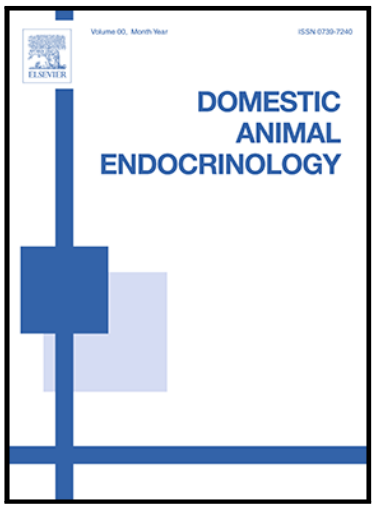

Shifts in uterine bacterial communities associated with endogenous progesterone and 17β-estradiol concentrations in beef cattle

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Highlights

- Progesterone concentrations may influence uterine bacterial diversity in heifers.
- Differing progesterone and estradiol concentrations may alter uterine *Corynebacterium*.
- Certain anaerobic bacteria (i.e., *Ruminococcus, Blautia*) may modulate uterine pH.

Shifts in uterine bacterial communities associated with endogenous progesterone and 17β-estradiol concentrations in beef cattle

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Abstract

The relation between circulating concentrations of progesterone and 17β-estradiol prior to insemination play a key role in optimizing fertility in cattle. This study aimed to determine the impact of endogenous progesterone (P_4) and estradiol (E_2) concentrations on uterine bacterial community abundance and diversity in beef cattle. Angus-influenced heifers were subjected to an industry standard estrous synchronization protocol. Uterine flushes were collected on day -2 (endogenous P_4) and day 0 (endogenous E_2) and used for targeting the V4 hypervariable region of 16S rRNA bacterial gene. Plasma was collected on days -2 and 0 for quantification of P_4 and E_2 concentrations by radioimmunoassay, respectively. Heifers were allotted to one of the following groups: High P_4 + High E_2 (H-H; n = 11), High P_4 + Low E_2 (H-L; n = 9), Low P_4 + High E_2 (L-H; n = 9), Low P_4 + Low E_2 (L-L; n = 11). Results indicated that Shannon's diversity index tended to be greater for H-L heifers compared to L-H heifers on day 0 (P = 0.10). For H-L heifers from day -2 to day 0, the relative abundance of Actinobacteria decreased and Tenericutes increased (P < 0.01). Within phylum

Actinobacteria, the relative abundance of *Corynebacterium* decreased from day -2 to day 0 in treatment groups H-H, H-L, and L-L ($P < 0.05$); however, did not differ by day for L-H heifers. Within phylum Tenericutes, the relative abundance of *Ureaplasma* increased from day -2 to day 0 for H-L heifers ($P = 0.01$). Additionally for H-L heifers, the relative abundance of Bacteroidetes tended to increase from day -2 to on day 0 ($P = 0.07$). For H-L heifers, uterine pH increased from day -2 to day 0 ($P = 0.05$). These results suggest that differing endogenous concentrations of P_4 and E_2 may be associated with shifts in uterine microbiota and pH, and this could ultimately impact fertility outcomes in beef cattle.

Keywords
microbiome, uterus, cattle, estradiol, progesterone

1. Introduction

Reproductive success is vital for the sustainability and profitability of cow-calf operations, and numerous factors can negatively impact this success such as nutrition, environmental stress, hormonal imbalance, or dysbiosis [1-4]. The processes of ovulation, formation of the corpus luteum (CL), and luteolysis are all essential for proper cyclicity, and required to have an adequate uterine environment conducive for establishing and maintaining pregnancy. Specifically, the relation between circulating concentrations of progesterone (P_4) and 17β-estradiol (E_2) prior to artificial insemination play a key role in optimizing reproductive success in cattle [5-9].

Low circulating P_4 during the growth of the pre-ovulatory follicle has been associated with the growth and development of a persistent follicle and reduced fertility in cattle [5,7,9]. Low plasma concentrations of P_4 (approximately 2 ng/mL) have been shown to result in the growth of a persistent follicle, elevate endogenous E_2 concentrations (>10 pg/mL), and decrease conception rates by approximately 25% when compared to greater plasma P_4 concentrations (>4 ng/mL) [5,7]. Additionally, cows with low circulating P_4 during the

growth of the pre-ovulatory follicle have an increased likelihood of pregnancy loss between day 35 and 56 of gestation [9].

The female reproductive tract is dynamic; constantly undergoing structural and hormonal changes. Similarly, bacterial communities within the reproductive tract undergo rapid changes, specifically shifting in abundance and phylogenetic diversity [10-14]. Otero et al. [10] used a culture-based method to evaluate vaginal microflora in cattle, specifically the genera *Lactobacillus* and *Enterococci*, and observed greater bacterial abundance during periods of elevated E_2 (i.e., proestrus and estrus) and reduced bacterial abundance during periods of elevated P_4 (i.e., metestrus and diestrus). This suggests that bacterial community abundances may shift throughout the estrous cycle in cattle. Recent studies utilizing 16S rRNA sequencing identified that bacterial diversity differs in vaginal samples collected either during the follicular and luteal phase, specifically P_4 concentrations appear to affect bacterial diversity [13,14]. Interestingly, another recent study in beef heifers demonstrated that differing E_2 concentrations (i.e., high, medium, and low) on day of artificial insemination resulted in no differences in the vaginal bacterial community profiles [15].

