poole et al., 2021). Due to the

importance of inflammatory balance in the uterus (Raghupathy, 2001), minor shifts in bacterial communities may disrupt this balance and negatively influence reproductive success.

Similar to cytokines and chemokines, fluctuations in bacterial communities of the reproductive tract occur between the luteal and follicular phases of the estrous cycle (Laguardia-Nascimento et al., 2015; Ault et al., 2019a; Ault et al., 2019b; Quereda et al., 2020). The concentration of both local and systemic hormones, such as progesterone and estrogen, is a major distinguishing factor between phases of the estrous cycle, but their direct effect on bacterial communities is not well understood. However, the effects of reproduction-associated steroid hormones on the uterine endometrium may indirectly impact the uterine bacterial community composition. Changes in both bacterial diversity and taxonomic composition have been observed over natural estrous cycles, estrus synchronization, and differences in pregnancy status (Laguardia-Nascimento et al., 2015; Ault et al., 2019a; Ault et al., 2019b; Quereda et al., 2020). Major differences observed in bacterial communities between pregnant and non-pregnant cows occurred prior to insemination during estrus synchronization when CL regression would begin to occur and the transition from a progesterone dominated to estrogen dominated environment (Ault et al., 2019a; Ault et al., 2019b). Evaluating the impact of bacteria on the uterus during different phases of the estrous cycle will contribute to the understanding of microbial impact on reproductive performance. Additionally, gene expression analyses indicated distinct separation of global gene expression profiles between the contralateral and ipsilateral uterine horns, relative to the dominant follicle or CL, potentially due to the presence of a hormone concentration gradient (Pope et al., 1982; Boos et al., 1986; Takahashi et al., 2016; Sánchez et al., 2018). Therefore, the local hormonal concentration may impact endometrial activity and immune response to bacteria differently depending on the uterine horn. Assessing the response of the contralateral and ipsilateral endometrium in each phase of the estrous cycle will determine the response to bacteria through the hormone concentration differences and varying responses across the uterus. By first evaluating the impact of potentially pathogenic bacterial component LPS on the uterus and uterine environment through the estrous cycle, future

studies may determine the direct influence of the reproductive tract microbiome on the uterus leading to differences in breeding success.

The objective of the current study was to evaluate the impact of LPS, as model for potentially pathogenic bacteria, on the endometrium throughout the uterus between the follicular and luteal phases of the estrous cycle. We hypothesized the immunological response in the endometrium to LPS will differ between both the luteal phase and follicular phase, and the ipsilateral and contralateral uterine horns.

Materials and methods

Collection and selection of reproductive tracts

Reproductive tracts were collected in late spring from the local abattoir immediately following slaughter. Only reproductive tracts with both ovaries attached and no abnormalities present on the ovaries were considered for sample collection. The reproductive tract and surrounding tissues were required to have moderate adiposity corresponding to a moderate BCS; tracts with no signs of adipose or excessive adiposity were not utilized. Tracts were then selected in the mid- to late luteal phase (progesterone dominated tract; $n = 7$), or in the follicular phase (estrogen dominated tract; $n = 7$). To determine the phases, gross ovarian measurements were evaluated to determine reproductive tract estrous cycle phase using standards previously described (Ireland et al., 1980; Miyamoto et al., 2000; Arosh et al., 2002). Example images of the contralateral and ipsilateral ovaries, relative to the dominant follicle or CL, on luteal and follicular phase tracts utilized for the current study are shown in Figures 1A–F. For the follicular phase (Figures 1A, B), a large, dominant, pre-ovulatory follicle (≥ 13 mm diameter) must be present on one ovary, and its closest uterine horn was considered the ipsilateral horn (Figure 1A). The second ovary had a small, regressing CL and/or small, non-dominant follicle(s), in which the closest uterine horn would be the contralateral horn (Figure 1B). For the luteal phase (Figures 1C–F), tracts were characterized by a large current CL on one ovary, and a small follicle(s) on either ovary; the uterine horn closest to the ovary with the CL was considered the ipsilateral horn. Luteal phase tracts were utilized if the CL was tan to orange in color externally and internally, ~ 1.5 to 2 cm in diameter, with some vasculature over apex and on periphery (Figures 1C, E). The follicles on either ovary were <10 mm in diameter, or not visible (Figures 1D, F). Reproductive tracts meeting these criteria for inclusion were transported on ice to the laboratory for processing within 1 hour (h).

Endometrial explant collection, culture, and treatment

Pinching the cervix closed, reproductive tracts were rinsed with ethanol and water. The uterus was then cut away from all other tissues and the contralateral and ipsilateral horns of the uterus were separated. Each horn was opened longitudinally for collection of endometrial explants. If any signs of placental membranes, infection, excessive mucus, or abnormalities were observed once either uterine horn was opened, the whole tract was discarded and excluded from the study.

The contralateral and ipsilateral horns were split into even halves, resulting in four sections per uterus. Endometrial explants were collected using an 8mm biopsy punch. Explants were taken from the intercaruncular area approximately 1-1/12" from the uterine body and approximately 1-1/12" past the uterine bifurcation towards the tip of the uterine horn of the contralateral and ipsilateral horns. Any visible myometrium obtained during the biopsy punch was trimmed from the explant. Three explants were taken from each section for three treatments [uncultured control (UC), cultured control (CC), and LPS] resulting in 12 endometrial explants per tract. Figure 2 depicts sections of each uterine horn and collected explants. Explants serving as uncultured controls were immediately snap frozen in liquid nitrogen and stored at -80°C . Explants for both culture treatments were placed in 20 mL of Hank's Balanced Salt Solution (HBSS; Gibco, Waltham, Massachusetts, USA) until all explants were collected. Explants were transferred from the first HBSS medium to a second conical of 20 mL of HBSS medium, and transferred to 20 mL HBSS containing 1% antibiotic and antimycotic media (ABAM; Gibco, Waltham, Massachusetts, USA) until plating for culture within 20 minutes.

Explants were removed from HBSS with 1% ABAM and plated into individual wells with 1 mL of Roswell Park Memorial Institute 1640 medium (RPMI; Gibco, Waltham, Massachusetts, USA) with 1% ABAM that was equilibrated to culture conditions in incubator. Plates with explants were incubated for 2 h at 38.8°C with 5% CO_2 and air for removal of cellular debris and red blood cells. After incubation, explants were transferred to a new plate into individual wells containing 1 mL of fresh equilibrated medium (RPMI with 1% ABAM) for cultured controls or fresh equilibrated medium containing 1 $\mu\text{g/mL}$ LPS (Ultrapure LPS, E. coli 0111:B4; *In vivo*Gen, San Diego, CA, USA; Dohmen et al., 2000; Herath et al., 2006). Following incubation at 38.8°C with 5% CO_2 and air for 8 h, explants were removed and snap frozen in liquid nitrogen, then stored at -80°C .

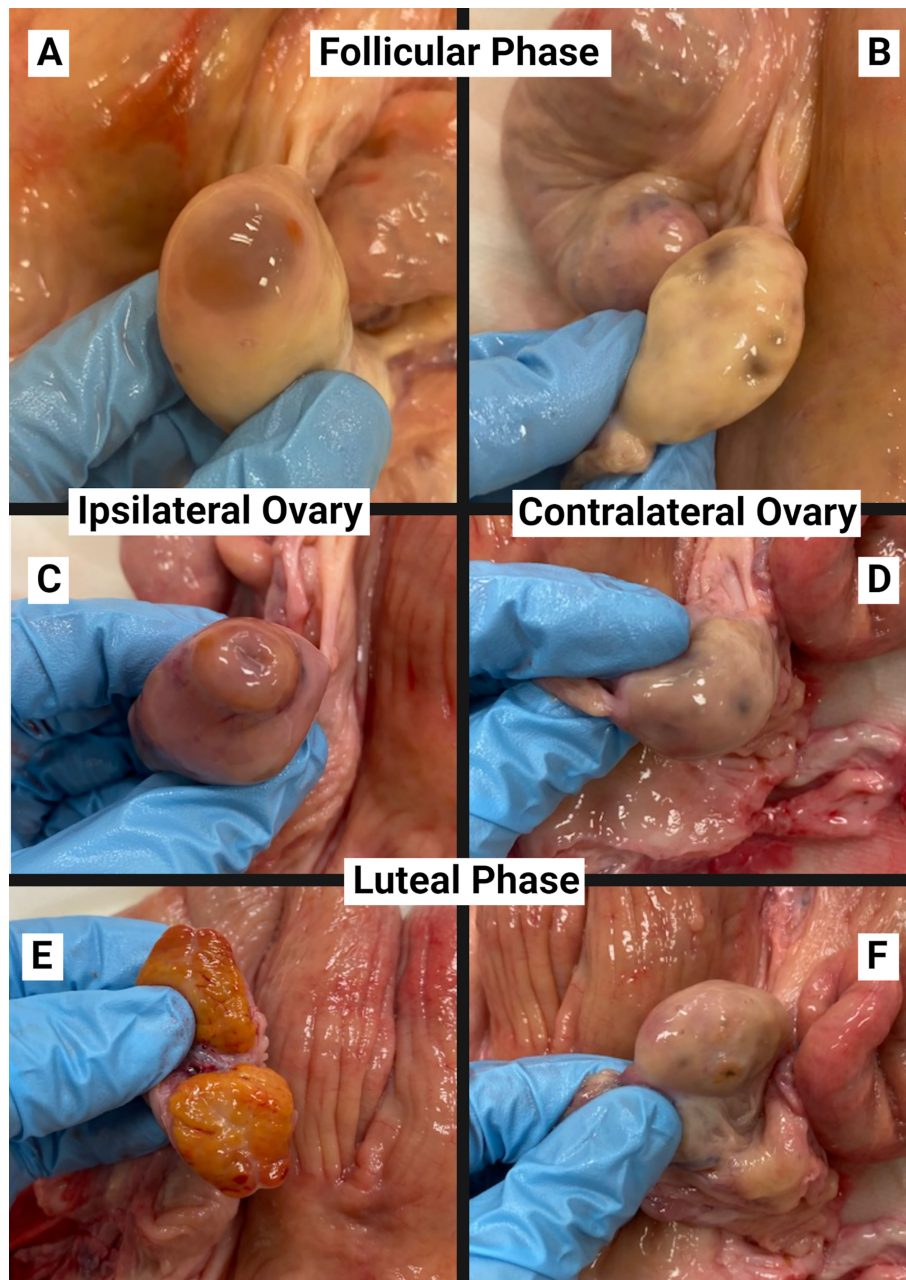


FIGURE 1

Examples of ovaries for staging of the estrous cycle on follicular phase (A, B) and luteal phase (C–F) reproductive tracts utilized for explant collection from the ipsilateral (A, C, E) and contralateral (B, D, F) uterine horn.

RNA extraction, cDNA synthesis, and targeted RNA sequencing

Total RNA was extracted from ~50 mg of endometrial explant tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) and chloroform (Thermo Scientific, USA) in combination with the Qiagen RNeasy kit (Qiagen, Hilden, Germany). Explants were homogenized in TRIzol for four minutes with a 5 mm steel

bead (Qiagen, Hilden, Germany) on the tissue homogenizer (Fisher). Following homogenization, 100 μ L chloroform was added to the homogenized sample and centrifuged at $12,000 \times g$ for 15 minutes at 4°C . The aqueous layer was then transferred to a new tube containing 1.5x volume of 100% ethanol. The resulting solution was added to a RNeasy column and proceeded with the Qiagen RNeasy kit according to manufacturer instructions. Quantity and quality of extracted RNA was

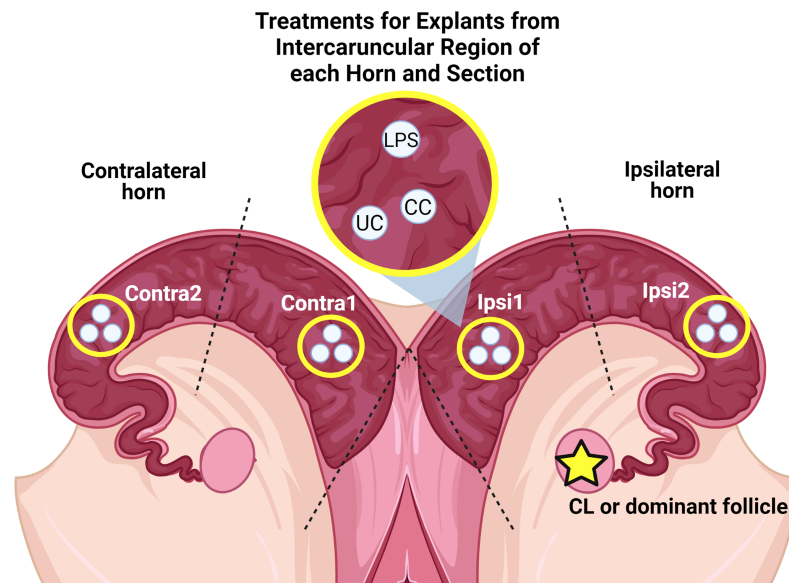


FIGURE 2

Sections of uterine horns with collected explants from the intercaruncular regions and their respective treatments. LPS: Cultured with complete media + LPS. CC: Control cultured in complete media without LPS. UC: Uncultured control.

evaluated *via* NanoDrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA) and Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA, USA). Extracted RNA with a RIN value < 6 and concentration < 35 ng/uL were not utilized for cDNA synthesis and further analyses. Synthesis of cDNA from extracted RNA was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA) according to manufacturer protocol with ~500 ng/mL of RNA.