Majority of studies in cattle have either focused on the vaginal microbiome or bacteria associated with metritis and endometritis, with few investigating the uterine microbiome in healthy cattle. Previous studies have identified shifts in both vaginal and uterine bacterial community diversity in beef cattle over the course of an estrous synchronization protocol, with bacterial community abundance and diversity having greater shifts over time within the uterus [11,12]. Moreover, taxonomic classification indicated that bacterial genera such as *Corynebacterium* and *Prevotella* within the uterus may potentially have a negative effect on fertility in beef cattle [12,16]. Interestingly, a bacterium that has been associated with a healthy uterine environment in cattle is *Ureaplasma* spp. [12]. Taken together, the present study was designed to examine the impact of endogenous P_4 and E_2 concentrations on uterine

bacterial community abundance and diversity in beef heifers. The hypothesis is that heifers with relatively high concentrations of P_4 and E_2 versus relatively low concentrations of P_4 and E_2 will differ in microbiota composition of the uterus, specifically with high concentrations having greater bacterial diversity and abundance.

2. Materials and Methods

This study was conducted at Texas A&M University O.D. Butler, Jr. Animal Science Complex Nutrition and Physiology Center in College Station, TX from November 2020 to March 2021 and performed under an approved protocol by the Institutional Animal Care and Use Committee of Texas A&M University (IACUC 2020-0077).

2.1 Experimental Animals, Reproductive Measurements, and Sample Collection

Angus-influenced heifers (n = 16) with an average weight of 376 ± 11 kg and with no clinical signs of uterine disease or reproductive tract abnormalities, were housed in an outdoor paddock and received *ad libitum* access to water and grass hay. An industry standard estrous synchronization protocol was implemented beginning with an intramuscular injection of gonadotropin releasing hormone (GnRH; Factrel, 100 μg; Zoetis Animal Health, Troy Hills, NJ; day -9) and seven days later followed by an intramuscular injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse, 5 mL; 5 mg/mL; Zoetis Animal Health, Troy Hills, NJ; day -2). Controlled internal release devices (CIDR) were not used in the study due to uterine flush collection methods. On day -2, estrus detection patches (Estrotect; Rockway, Inc. Spring Valley, WI) were applied. Two days following $PGF_{2\alpha}$ administration (day 0), another intramuscular injection of GnRH was administered. Heifers were evaluated 2 times daily for estrus activity from days -1 to 1. This protocol was replicated 2 more times on the same group of heifers with a rest period of 28 days between replications.

Uterine flushes were collected on day -2 and day 0 and used for bacterial DNA extraction and sequencing and pH as previously described [11,12,16]. Briefly, the perineal area was cleaned and disinfected prior to flushing. To obtain uterine flush samples, 180 mL 0.9% sterile saline (Vetivex) was flushed through a Foley catheter into the uterus by a single technician. When placing the Foley catheter, a sterile chemise (WTA; College Station, TX) was used to prevent contamination (i.e., vaginal contamination) while placing the catheter in the uterus. Resulting uterine flush fluid was collected by rectal massage. Two blank control flushes were collected through sterile Foley catheters, and these had minimum quality-filtered reads (7 and 95), thus contamination was considered minimum to absent. The pH of the flush samples was measured by Orion Star™ pH portable meter (Thermo Fisher Scientific Inc., Waltham, MA) and recorded immediately following collection. Samples were snap-frozen in liquid nitrogen and stored at -80 ℃ until analysis.

2.2 Blood Sampling and Radioimmunoassay

Blood samples were collected on days -2 and 0 by tail venipuncture into a 10 mL K_2EDTA vacutainer tube (BD Vacutainer, Becton, Dickinson and Company, NJ) and placed on ice until transported to the laboratory for processing. Samples were centrifuged at 1,500 × *g* at 4 ℃ for 15 minutes. Plasma was immediately collected and transferred into 1.5 mL tubes then stored at -20 ℃ until analysis.

Estradiol concentrations were evaluated using an RIA protocol described by Kirby et al. [17] with antibody and 3-Ido-Estradiol-17β Tracer from MP Biomedicals (Santa Ana, CA). Standard curves and high/low control serum samples were run at the beginning and end of the assays. The inter- and intra-assay CVs were 6.07% and 5.63%, respectively. Progesterone concentrations were quantified according to the previously described protocol [18] using a double-antibody RIA kit (MP Biomedicals, Santa Ana, CA). A standard curve was used to

calculate sample concentrations in a single assay with high/low controls for quality control. The intra-assay CV was 2.66%.

2.3 Treatment Group Classification

Transrectal ultrasound was conducted by a single technician at each time point (day -9, -2, and 0) to observe ovarian structures present. All heifers of the following criteria for the study were submitted for sequencing and further analysis as previously described [11,12]. The criteria for inclusion included: 1) response to GnRH on day -9 as assumed by the presence of a CL on day -2 and 2) ovulatory follicle present on day 0. Once heifers were identified by the previous criteria, they were sorted based on their day -2 plasma P_4 concentrations and day 0 plasma E_2 concentrations (Table 1). For P_4 and E_2 concentrations, the overall mean concentration (3.25 ng/mL for P_4 and 3.38 pg/mL for E_2) and standard deviation (SD) was determined. High concentrations were designated as within mean plus three SD and low concentrations were designated as within mean minus three SD. Heifers were allotted to one of the following groups: 1. High P_4 + High E_2 group (H-H; n = 11), 2. High P_4 + Low E_2 group (H-L; n = 9), 3. Low P_4 + High E_2 group (L-H; n = 9), 4. Low P_4 + Low E_2 group (L-L; n = 11).

2.4 DNA Extraction and Sequencing

Flush samples were selected based on inclusion criteria previously outlined in Treatment Group Classification. Samples were sent to FERA Diagnostics and Biologicals Corp. (College Station, TX) for DNA extraction and 16S rRNA gene amplicon sequencing. Samples were transferred to a 96-well plate and DNA extraction was performed using Mag-Bind® Universal Pathogen 96 Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's instructions.