Genes of interest related to immune response and associated with reproductive processes were selected for targeted RNA sequencing and identified using the NCBI Gene database from the *Bos taurus* genome. A total of 40 genes were selected including interleukin family genes (*IL-1 α* , *IL-1 β* , *IL-4*, *IL-6*, *IL-10*, *IL-17A*, *IL-36RA*, *LIF*), various cytokines (*TGF β* , *TNF α* , *VEGFA*), CXCL chemokines (*CXCL1*, *CXCL2*, *CXCL8*, *CXCL10*), CCL chemokines (*CCL2*, *CCL3*, *CCL4*), mucins (*MUC1*, *MUC4*, *MUC13*, *MUC15*), steroid receptors (*PGR*, *ESR1*, *ESR2*), interferon associated genes (*IFN γ* , *MX1*, *MX2*, *ISG15*, *OAS2*, *IRF1*, *IRF3*, *IRF8*), colony stimulating factors (*CSF2*, *CSF3*), prostaglandin pathway genes (*PTGES*, *PTGES2*, *PTGESR2*), and toll-like receptor pathway genes (*TLR4*, *NF κ B1*). For all genes of interest, primers were designed in NCBI Primer BLAST to span across an intron and result in amplicons of 90–110 base pairs. Quantification of housekeeping gene *GAPDH* was included for sample normalization. Table 1 lists genes of interests with their respective accession numbers from the NCBI database and resulting primers. Amplification of cDNA was

performed in duplicate *via* Hi-Plex approach optimized by Floodlight Genomics (Knoxville, TN, USA), followed by multiplex sequencing using a 2 × 150 kit on an Illumina HiSeq X (San Diego, CA, USA). Resulting reads were mapped to the *Bos taurus* genome using CLC Genomics Workbench version 9.5.2 (Qiagen, Bethesda, MD, USA). Reads were standardized according to total read count per sample to obtain the proportion of reads for each gene. Values for each sample duplicates were averaged among each gene within each sample for further analyses (Mihelic et al., 2020).

Statistical analyses

Resulting gene expression values were imported to Metaboanalyst 5.0 and normalized according to the expression of housekeeping gene *GAPDH*, which indicated stable expression among treatment, estrous cycle phase, and uterine horn. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were performed in Metaboanalyst 5.0 to evaluate the overall gene expression differences between groups. Variable importance in projection (VIP) plots were generated to determine genes contributing to clustering differences between groups in the OPLS-DA. Resulting normalized expression values from Metaboanalyst 5.0 were also utilized for a GLIMMIX procedure in SAS 9.4 with effects of treatment (uncultured and cultured controls, and LPS), estrous cycle phase (follicular

TABLE 1 Genes of interest and NCBI gene accession number with resulting forward and reverse primers for targeted RNA sequencing.

Gene	Accession #	Forward Primer	Reverse Primer
<i>TLR4</i>	NM_174198.6	CCTGAGAACCGAGAGTTGGG	TATGGGGATGTTGTCGGGGA
<i>IL1α</i>	NM_174092.1	AGGTCCATACCTGACGGCTA	AGAGTCACAGGAAGCTGAGAA
<i>IL1β</i>	NM_174093.1	AAAAATCCCTGGTGCTGGCT	GCACAAAGCTCATGCAGAACA
<i>TNFα</i>	NM_173966.3	TCACATACCCTGCCACAAGG	CCACATCCCGGATCATGCTT
<i>IFNγ</i>	NM_174086.1	TCTTATGGCCAGGGCCAATTT	AGGCCACCCCTTAGCTACAT
<i>IL10</i>	NM_174088.1	GGCCTGACATCAAGGAGCAC	GCCTTGCTCTTGTTTTCGCA
<i>IL6</i>	NM_173923.2	AAGCGCATGGTCGACAAAAAT	TTCTGCCAGTGTCTCCTTGC
<i>MUC15</i>	NM_176631.1	TCCACCCCTGAAATTCACCA	AGCACCTAAAAATGGCCCCAA
<i>MUC13</i>	XM_002684796.6	AGCCAGAGAACCCCTACACA	ATGAGTTGAGGGGCTGTAGA
<i>MUC1</i>	NM_001372018.1	CAGTGCCCATTTCCITCCT	ATAGATGATGGCCAGCGCAA
<i>MUC4</i>	XM_024994862.1	CCGATTCACAAGTCACCCCA	CCCGTAGGGGAAGAGGAGAA
<i>MX1</i>	NM_173940.2	AGATGGTTTCTTCTGACTTGGGT	TTGGCTGTACAGGTTGCTCT
<i>MX2</i>	NM_173941.2	CAAGAGTTCCGTGCTGGAGG	CCCGCTTTGTCAGTTTCAGC
<i>ISG15</i>	NM_174366.1	AGGAGCGTGTACAAGCAGAC	CCCCTTCATGAGGCCGTATT
<i>OAS2</i>	NM_001024557.1	ACAGCGCAATTTTGTTGCT	GCAGAGACCCCTTTTGCTTC
<i>IRF1</i>	NM_001191261.2	CATGCCCTCCACCTCTGAAG	CCACCTGTTTGCTCCAAGA
<i>IRF3</i>	NM_001029845.3	TGTGCAGCTGAGAGTTCGAG	GTCTGGCCTAAGTGTTGGGT
<i>IRF8</i>	NM_001083769.2	CGGACTGGTGGGTGCAG	GACCATCTGGGAGAAGGCTG
<i>PTGES2</i>	NM_001166554.1	TTATGGCCCAGGAAGGAGAGA	TGATGATGTCTGCCAGGGGT
<i>PTGER2</i>	NM_174588.2	ATGACCATCACCTTCGCCGT	CTAAGAGCTTGAGAGTCCAC
<i>PTGES</i>	NM_174443.2	CCTCCAGTATTGCCGGAACG	AGACAAAGCCCAGGAACAGG
<i>NFκB</i>	NM_001076409.1	TTGGGAGGGCGTGAACAAAT	GGCCATCTGCTGTTGGTAGT
<i>CXCL8</i>	NM_173925.2	GACTTCCAAGCTGGCTGTTG	ATGCATTGGCATCGAAGTTCTG
<i>CCL2</i>	NM_174006.2	AGACCCCAAGCAGAAATGGG	TTGGGTTGTGGAGTGAAGTC
<i>TGFβ</i>	NM_001166068.1	AACCTGTGTGCTCTCTCGG	GAGGTAGCGCCAGGAATTGT
<i>CCL3</i>	NM_174511.2	CAGCATCATGAAGTTCGCCG	TTGGGTTGTACAGCCCAAAT
<i>CSF2</i>	NM_174027.2	ACTTCCTGTGGAACCCAGTT	TGGTTCACAGCAGTCAAAGG
<i>CSF3</i>	NM_174028.1	TACACGGCGGCCTCTTCTC	TCCAGCTGCAGTGTGTCCAAG
<i>CXCL1</i>	NM_175700.2	ACCGCCCCATGGTTAAGAA	CACTGAGGCTGCTGGAGTATC
<i>CXCL2</i>	NM_174299.3	GCCGCTCCCATGGTTAAGAA	TGGAACAGCCATCCAAGAGC
<i>CXCL10</i>	NM_001046551.2	GGAATACACGCTGTTCTCTGC	CACGTGGGCAGGATTGACTT
<i>LIF</i>	NM_173931.1	AACTGCCGCATCTAAGGTC	AGTGGAGAACCAGCAGCAAG
<i>IL4</i>	NM_173921.2	GCCACACGTGCTTGAACAAA	TGTGCTCGTCTTGGCTTCAT
<i>IL17A</i>	NM_001008412.2	ACATCGTTAACCGAGCACA	CTCTCAGGGTCCTCATTGCG
<i>IL36RN</i>	NM_001075775.1	CTCAAGATGGTCTGAGCGG	TGTAGGCCTCCGGCTAGAAG
<i>CCL4</i>	NM_001075147.2	GCGGAAGATTCTCGCAACT	CCTGCCCTTTTGTGCTGGA
<i>VEGFA</i>	NM_001316955.1	GGCGAGGCAGCTTGAGTTA	CTGGTTCCCGAAACCCTGAG
<i>PGR</i>	NM_001205356.1	AGAAAGTGCTGTCAGGCTGG	GGAGGGCAACAGCATCTAGT
<i>ESR1</i>	NM_001001443.1	TCTGGAAAAGACGCTCTTGATCC	GTCATGGTCATGATGAGCGG
<i>ESR2</i>	NM_174051.3	CGACGTAAAAGCTGTCAGGC	ATGCGGTACCCACACCTTTC
<i>GAPDH</i>	NM_001034034.2	TGGTGAAGGTCGGAGTGAAC	ATGGCGACGATGTCCACTTT

and luteal), uterine horn (contralateral and ipsilateral), and their interaction, with random effect of uterus. Section of the uterine horn from which the explant was collected was evaluated but did not affect any individual gene expression or overall gene expression *via* OPLS-DA and was removed from all analyses. Normal distribution of gene expression values

were evaluated by the UNIVARIATE procedure and genes with a Shapiro-Wilk score < 0.85 were log transformed. Gene expression values are reported as the mean normalized total count \pm standard error of the mean (SEM). Significance for all analyses was determined by a $P \leq 0.05$, and tendencies with $P \leq 0.10$.

Results

Estrous cycle phase

Overall expression of targeted genes of interest was evaluated by OPLS-DA. Distinct clustering of samples was observed between luteal and follicular phase samples with slight overlap ($P < 0.001$; **Figure 3A**). Clustering within each phase indicates similar overall expression of targeted genes within each phase. The overlap observed between phases indicates some similarity of gene expression not impacted by phase of the estrous cycle. The five most influential genes contributing to observed clustering within each phase, determined from VIP scores, were *MUC13*, *MUC1*, *IFN γ* , *ISG15*, and *ESR1* (**Figure 3B**). Of these genes, *MUC13*, *MUC1*, *ISG15*, and *ESR1* had greater expression in explants of luteal than follicular phase, while *IFN γ* had greater expression in the follicular than luteal phase (**Figure 3B**).

Differences in the expression of individual genes by estrous cycle phase was determined by ANOVA. Estrous cycle phase affected expression of genes *ESR1*, *ISG15*, *IL-1 α* , *PTGER2*, *PGR*, *MUC1*, *MUC13*, and *NF κ B1* ($P < 0.05$), and tended to affect *CXCL8* and *IL-1 β* ($P = 0.09$; **Table 2**). All genes impacted by estrous cycle phase had greater expression in explants from the luteal phase tissue compared with follicular phase tissues (**Table 2**).

Explant culture and LPS treatment

Treatment of explants indicated clustering, with complete separation of uncultured controls from cultured controls and LPS, which also clustered, but with slight overlap ($P < 0.001$;

Figure 4A). Scores from VIP determined *CXCL8*, *IL-6*, *IL-1 β* , *CXCL2*, and *IL-1 α* to contribute the most to differences between treatment groups (**Figure 4B**). Cytokines *CXCL8*, *IL-6*, *IL-1 β* , and *CXCL2* had greater expression in cultured control explants, with the least expression in uncultured control explants. Expression of *IL-1 α* was greatest in LPS treated and the least in uncultured controls (**Figure 4B**). Due to observed difference between uncultured and cultured explants, cultured explants serve as a better control to determine the specific effect of LPS. Therefore, an OPLS-DA was conducted with uncultured controls removed. In comparison of cultured controls and LPS treated explants, clear separation of overall gene expression between the groups was observed ($P < 0.001$; **Figure 4C**). The VIP plot indicated *CSF2*, *CCL3*, *LIF*, *IL-10*, and *OAS2* contributed the most to the separation of cultured controls and LPS treated explants, with greater expression in LPS explants except *LIF* which was greater in cultured controls (**Figure 4D**).

The expression of 29 genes were affected by treatment ($P < 0.05$, **Table 3**). Of these genes, 15 were affected between uncultured versus cultured explants. Expression of *CCL4*, *TNF α* , *ESR1*, *IRF3*, *IL-1 β* , *VEGFA*, *IL-6*, *TLR4*, *CXCL2*, *ISG15*, *PTGER2*, *ESR2*, and *NF κ B1* was the least in uncultured explants, with greater expression in cultured tissue and did not differ between cultured controls and LPS explants ($P < 0.05$, **Table 3**). In contrast, *IFN γ* ($P = 0.003$) and *IL-4* ($P < 0.0001$) had the greatest expression in uncultured explants, with lesser expression in cultured tissue but similar expression between cultured controls and LPS explants. Explants from cultured control treatment group had greater expression of *CXCL8*, *TGFB*, *CSF3*, *PGR*, and *MX1* relative to the LPS and uncultured control groups ($P < 0.05$, **Table 3**). Six genes increased expression in response to LPS including *IL-10*, *IL-*

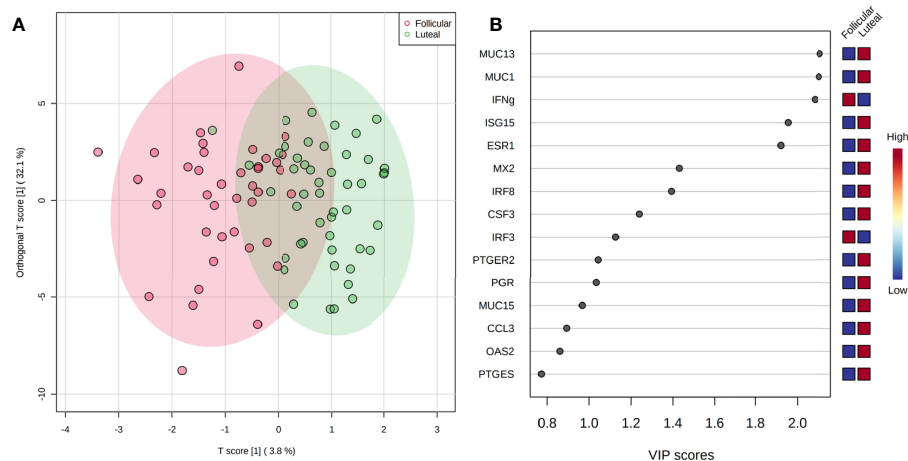


FIGURE 3
Overall gene expression clustering of explants between the estrous cycle phases ($P < 0.001$) by OPLS-DA (**A**) with corresponding VIP plot (**B**) indicating genes contributing to observed clustering.

TABLE 2 Expression of genes affected by phase of the estrous cycle.