The 16S amplicons were amplified by PCR for individual metagenomic DNA samples according to previously described methodology [19]. The V4 hypervariable region of bacterial/archaeal 16S rRNA gene were amplified with 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers using methods optimized for the Illumina MiSeq platform [20].

2.5 Bioinformatic Analysis

FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess the quality of raw fastq datasets. The QIIME2 pipeline (https://qiime2.org/) was used to process all samples [21]. The raw fastq sequences of all samples were imported into QIIME2 for taxon analysis. DADA2 was used for removing the low-quality sequences based on the quality plots [22]. The quality seemed to trail down below Q20 at a length of 290, hence 290 was set as the truncation length. The number of sequences for each operational taxonomic unit (OTUs) and the representative sequences for the same were obtained. Taxonomy assignment was performed for the representative sequences using the SILVA 138 release database's pre-trained classifiers for the 99% OTUs (https://data.qiime2.org/2021.11/common/silva-138-99-nb-classifier.qza). For further statistical analysis, BIOM file was exported, and metadata and taxonomy information were added.

2.6 Statistical Analysis

To calculate the phylogeny related metrics such as weighted and unweighted UniFrac distances, phylogenetic trees were constructed and a file was generated using ‘mafft’ [23] and ‘FastTree’ [24], both of which are an integral part of QIIME2. Before calculating diversities, sample rarefaction was done using a sampling depth of 1132. Samples with a lower sampling depth were discarded from the analysis. Kruskal-Wallis test was performed for alpha

diversity. Similarly, PERMANOVA was performed for beta diversity [25]. All plots were generated using R packages phyloseq [26] and ggplot2 [27].

Nonparametric ANOVA in SAS 9.4 was used for all bacterial abundance data with the independent variables of day or treatment group. Significance was determined by Wilcoxon exact test for comparisons between treatment groups and Kruskal-Wallis test for comparisons among days with the Benjamini–Hochberg FDR multiple test correction [28]. Replicate and individual heifer was not included in final model as they were determined to have no significant effect. Uterine pH data was analyzed using PROC MIXED in SAS 9.4 with fixed effects of day, treatment and the interaction, and random effects of heifer within day. A statistical significance was reported at $P \leq 0.05$. A tendency was reported at $P > 0.05$ and ≤ 0.10.

3. Results

3.1 Sequence Information

A total of 1,334,404 raw reads were observed with a maximum number of 79,108 reads and a minimum of 87 reads observed. Sequences with a length of 290 bp that fell below the Q20 criterion were identified and removed using the interactive quality plot. After quality control filtering and removal, 590,557 cleaned reads were kept for further analysis. In this study, the sampling depth was set at 1132, which resulted in the discarding of 10 samples with fewer than 1132 reads, leaving 63 samples for further diversity analysis. Table 2 indicates that average quality-filtered reads and standard error of the mean (SEM) per treatment group for each day. All samples contained a total of 9,288 characteristics (OTUs).

3.2 Alpha Diversity

For all alpha diversity metrics, there were no statistical differences observed between trial days regardless of treatment group ($P > 0.05$; Supplementary Figure S1). Similarly, there were no statistical differences for all alpha diversity metrics by treatment groups regardless of trial day using the Kruskal-Wallis test ($P > 0.05$; Supplementary Figure S2); however, a pairwise analysis identified a statistical difference for the alpha diversity metric, Shannon's diversity index, being greater for H-L heifers when compared to L-L heifers (4.69 ± 0.11 vs. 4.26 ± 0.15, respectively; $P = 0.05$, $Q = 0.29$).

On day -2, there were no statistical differences for all alpha diversity metrics by treatment groups using the Kruskal-Wallis test ($P > 0.05$; Supplementary Figure S3); however, a pairwise analysis identified statistical differences for the alpha diversity metrics, Chao1 ($P = 0.03$, $Q = 0.18$) and Shannon's diversity index ($P = 0.02$, $Q = 0.14$), being lower for H-H heifers when compared to H-L heifers (Table 2).

On day 0, there were no statistical differences for all alpha diversity metrics by treatment groups using the Kruskal-Wallis test ($P > 0.05$; Supplementary Figure S4); however, a pairwise analysis identified statistical differences for the alpha diversity metric, Chao1, being greater for H-H heifers when compared to L-H heifers and L-L heifers ($P = 0.03$, $Q = 0.20$ and $P = 0.09$, $Q = 0.26$, respectively; Table 2). Additionally, a pairwise analysis identified a tendency for the alpha diversity metric, Shannon's diversity index, being greater for H-L heifers when compared to L-H heifers ($P = 0.10$, $Q = 0.36$; Table 2).

3.3 Beta Diversity

For day -2, there was no significant clustering among samples by treatment groups for both unweighted and weighted UniFrac ($P > 0.05$; Figure 1A and Figure 1B, respectively). For the day 0 unweighted UniFrac (Figure 1C), PERMANOVA pairwise analysis identified a qualitative difference between H-H and H-L heifers ($P = 0.05$, $Q = 0.15$) and between H-L

and L-H heifers (P = 0.02; Q = 0.11). Additionally for the day 0 weighted UniFrac (Figure 1D), PERMANOVA pairwise analysis identified a qualitative difference between H-L and L-H heifers (P = 0.006; Q = 0.04).

There was no significant clustering among samples by treatment groups regardless of trial day (P > 0.05; Supplementary Figure 5). Despite no obvious clustering among the different treatment groups for the unweighted UniFrac, PERMANOVA pairwise analysis identified a qualitative difference between H-H and H-L heifers (P = 0.05, Q = 0.31). There was a tendency for clustering among samples by trial day regardless of treatment group for the unweighted UniFrac. The PERMANOVA pairwise analysis identified a qualitative difference in beta diversity between day -2 and day 0 (P = 0.06, Q = 0.06; Supplementary Figure S5).