Estrous Cycle Phase ¹			
Gene	Luteal (n=48)	Follicular (n=40)	P Value ²
<i>ESR1</i>	1,573.44 ± 27.92 ^a	1,449.07 ± 29.84 ^b	0.003
<i>CXCL8</i>	1,039.29 ± 50.79	943.19 ± 52.27	0.09
<i>IL1β</i>	1,112.56 ± 48.93	989.17 ± 51.19	0.09
<i>ISG15</i>	1,017.12 ± 48.84 ^a	837.82 ± 50.59 ^b	0.01
<i>IL1α</i>	718.29 ± 33.29 ^a	607.44 ± 35.87 ^b	0.03
<i>PTGER2</i>	969.21 ± 14.25 ^a	923.66 ± 15.25 ^b	0.03
<i>PGR</i>	838.42 ± 15.24 ^a	772.94 ± 15.90 ^b	0.004
<i>MUC1</i>	561.75 ± 45.93	430.07 ± 48.09	0.05
<i>MUC13</i>	21.66 ± 0.62 ^a	19.30 ± 0.66 ^b	0.01

¹ n = number of explants; Gene expression presented as mean normalized total counts ± SEM.
² ^{ab} denotes P < 0.05 between groups within each gene.

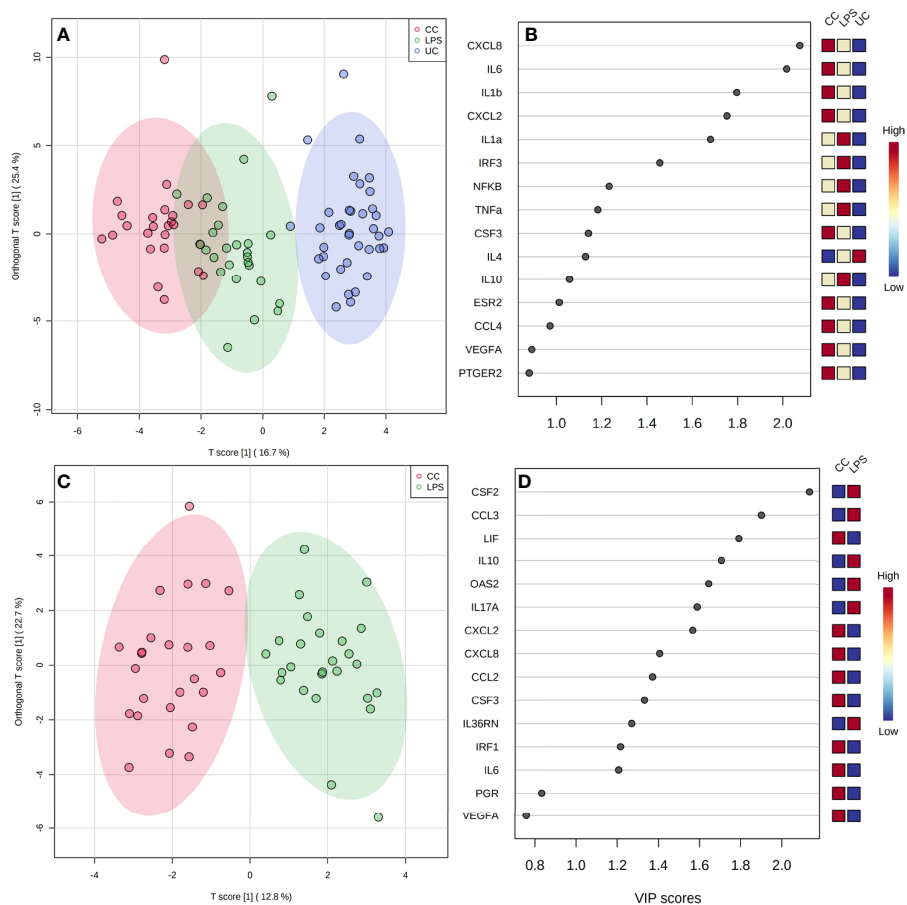


FIGURE 4 Overall gene expression clustering of explants between all treatments [(A); $P < 0.001$], and between LPS and cultured control treatments (C), by OPLS-DA, with corresponding VIP plots (B, D) indicating genes contributing to observed clustering. CC, Cultured Controls; LPS, Lipopolysaccharide; UC, Uncultured Controls.

TABLE 3 Expression of genes affected by treatment group.

Gene	Treatment ¹			P Value ²
	Uncultured Control (n=37)	Cultured Control (n=25)	LPS (n=26)	
<i>CCL4</i>	1,316.93 ± 43.79 ^b	1,541.43 ± 50.57 ^a	1,513.66 ± 50.06 ^a	0.0004
<i>TGFβ</i>	1,299.32 ± 14.31 ^b	1,378.40 ± 16.81 ^a	1,338.86 ± 16.65 ^{ab}	0.001
<i>TNFα</i>	1,123.45 ± 53.36 ^b	1,460.42 ± 62.55 ^a	1,548.96 ± 61.96 ^a	<0.0001
<i>ESR1</i>	1,425.29 ± 29.82 ^b	1,589.43 ± 35.54 ^a	1,519.04 ± 35.25 ^a	0.002
<i>CXCL8</i>	187.64 ± 45.02 ^c	1,465.80 ± 50.25 ^a	1,320.29 ± 49.72 ^b	<0.0001
<i>IRF3</i>	1,219.56 ± 14.96 ^b	1,374.36 ± 17.43 ^a	1,364.81 ± 17.26 ^a	<0.0001
<i>IL1β</i>	568.56 ± 47.49 ^b	1,268.78 ± 54.87 ^a	1,315.25 ± 54.31 ^a	<0.0001
<i>VEGFA</i>	1,219.80 ± 13.22 ^b	1,285.93 ± 14.83 ^a	1,260.95 ± 14.67 ^a	0.0004
<i>IL6</i>	469.38 ± 37.98 ^b	1,256.57 ± 43.1 ^a	1,193.44 ± 42.65 ^a	<0.0001
<i>IRF1</i>	1,213.49 ± 18.46 ^a	1,175.54 ± 21.77 ^a	1,102.33 ± 21.58 ^b	0.0005
<i>TLR4</i>	969.47 ± 12.84 ^b	1,037.94 ± 15.20 ^a	1,027.26 ± 15.06 ^a	0.0008
<i>CXCL2</i>	633.10 ± 45.37 ^b	1,135.62 ± 50.74 ^a	1,056.34 ± 50.21 ^a	<0.0001
<i>IL10</i>	581.36 ± 74.50 ^c	1,009.59 ± 84 ^b	1,648.08 ± 83.11 ^a	<0.0001
<i>CSF3</i>	1,018.05 ± 62.36 ^c	1,448.04 ± 72.30 ^a	1,240.03 ± 71.58 ^b	<0.0001
<i>ISG15</i>	805.27 ± 44.96 ^b	974.86 ± 50.94 ^a	1,002.29 ± 50.41 ^a	0.0014
<i>IL1A</i>	162.50 ± 36.76 ^c	846.36 ± 44.23 ^b	979.74 ± 43.92 ^a	<0.0001
<i>PTGER2</i>	895.92 ± 15.30 ^b	979.54 ± 18.26 ^a	936.94 ± 18.12 ^a	0.0009
<i>ESR2</i>	1,161.76 ± 37.99 ^b	1395.44 ± 44.01 ^a	1,327.15 ± 43.57 ^a	<0.0001
<i>PGR</i>	759.80 ± 14.60 ^c	854.47 ± 16.79 ^a	802.77 ± 16.62 ^b	<0.0001
<i>OAS2</i>	795.99 ± 67.59 ^b	533.07 ± 76.48 ^c	1,021.34 ± 75.67 ^a	<0.0001
<i>CCL2</i>	868.76 ± 9.98 ^a	846.85 ± 11.74 ^a	785.06 ± 11.63 ^b	<0.0001
<i>NFκB</i>	701.78 ± 31.58 ^b	951.54 ± 38.13 ^a	973.68 ± 37.88 ^a	<0.0001
<i>IL17A</i>	74.76 ± 89.79 ^c	303.45 ± 95.31 ^b	771.90 ± 94.56 ^a	<0.0001
<i>MX1</i>	539.09 ± 13.67 ^b	621.27 ± 16.51 ^a	581.08 ± 16.40 ^{ab}	0.001
<i>CSF2</i>	16.77 ± 25.81 ^c	166.01 ± 28.93 ^b	554.05 ± 28.62 ^a	<0.0001
<i>CCL3</i>	3.99 ± 12.32 ^c	57.53 ± 13.32 ^b	186.43 ± 13.20 ^a	<0.0001
<i>IFNγ</i>	6.44 ± 1.15 ^a	1.96 ± 1.30 ^b	3.14 ± 1.29 ^b	0.003
<i>IL4</i>	19.75 ± 2.33 ^a	5.15 ± 2.59 ^b	7.84 ± 2.56 ^b	<0.0001
<i>IL36RN</i>	-0.21 ± 1.81 ^c	0.81 ± 2.07 ^b	11 ± 2.04 ^a	<0.0001