3.4 Bacterial Community Composition - Phylum

The relative abundance of bacteria derived from each individual uterine flush sample was assigned to 31 different phyla, of which Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Tenericutes were the most abundant (greater than 1% relative abundance; Figure 2). Differences of phyla less than 1% relative abundance are listed in Supplementary Tables S1, S2 and S3.

There were no significant differences detected in the relative abundance of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, or Tenericutes for treatment groups H-H, L-H, and L-L between days -2 and 0 (P > 0.05). For H-L heifers, the relative abundance of Actinobacteria and Tenericutes significantly changed from day -2 to day 0. Relative abundance of Actinobacteria decreased from 12.26 ± 2.96% on day -2 to 3.91 ± 0.49% on day 0 (P = 0.01), whereas the relative abundance of Tenericutes increased from 0.93 ± 0.18% on day -2 to 8.16 ± 4.43% on day 0 (P = 0.004). Additionally for H-L heifers, the relative abundance of Bacteroidetes tended to increase from 16.31 ± 1.26% on day -2 to 21.23 ±

2.66% on day 0 (P = 0.07). There was a tendency in L-L heifers for the relative abundance of Actinobacteria to decrease from 9.34 ± 0.98% on day -2 to 6.69 ± 1.30% on day 0 (P = 0.08). There were no differences detected in the relative abundance of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, or Tenericutes between treatment groups on day -2 (P > 0.05). On day 0, the relative abundance of Bacteroidetes significantly differed (P = 0.04) by treatment group with H-H (21.37 ± 2.68%) and H-L (21.23 ± 2.66%) heifers being greater compared to L-H (13.76 ± 1.95%) and L-L (15.96 ± 2.29%) heifers. Additionally on day 0, the relative abundance of Tenericutes tended to differ (P = 0.10) by treatment group with H-L (8.16 ± 4.43%) and L-H (6.63 ± 5.53%) heifers being greater compared to H-H (1.18 ± 0.20%) and L-L (2.85 ± 1.31%) heifers.

3.5 Bacterial Community Composition - Genus

The relative abundance of bacteria derived from each individual uterine flush sample was assigned to 842 different genera, of which 19 genera were greater than 1% relative abundance (Table 3). Differences of genera less than 1% relative abundance on day -2 and 0 are listed in Supplementary Tables S4 and S5, respectively.

For H-H heifers, the relative abundance of *Clostridium* (P = 0.03) and *Agrobacterium* (P = 0.01) increased from day -2 to day 0, whereas *Corynebacterium* (P = 0.0002) decreased from day -2 to day 0. For H-L heifers, the relative abundance of *Blautia* (P = 0.01), *Pedobacter* (P = 0.06), *Ruminococcus* (P = 0.08), and *Ureaplasma* (P = 0.01) increased from day -2 to day 0, whereas *Corynebacterium* (P = 0.05) decreased day -2 to day 0. For L-H heifers, the relative abundance *Agrobacterium* (P = 0.01) increased from day -2 to day 0, whereas *Oscillospira* (P = 0.04) decreased from day -2 to day 0. For L-L heifers, the relative abundance of *Mycoplasma* (P = 0.10) tended to increase from day -2 to day 0, whereas *Corynebacterium* (P = 0.004) and *Caloramator* (P = 0.06) decreased from day -2 to day 0.

3.6 Uterine pH

On day -2, uterine pH was significantly lower in H-L heifers when compared to L-L heifers (6.40 vs. 6.73 ± 0.12, respectively; $P = 0.05$). On day 0, there were no differences in pH between treatment groups ($P > 0.05$). There were no significant differences detected in uterine pH for treatment groups H-H, L-H, and L-L between days -2 and 0 ($P > 0.05$). For H-L heifers, uterine pH increased from day -2 to day 0 (6.40 vs. 6.75 ± 0.12; $P = 0.05$).

4. Discussion

The relationship between low concentrations of P_4 with the development of a persistent follicle and elevated E_2 secretion is a phenomenon that has been extensively investigated for decades and widely accepted as one cause of reduced fertility (i.e., conception rates) observed in cattle [5-9]. In recent years, certain bacterial community abundances within the uterus prior to breeding also appear to potentially affect fertility outcomes in beef cattle [11,12]. Based on this research, it was suggested that hormonal concentrations, such as P_4 and E_2, may shift uterine bacterial communities toward either a pathogenic or healthy/commensal microbiome, thus subsequently impacting reproductive success. In the present study, P_4 concentrations seem to be the driver for most differences observed in uterine bacterial community diversity. Additionally, heifers classified as low endogenous P_4 (< 2 ng/mL) and high endogenous E_2 (> 6 pg/mL; L-H) appear to exhibit bacterial community taxonomic profiles that have previously been associated with reduced fertility [12,16].

The association between reproductive tract health and microbial diversity has been extensively investigated in humans [29]. Specifically, a lower phylogenetic diversity of bacteria in the reproductive tract in humans is favorable, with the genus *Lactobacillus* accounting for over 90% of the vaginal microbiome in healthy, fertile women [29-31].