¹LPS: Lipopolysaccharide; n = number of explants; Gene expression presented as mean normalized total counts ± SEM.

²abc denotes P < 0.05 between groups within each gene.

1α, *IL-17A*, *CSF2*, *CCL3*, and *IL-36RN*, with greatest expression in LPS explants, intermediary expression in cultured control explants, and lowest expression in uncultured explants ($P < 0.05$, Table 3). Conversely, LPS explants had lower expression of *IRF1* ($P = 0.005$) and *CCL2* ($P < 0.0001$) compared to uncultured and cultured control explants which were similar in expression (Table 3).

Uterine horn

Separation of overall gene expression was not observed by uterine horn as profiles completely overlapped in the OPLS-DA ($P = 0.41$). However, expression of individual genes differed by uterine horn including *ESR1*, *IL-10*, *OAS2*, *MUC1*,

MUC13, *IFNγ*, and *MUC15* ($P \leq 0.05$), with tendencies for *CCL4*, *IRF8*, *OAS2*, *NFκB1*, and *VEGFA* ($P \leq 0.10$; Table 4). The ipsilateral horn had the greatest expression of all affected genes (Table 4).

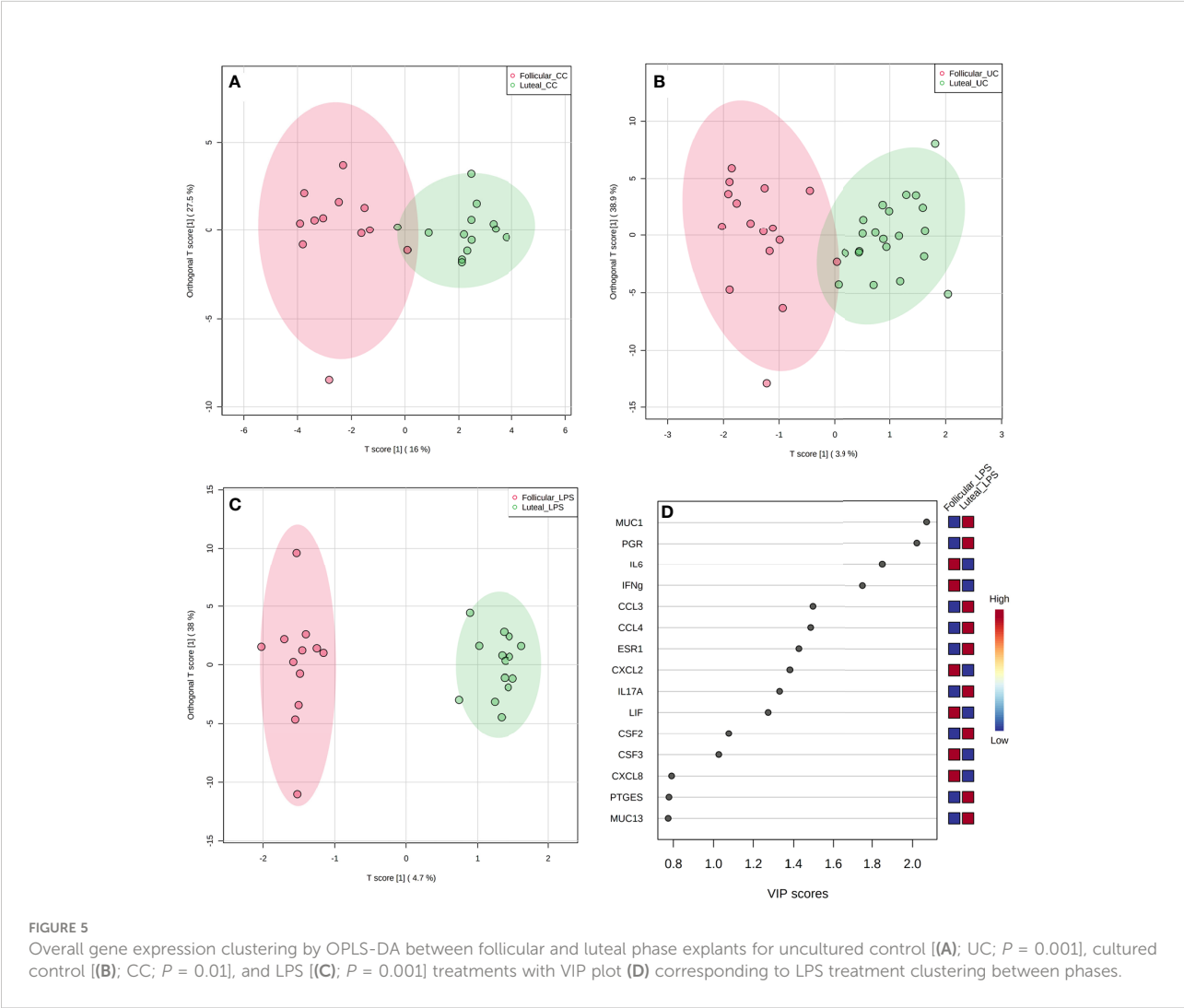
Interactions of estrous cycle phase, treatment, and uterine horn

The relationship between phase of the estrous cycle and treatment is depicted in Figures 5A–C. Separation with slight overlap of follicular and luteal phase explants in uncultured ($P = 0.001$, Figure 5A) and cultured controls ($P = 0.01$, Figure 5B) was observed. However, distinct separation with no overlap of follicular and luteal phase explants in response to LPS

TABLE 4 Expression of genes affected by uterine horn.

Gene	Uterine Horn ¹		P Value ²
	Ipsilateral (n=42)	Contralateral (n=46)	
CCL4	1,498.72 ± 41.97	1,415.97 ± 40.06	0.10
ESR1	1,550.40 ± 28.60 ^a	1,472.11 ± 26.94 ^b	0.04
IRF8	1,347.87 ± 50.08	1,231.23 ± 47.49	0.07
IL10	1,174.49 ± 71.58 ^a	984.86 ± 68.95 ^b	0.02
OAS2	844.16 ± 64.91	722.78 ± 62.45	0.09
NFκB1	914.79 ± 30.34	836.54 ± 28.40	0.06
MUC1	556.05 ± 42.86 ^a	435.77 ± 40.89 ^b	0.02
MUC13	21.47 ± 0.65 ^a	19.49 ± 0.61 ^b	0.03
IFNγ	4.88 ± 1.11 ^a	2.81 ± 1.07 ^b	0.05
MUC15	9.85 ± 4.62 ^a	8.46 ± 4.42 ^b	0.02
VEGFA	0.14 ± 0.01	0.10 ± 0.01	0.06

¹n = number of explants; Gene expression presented as mean normalized total counts ± SEM.
^{2ab} denotes P < 0.05 between groups within each gene.



treatment was observed ($P = 0.001$, Figure 5C), indicating differing responses between estrous cycle phases to LPS than other treatments. The VIP for the genes contributing to differences in observed clustering between phases specifically for LPS treatment is depicted in Figure 5D.

For individual gene expression differences, the interaction of treatment \times phase was observed for *IL-1 α* ($P < 0.01$) and *PGR* ($P = 0.05$), with tendencies for *ISG15* ($P = 0.10$) and *NF κ B1* ($P = 0.07$). For *IL-1 α* , the least expression was observed for uncultured explants of luteal and follicular phase, with the greatest expression in LPS explants of luteal and follicular phase. Similarly, luteal and follicular uncultured explants had the least expression of *PGR*. However, follicular LPS and cultured control explant *PGR* expression did not differ from uncultured controls, though expression was higher in luteal cultured control explants than all other groups.

No interactions between treatment \times horn were observed, but the interaction of phase \times horn was detected for *ESR1* and *MUC13* ($P = 0.05$). Expression of *ESR1* and *MUC13* followed the same pattern, with greatest expression detected in the ipsilateral horn of luteal phase explants, with similar expression in the luteal contralateral horn and follicular ipsilateral horn. However, less expression of *ESR1* and *MUC13* was observed in the follicular contralateral horn ($P = 0.05$). No genes were affected by the triple interaction of treatment, phase, and horn.

Discussion

Potentially pathogenic bacteria are present in healthy microbial communities, including the uterine microbiome, but can increase in abundance and lead to a diseased state if provided the necessary shift in their environment (Karstrup et al., 2017). Various stimuli such as diet and the external environment can influence microbiome composition and its respective environment, leading to greater abundances of potentially pathogenic bacteria (Li et al., 2012; Monteiro and Faciola, 2020). Recently, shifts in the abundance of common potential pathogens in a healthy uterus through the estrous cycle were associated with influencing breeding success (Ault et al., 2019a). Interactions of bacteria with the uterine environment leading to changes in reproductive outcomes must be explored to better understand their role in reproductive efficiency. Therefore, the current study utilized LPS as a model for potentially pathogenic bacteria to evaluate its effects on the local immune response of the uterine endometrium between phases of the estrous cycle and across the uterine horns. Differences in the expression of LPS binding pattern recognition receptor *TLR4* and transcription factor *NF κ B1* were not detected in LPS treated explants. Previous studies have reported the ability of LPS to increase expression of *TLR4*, with other studies reporting minimal to no difference

between control and LPS challenged tissues, potentially due to differences in LPS concentration and duration of exposure (Cronin et al., 2012; Swangchan-Uthai et al., 2012; Fu et al., 2013; Ding et al., 2020). The *TLR4* receptors are present on endometrial tissue, including epithelial and stromal layers, and resident immune cells, such as macrophages, without LPS challenge in preparation for bacterial response (Herath et al., 2006; Dadarwal et al., 2017). Although tissues were selected based on negative signs of pregnancy and infection, level of *TLR4* expression may be influenced by the state of the uterine microbiome, previous infection, or reproductive status prior to tissue collection.

No differences in the individual cytokine gene expression were detected in commonly LPS-stimulated cytokines *TNF α* , *IL-6*, and *IL-1 β* of the current study. The LPS concentration used has been indicated to be present in the bovine postpartum uterus and validated to elicit a response (Dohmen et al., 2000; Herath et al., 2006), with responses to LPS occurring as early as 3 h, up to 24 h (Borges et al., 2012; Swangchan-Uthai et al., 2012; Fu et al., 2013). However, the early response to LPS may result in various patterns of cytokine concentrations. Concentration of *TNF α* peaked by 1 h in bovine endometrial culture with LPS, while *IL-1 β* did not reach peak concentration until 3 to 6 h post LPS, with both decreasing up to 24 h (Swangchan-Uthai et al., 2012; Fu et al., 2013). Other pro-inflammatory cytokines *IL-1 α* and *IL-17A*, and chemokine *CCL3*, however, had greater expression in LPS treated explants of the current study. Similarly, bovine endometrial epithelial cells treated with LPS indicated *IL-1 α* to be the most upregulated pro-inflammatory cytokine gene, and one of the most upregulated genes among all immune response genes (Oguejiofor et al., 2015). A greater response of *IL-1 α* than *IL-1 β* and *IL-6* relative to controls was observed in LPS treated endometrial cells, suggesting *IL-1 α* aids in upregulating the inflammatory response to LPS (Healy et al., 2014). The *IL-17* signalling pathway was also identified as a major inflammatory pathway in LPS stimulation (Ding et al., 2020). The chemokine *CCL3* is produced by immune cells, such as macrophages and dendritic cells, to upregulate an inflammatory response by recruiting additional immune cells for LPS clearance (Jing et al., 2003). Therefore, an *in vivo* model may result in greater overall cytokine and chemokine concentrations due to increased immune cell infiltration from other locations to the endometrium. In addition to these increases in pro-inflammatory cytokine expression, regulatory cytokine *IL-10* also had greater expression in explants treated with LPS. Regulatory cytokine expression, such as *IL-10*, can increase in response to elevated pro-inflammatory cytokine concentrations to mediate the level of inflammation (Dadarwal et al., 2017). An increased ratio of *IL-1 α* and *IL-1 β* to *IL-10* concentration in postpartum cows with uterine disease was associated with failure to rebreed (Herath et al., 2009). Increases in regulatory and anti-inflammatory cytokines can

result from a transition in macrophage activation or by production of the affected tissue cells. Stromal cells of the endometrium have indicated greater production of IL-10 when exposed to LPS compared to controls and epithelial cells treated with LPS (Koh et al., 2018). Therefore, treatment of LPS on endometrial explants in the current study resulted in increased pro-inflammatory cytokine expression, but concurrent upregulation of *IL-10* expression may indicate an attempt to balance the inflammatory state of the endometrium.