Studies conducted in livestock identified that the reproductive tract of healthy animals have a greater bacterial species diversity when compared to humans [11,13,16,32]. When evaluating diversity, there are two predominant methods: alpha and beta diversity. Alpha diversity is used when evaluating the different number of bacterial species within a sample and can be further subdivided to evaluate the richness and/or evenness of distribution [33]. The richness of a sample, represented as Chao1 metrics, refers to the number of different bacterial species detected whereas the evenness of distribution refers to how balanced the species are within a given sample and the Shannon's diversity metric captures both the richness and evenness [33-35]. Based on the current data, E_2 concentrations do not appear to influence alpha diversity metrics, and this was similarly observed by previous researchers [15]. Most notably in the present study, samples collected on day 0 from heifers with previously high P_4 concentrations (H-H and H-L groups) had greater bacterial species richness and evenness when compared to samples from heifers with previously low P_4 concentrations (L-H and L-L groups). These results align with previous studies suggesting that elevated P_4 concentrations allows for an increase in bacteria abundance and diversity [13,14]. While this has been previously associated with pathogenic bacterial contamination and uterine disease, this high P_4 uterine environment may also allow for the growth and proliferation of commensal bacterial species. Several studies investigating the gut and respiratory microbiome have shown that commensal bacteria have a protective function and promote resistance to several pathogens by either direct action (i.e., directly inhibiting pathogens and/or competing for colonization) or through host-mediated immunity [36-38]. Moreover, previous data from this group demonstrated that prior to breeding (day -2) in cattle that establish and maintain pregnancy, P_4 concentrations were positively associated with both an anti-inflammatory uterine environment and potential commensal bacteria abundance (i.e., *Ureaplasma* spp.) within the uterus [39].

Beta diversity is a method used to calculate the differences in diversity between samples and is presented as a distance or dissimilarity matrix [33]. Unweighted and weighted UniFrac measures are phylogeny-based assessment of differences that were used to perform beta diversity analysis in the present study. Unweighted UniFrac is a qualitative measure of beta diversity that solely evaluates the presence or absence of a microbe, whereas weighted UniFrac evaluates presence or absence of a microbe and considers abundance [33,40,41]. In the current study, the analysis of community structure using unweighted UniFrac distance demonstrated differences between samples collected on day -2 and day 0 (Supplementary Figure S5). This observation was similarly reported by previous researchers also using unweighted Unifrac distance showing vaginal bacterial community structure differences between the follicular and luteal phases [13]. Collectively, these results further indicate the potential influence of E_2 and P_4 secretion on reproductive tract bacterial community abundance and diversity. Moreover, using both unweighted and weighted UniFrac distance analyses, differences existed between H-L and L-H samples on day 0 in the present study. Interestingly, previous studies have demonstrated differences in unweighted UniFrac distance based on fertility outcomes (pregnant vs. nonpregnant) from uterine samples collected prior to breeding [11,42]. Potentially, the differences observed on day 0 could indicate that one hormonal profile (H-L vs. L-H) results in a more favorable uterine microbial community structure, thus influencing fertility outcomes.

In the current study, an average of 9,322 quality-filtered reads per sample is similar to previous studies in beef heifers in which the V4 hypervariable region of the 16S rRNA gene was amplified (approximately 14,000 per sample [15]). Interestingly, studies that used postpartum beef cows had a greater average quality-filtered reads per sample (approximately 90,000 per sample); however, it should be noted that the V1 to V3 hypervariable regions of the 16S rRNA gene were amplified in these studies [11,16]. Across similar studies

investigating the uterine microbiome in cattle, the phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Tenericutes are the most abundant [12,16,42,43]. In the current study, the relative abundance of Bacteroidetes tended to increase by approximately 5% from day -2 to day 0 for H-L heifers. This observation has been similarly reported with an approximate 6% increase in the relative abundance of Bacteroidetes in the uterus prior to breeding (day -3) being observed in heifers that establish and maintain pregnancy [42]. Interestingly, these results contradict previous literature indicating that an increase in the relative abundance of Bacteroidetes in the vagina and uterus is associated with uterine disease [19,44,45]. These discrepancies may indicate specific species-level differences within the phylum Bacteroidetes in which certain bacterial species are opportunistic pathogens within the reproductive tract resulting in disease while others are symbiotic and/or mutualistic bacterial species.

Unlike the discrepancies for Bacteroidetes, bacteria in the genus *Corynebacterium* within phylum Actinobacteria have repeatedly been shown to be pathogenic in the uterus and associated with uterine disease and infertility [12,16,44,46,47]. In the current study, the relative abundance of Actinobacteria as well as *Corynebacterium* decreased from day -2 to day 0 for H-L heifers. The same decrease in *Corynebacterium* was also observed for H-H and L-L heifers; however, not for L-H heifers. This is notable because a previous study found that the relative abundance of uterine *Corynebacterium* of cows that were able to develop a pregnancy decreases throughout an estrous synchronization protocol and is less than 1% relative abundance two days prior to breeding [12]. In addition, the relative abundance of Tenericutes as well as *Ureaplasma* increased from day -2 to day 0 for H-L heifers. In recent years, multiple studies have indicated that an increase in *Ureaplasma* is associated with a healthy uterine environment and positive fertility outcomes in cattle [12,39,42,45]. Further research is necessary to determine the specific *Ureaplasma* species contributing to a healthy,

optimal uterine environment suitable for pregnancy establishment in cattle. It is important to note that the current study cannot rule out effects of repeated flushing of the reproductive tract from day -2 to day 0. Previous studies in horses and dairy cattle have shown that repeated uterine lavage can be an important technique used to treat uterine inflammation [48,49]. Particularly in dairy cattle, the number of polymorphonuclear cells (i.e., immune cells) decrease after uterine lavage to help in treatment of subclinical endometritis [49]. A link between the local uterine immune response and the microbiome cannot be ignored and future research is needed to further explore this relationship. Collectively, these sequencing-based results further indicate that endogenous P_4 and E_2 may influence the abundance of either pathogenic (i.e., *Corynebacterium* spp.) or commensal (i.e., *Ureaplasma* spp.) bacteria within the uterus of cattle; however, future studies are needed to elucidate the live bacteria that may fluctuate in response to endocrine changes and impact fertility outcomes.