Steroid hormones estradiol and progesterone dominate specific phases of the estrous cycle to prepare the uterus for reproductive events such as pregnancy development and maintenance. As expected, differences in the gene expression of *PGR* and *ESR1* were observed between phases, with *PGR* expression highest during the luteal phase of the current study. The abundance of progesterone receptors on the endometrium epithelial cells peaks during mid-luteal phase with increasing progesterone secretion (Robinson et al., 2001; Okumu et al., 2010). Following mid-luteal phase, nuclear progesterone receptors begin to decrease in the surface endometrial epithelial cells, a prerequisite of uterine receptivity and the endometrial luteolytic mechanism, but remain present in stromal cells (Kimmins and MacLaren, 2001; Okumu et al., 2010). Additionally, *PTGER2* expression was greater in luteal phase explants of the current study. Secretion of PGE2 and its receptor *PTGER2* expression has previously shown to be greater during mid-diestrus when progesterone secretion is greatest, then decreases around the time of luteolysis (Arosh et al., 2003; Zhang et al., 2017). Therefore, greater expression of *PGR* and *PTGER2* in the current study, in addition to careful selection of reproductive tracts after staging the estrous cycle, would indicate successful selection of uteri during the mid-luteal phase when maternal blood progesterone concentrations are high. Due to the importance of controlling the maternal immune system response to prevent rejection of the fetus, progesterone dominance is typically classified as an anti-inflammatory period (Raghupathy, 2001; Schjenken et al., 2012). However, IL-1 α , IL-1 β , and CXCL8, which are classically categorized as pro-inflammatory, were detected in greater abundance in luteal phase explants in the current study. High progesterone supplementation on bovine endometrial epithelial cells has indicated increases in pro-inflammatory cytokine expression (Cui et al., 2020). But when separated by estrous cycle stage, concentrations of IL-1 α , IL-1 β , and CXCL8 were greater in early to mid-diestrus, but decreased by late-diestrus in non-pregnant animals, suggesting preparation for pregnancy beginning around the time of maternal recognition (Oliveira et al., 2013). Therefore, fluctuations of individual inflammatory cytokines occur throughout the estrous cycle, reaching an anti-inflammatory state by the time of pregnancy establishment (Oliveira et al., 2013). However, these differences across the cycle may indicate a greater susceptibility when exposed

to stressors such as pathogenic bacteria and their LPS membrane component.

The inflammatory response to a stressor is not based on the expression of a single immune response mediator, but the cohesive actions of multiple cytokines, chemokines, and other immune response related proteins from a variety of immune cells and tissues. Therefore, the overall profile of immune-related gene expression may be a better representation of the response to LPS and differences between estrous cycle phases. Interestingly, profiles grouped by estrous cycle phases but with similar gene expression of both control treatment groups, but distinct profiles were observed in response to LPS treatment between the follicular and luteal phase. The mucin-family genes, *MUC1* and *MUC13*, were affected by estrous cycle phase with greater expression in the luteal than follicular phase, and were determined by VIP as the top contributors to differences in overall gene expression between phases and phase response to LPS. Mucins are anti-adhesive proteins that serve as a defense mechanism for the innate immune system, especially in the reproductive tract (Brayman et al., 2004). Greater expression of mucins has been associated with uterine disease and infertility, potentially preventing trophoblast attachment (Horne et al., 2005; Kasimanickam et al., 2014). Decreases in endometrium epithelial *PGR* expression during the late luteal phase, especially before maternal recognition of pregnancy in pregnant animals, is associated with reductions in mucin expression to prepare the uterus for conceptus attachment and implantation (Carson et al., 2000; Spencer et al., 2006). As mucins are a surface protein in the uterine lumen, the greater expression of *PGR*, *MUC1*, and *MUC13* in luteal phase explants of the current study indicate mid-luteal phase uteri prior to the loss of *PGR* on endometrial epithelial cells. Although individual mucin expression was not affected by LPS treatment, OLPS-DA analysis indicated *MUC1* to contribute the most to the difference between luteal and follicular phase response to LPS. Additionally, pro-inflammatory IL-6 and IFN γ were important contributors to phase response to LPS, being higher in follicular phase explants, although they did not differ in individual expression by treatment. Due to the protective role of mucins to bind bacteria and prevent invasion into uterine tissue (Brayman et al., 2004; Sando et al., 2009), mucin expression differences between estrous phases may be a result of the total cytokine and other immune factor responses, therefore affecting uterine response to bacteria.

Greater progesterone concentrations have been previously observed in the cranial portion of the ipsilateral horn closest to the CL, compared to the contralateral horn, suggesting a hormonal gradient throughout the uterus potentially impacting the uterine environment in response to progesterone actions (Pope et al., 1982; Weems et al., 1988; Takahashi et al., 2016). Global gene expression analysis has indicated greater variation

between the contralateral and ipsilateral horns during the early estrous cycle that decreased towards the period of uterine receptivity to pregnancy (Sánchez et al., 2018). All mucins evaluated in the current study had greater expression in the ipsilateral than contralateral horn. The strong influence of steroid hormones on the expression of mucins potentially contributed to their observed differences by uterine horn, although divisions of each horn did not impact expression in the current study. In addition to mucins, multiple other genes were affected by explants taken from the contralateral and ipsilateral horn. Expression of major angiogenic cytokine *VEGFA* was increased in the ipsilateral horn compared to the contralateral horn. Blood flow to the ipsilateral horn, especially in preparation for pregnancy is crucial to supply the early embryo with nutrients for survival (Ford et al., 1979). Changes in *VEGFA* suggest vascular development cytokines begin to stimulate these changes in the estrous cycle of non-pregnant animals. Additionally, both regulatory cytokine *IL-10* and pro-inflammatory *IFN γ* had greater expression in the ipsilateral horn. Previous studies indicated altered response of the ipsilateral or contralateral uterine tissue to culture with a conceptus (Bagés-Arnal et al., 2020). Due to the importance of a controlled immune response for pregnancy development (Raghupathy, 2001), differences in cytokine expression between uterine horns may indicate regulation of immune status is necessary in the ipsilateral horn for pregnancy establishment and maintenance. Therefore, the majority of genes that differed between the contralateral and ipsilateral uterine horns are important for pregnancy establishment and maintenance, however further investigation is needed to determine how they may impact breeding success.

In conclusion, estrous cycle phase resulted in differing overall endometrial gene expression profiles of immune response to LPS treatment. Multiple immune response genes differed between the follicular and luteal phases. Mucins are greatly impacted by the dominant steroid hormone present of each estrous cycle phase and their protective role during high abundance of the luteal phase likely impacts the resulting immune response to a stimulus such as LPS. However, overall gene expression profile did not differ between uterine horns and did not influence response to LPS treatment. Further research is needed to understand how these differences in estrous cycle response to LPS correlate to changes in the uterine bacterial communities and their direct effects on pregnancy establishment.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, PRJNA824812.

Ethics statement

Ethical review and approval was not required for the animal study because no live animals were utilized for the research.

Author contributions

TA-S was responsible for primary manuscript preparation, experimental design, sampling, RNA extraction, and data analyses. RP assisted with laboratory analyses and manuscript preparation. KL was responsible for targeted RNA sequencing. BV and ES assisted with targeted RNA sequencing preparation and data analyses. SM, KP, FS, DM, KM, and PM were responsible for experimental design development and manuscript preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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