Reproductive tract environment, including uterine pH, has been shown to play an important role in proper sperm transport for successful fertilization [50,51]. It has been shown that steroid hormones, specifically E_2, can influence uterine pH in cattle [52-54]. In the current study, the only changes from day -2 to day 0 in uterine pH was observed in H-L heifers with the pH increasing to approximately 6.75 on day 0. This pH is similar to previous studies with uterine pH at estrus ranging from 6.7 to 6.8 [52-54]. Additionally, this uterine pH is similar to the pH of bull semen, and previous studies have demonstrated that compatible uterine and semen pH enhance sperm viability and increase the likelihood of pregnancy establishment [52,54,55]. Few studies have shown that bacteria can influence uterine pH, with anaerobic bacterial growth impacting reproductive tract pH with an average pH value of 6.72 [56,57]. Interestingly, in the current study, common anaerobic bacteria (i.e., *Ruminococcus* spp. and *Blautia* spp. [58]) increased in relative abundance in H-L heifers from day -2 to day 0. In addition, an increase in uterine *Ruminococcus* prior to breeding has been observed in cattle

that establish and maintain pregnancy [12,42]. Potentially, a high P_4 and low E_2 hormonal profile influences anaerobic bacteria abundance, thus modulating uterine pH to a suitable environment for proper sperm transport and pregnancy establishment.

These results provide evidence that differing endogenous concentrations of P_4 and E_2 may alter uterine bacterial community abundance and diversity in beef heifers. Specifically, low P_4 and high E_2 upon ovulation could shape the uterine bacterial community structure to one that has previously been associated with reduced fertility. Whereas high P_4 and low E_2 at the same stage could shape the uterine bacterial community structure to one that has been previously documented as a more suitable uterine environment for pregnancy establishment. Continued investigation towards understanding the reproductive microbiome is imperative, as this information can lead to strategies that will improve reproductive efficiency in cattle.

Declaration of Interest

The authors have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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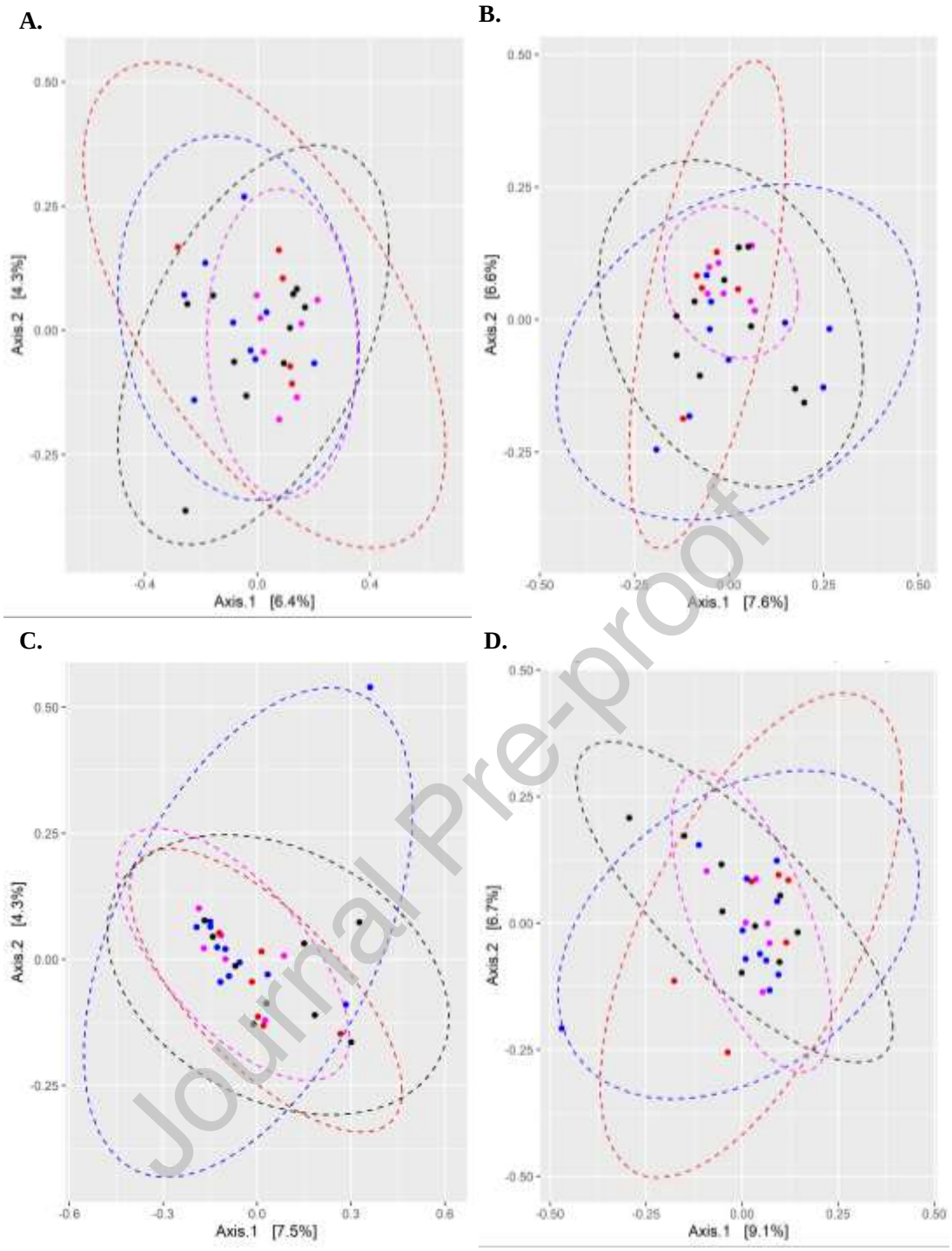

Figure 1. Principal coordinate analyses using UniFrac metrics A) Unweighted between treatment groups on day -2 did not differ (P = 0.77), B) Weighted between treatment groups on day -2 did not differ (P = 0.83), C) Unweighted between treatment groups on day 0 with a difference between H-H and H-L heifers (P = 0.05) and H-L and L-H heifers (P = 0.02), and D) Weighted between treatment groups on day 0 with a difference between H-L and L-H

heifers (P = 0.006). Treatment group legend: blue circles, H-H: High P_4 + High E_2 (n = 11); pink circles, H-L: High P_4 + Low E_2 (n = 9); red circles, L-H: Low P_4 + High E_2 (n = 9); black circles, L-L: Low P_4 + Low E_2 (n = 11).

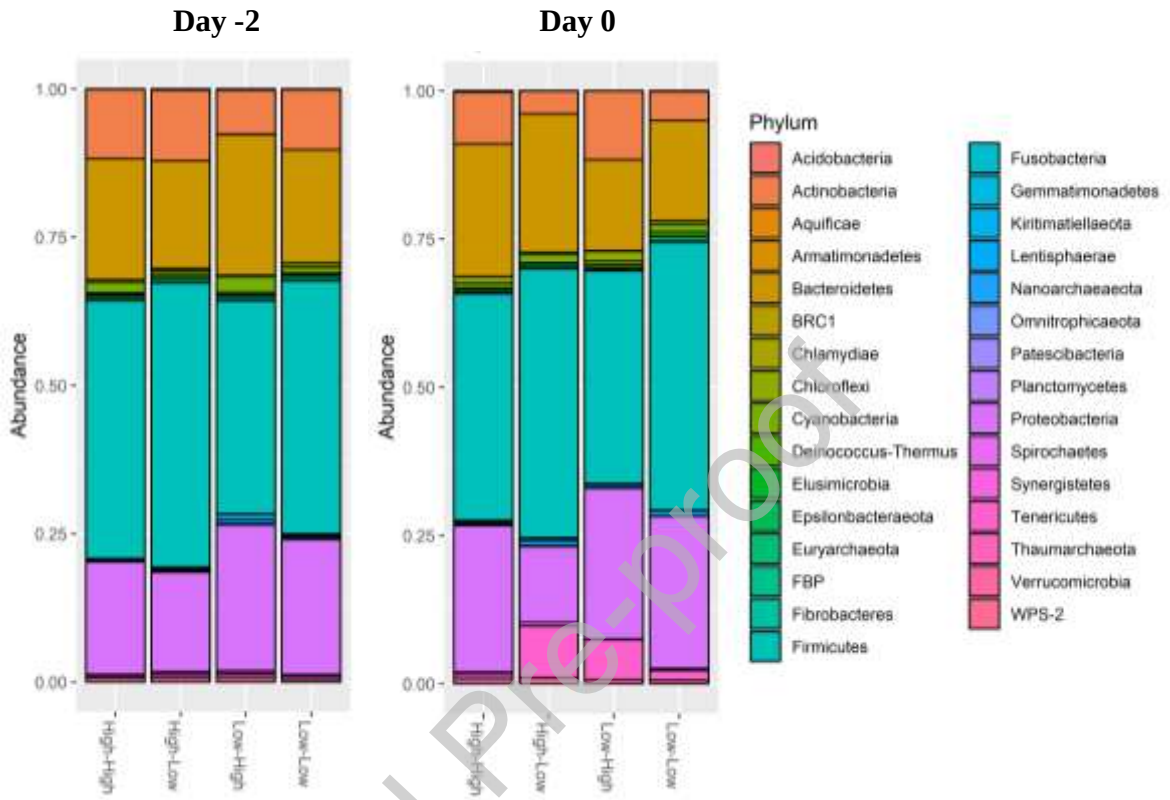

Figure 2. Impact of high or low endogenous progesterone (P_4) and estradiol (E_2) concentrations on uterine bacterial phyla relative abundance. A total of 31 different phyla were detected, of which Firmicutes (teal), Bacteroidetes (yellow), Proteobacteria (purple), Actinobacteria (orange), and Tenericutes (pink) were the most abundant. For High-Low heifers, the relative abundance of Actinobacteria (P = 0.01) and Tenericutes (P = 0.004) significantly changed from day -2 to day 0. Treatment group abbreviations: High-High: High P_4 + High E_2 (n = 11), High-Low: High P_4 + Low E_2 (n = 9), Low-High: Low P_4 + High E_2 (n = 9), Low-Low: Low P_4 + Low E_2 (n = 11). All phyla relative abundances less than 1% that significantly differ are presented in Supplementary Tables S1, S2, and S3.

Table 1. Plasma concentrations (mean ± SEM) of day -2 progesterone (P_4) and day 0 estradiol (E_2) for each treatment group of Angus-influenced heifers.

Treatment[1]	Progesterone (ng/mL)	Estradiol (pg/mL)
H-H (*n* =11)	4.68 ± 0.55	4.64 ± 0.31
H-L (*n* =9)	6.33 ± 0.96	1.57 ± 0.39
L-H (*n* =9)	1.43 ± 0.10	6.02 ± 1.06
L-L (*n* =11)	0.57 ± 0.20	1.27 ± 0.27

[1]Treatment group abbreviations: H-H: High P_4 + High E_2, H-L: High P_4 + Low E_2, L-H: Low P_4 + High E_2, L-L: Low P_4 + Low E_2

Table 2. Sequence and alpha diversity statistics between treatment groups of Angus-influenced heifers on days -2 and 0 (mean ± SEM).

Trial Day	Treatment[1]	Quality-filtered reads	Chao1[2]	Shannon's diversity index[3]	Simpson's diversity index[4]
Day -2	H-H	6,413 ± 1,885	117.7 ± 23.6^{a}	4.20 ± 0.19^{a}	0.977 ± 0.004
	H-L	12,105 ± 2,180	174.9 ± 20.2^{b}	4.74 ± 0.12^{b}	0.988 ± 0.002
	L-H	10,313 ± 2,567	161.8 ± 30.6^{ab}	4.56 ± 0.27^{ab}	0.983 ± 0.005
	L-L	7,826 ± 1,692	138.3 ± 20.7^{ab}	4.36 ± 0.20^{ab}	0.979 ± 0.005
Day 0	H-H	10,428 ± 1,612	$164.8 \pm 26.3^{a\,x}$	4.41 ± 0.30^{xy}	0.971 ± 0.014
	H-L	14,096 ± 3,669	184.3 ± 33.5^{ab} xy	4.63 ± 0.21^{x}	0.983 ± 0.004
	L-H	8,257 ± 2,104	119.0 ± 19.4^{b} xy	4.10 ± 0.23^{y}	0.969 ± 0.007
	L-L	7,243 ± 2,168	118.6 ± 26.4^{ab} y	4.14 ± 0.24^{xy}	0.973 ± 0.006

[1]Treatment group abbreviations: H-H: High P_4 + High E_2 (*n* = 11), H-L: High P_4 + Low E_2 (*n* = 9), L-H: Low P_4 + High E_2 (*n* = 9), L-L: Low P_4 + Low E_2 (*n* = 11)
[2]Chao1: estimates the total richness
[3]Shannon's diversity index: measurement of richness (number of species per sample) and evenness of distribution (measure of the relative abundance of the different species)
[4]Simpson's diversity index: measurement of richness and evenness, ranges between 0 and 1 with the greater the value, the greater the sample diversity
[ab]Between treatment in each column for each day indicates $P \leq 0.05$
[xy]Between treatment in each column for each day indicates $0.05 > P \leq 0.10$

Table 3. Impact of high or low endogenous progesterone (P_4) and estradiol (E_2) concentrations on differences in uterine bacterial genera relative abundance greater than 1% in Angus-influenced heifers (mean ± SEM)[1]

	H-H[2]		H-L[2]		L-H[2]		L-L[2]	
	Day -2	Day 0	Day -2	Day 0	Day -2	Day 0	Day -2	Day 0
Ruminococcus	5.35 ± 0.87	7.29 ± 1.22	5.69 ± 0.95^{x}	8.21 ± 1.34^{y}	5.14 ± 1.65	5.39 ± 0.69	6.63 ± 1.09	6.11 ± 1.20
Pedobacter	3.92 ± 0.47	4.78 ± 0.72	3.39 ± 0.57^{x}	5.14 ± 0.92^{y}	4.08 ± 1.03	3.39 ± 0.49	4.44 ± 0.79	3.32 ± 0.58
Corynebacterium	4.96 ± 0.93^{a}	1.42 ± 0.23^{b}	5.33 ± 1.53^{a}	1.42 ± 0.16^{b}	2.53 ± 0.47	3.60 ± 1.08	4.12 ± 0.60^{a}	1.79 ± 0.45^{b}
Clostridium	3.11 ± 0.29^{a}	4.15 ± 0.51^{b}	3.61 ± 0.51	3.83 ± 0.50	3.19 ± 0.71	2.75 ± 0.45	3.92 ± 0.59	3.54 ± 0.47

Agrobacterium	0.04 ± 0.02[a]	8.79 ± 8.61[b]	0.26 ± 0.13	0.15 ± 0.07	0.02 ± 0.01[a]	6.87 ± 6.71[b]	0.17 ± 0.05	0.32 ± 0.15
Blautia	2.73 ± 0.38	2.64 ± 0.43	2.27 ± 0.19[a]	3.41 ± 0.22[b]	2.62 ± 0.56	1.81 ± 0.14	2.24 ± 0.30	2.78 ± 0.36
Ureaplasma	0.11 ± 0.10	0.07 ± 0.07	0.06 ± 0.04[a]	7.32 ± 4.91[b]	0.04 ± 0.03	0.15 ± 0.14	0.05 ± 0.03	1.36 ± 1.31
Mycoplasma	0.29 ± 0.07	0.27 ± 0.07	0.20 ± 0.04	0.58 ± 0.12	0.48 ± 0.16	6.40 ± 6.00	0.24 ± 0.06[x]	1.09 ± 0.62[y]
Caloracator	1.43 ± 0.26	1.41 ± 0.24	1.36 ± 0.32	1.44 ± 0.28	1.63 ± 0.67	0.98 ± 0.18	1.66 ± 0.26[x]	1.01 ± 0.19[y]
Oscillospira	2.12 ± 0.37	1.81 ± 0.28	1.47 ± 0.23	1.79 ± 0.41	2.27 ± 0.40[a]	1.54 ± 0.58[b]	1.97 ± 0.29	1.72 ± 0.29

[ab]Between day in each row for each treatment separately indicates $P \leq 0.05$

[xy]Between day in each row for each treatment separately indicates $0.05 > P \leq 0.10$

[1]All genera relative abundances less than 1% that significantly differ are presented in Supplementary Tables S4 and S5.

[2]Treatment group abbreviations: H-H: High P_4 + High E_2, H-L: High P_4 + Low E_2, L-H: Low P_4 + High E_2, L-L: Low P_4 + Low E_2