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Reproduction

REPRODUCTION

Uterine and vaginal bacterial community diversity prior to artificial insemination between pregnant and nonpregnant postpartum cows[1]

Taylor B. Ault,[*] Brooke A. Clemmons,[*] Sydney T. Reese,[*,†] Felipe G. Dantas,[*] Gessica A. Franco,[*,†] Tim P. L. Smith,[‡] J. Lannett Edwards,[*] Phillip R. Myer,[*] and Ky G. Pohler[*,†,2]

[*]Department of Animal Science, University of Tennessee, Knoxville, TN 37996, †Department of Animal Science, Texas A&M University, College Station, TX 77843-2471, and ‡U.S. Meat Animal Research Center, Agricultural Research Service, United States Department of Agriculture, Clay Center, NE 68933

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[2]Corresponding author: kpohler@tamu.edu

ORCiD number: 0000-0002-1980-2105 (Phillip R. Myer).

Abstract

The present study evaluated the bovine vaginal and uterine bacterial community diversity and its relationship to fertility. Postpartum beef cows (n = 68) were synchronized beginning on day –21 and ending with timed artificial insemination (**TAI**) on day 0. Pregnancy was diagnosed 30 d after TAI. Uterine and vaginal flushes were collected on day –21, –9, and –2 for bacterial DNA extraction to sequence the V1 to V3 hypervariable regions of the 16S rRNA gene. Results indicated a decrease in the number of bacterial species over time in the uterus of resulting pregnant and nonpregnant beef cows ($P < 0.0001$). Principal coordinate analyses (PCoA) depicted clustering of samples, indicating closely related bacterial communities, by day in the uterus and vagina ($P < 0.0001$). At day –2, uterine samples from nonpregnant and pregnant animals clustered separately ($P < 0.0001$), with nonpregnant animal samples clustering tightly together. Overall, the current study suggests the shift in the reproductive bacterial communities' diversity and phylogenetic relationship leading up to the time of breeding may contribute to successful pregnancy establishment.

Key words: cow, fertility, reproductive microbiome, uterus, vagina

Introduction

Bacteria reside in multiple systems throughout the body contributing vital roles to health as part of the normal microbiome. Collectively, the microbiome is defined as all microbial genetic material present in a specific environment. Functions of bacteria in the microbiome include modulating pH of their environment, producing nutrients, and providing protection against harmful pathogens (NIH et al., 2009; Beecher et al., 2014). An imbalance, termed dysbiosis, in the normal microbiome of the gastrointestinal tract has been determined to result in increased risk for various health issues such as

inflammatory bowel disease and obesity (Ojetti et al., 2009; Zhang et al., 2015). The reproductive tract contains its own distinct microbiome with fewer species and lower phylogenetic diversity than the gastrointestinal tract (The Human Microbiome Project, 2012; Clemmons et al., 2017). Human studies have shown a dysbiosis resulting in a high diversity of bacteria in the reproductive tract can affect establishment and maintenance of pregnancy. Specifically, *Lactobacillus* accounts for over 90% of the vaginal reproductive microbiome in healthy women, with reduced *Lactobacillus* dominance associated with disease and compromised fertility (Rönnqvist et al., 2006; Sirota et al., 2014; Moreno et al., 2016).

Although many human studies have indicated the importance of microbial diversity on reproductive tract health and fertility, it is relatively unknown in bovine. Studies characterizing various livestock reproductive tracts in a nondiseased state have identified a greater species diversity compared to humans (Otero et al., 1999, 2000; Swartz et al., 2014). Our group recently characterized the microbiome of nonpregnant postpartum beef cows 2 d prior to ovulation to determine the difference in the bacterial communities between the uterus and vagina. Results indicated the bacterial phylogenetic diversity and the number of species present in the vagina is significantly greater than in the uterus (Clemmons et al., 2017). Laguardia-Nascimento et al. (2015) reported that the vaginal microbiome in *Bos indicus* heifers and cows had reduced bacterial species diversity in pregnant animals compared to nonpregnant animals. The bovine reproductive microbiome diversity's relationship to fertility, however, has not been determined. The objective of the current study was to evaluate the bacterial community diversity of the uterus and vagina of postpartum cows during implementation of an ovulation synchronization protocol and compare between resulting pregnancy statuses 30 d following timed artificial insemination (**TAI**). We hypothesized the diversity of the bacterial communities differ in the uterus and the vagina of cows that are pregnant 30 d after TAI versus cows that are not pregnant 30 d after TAI and that the bacterial communities will change over time during the synchronization protocol.

Materials and Methods

This study was performed under an approved protocol by the Institutional Animal Care and Use Committee of the University of Tennessee, Knoxville. The following methods are as described in Clemmons et al. (2017) with slight modifications.

Experimental Design

Beef cows (n = 68) located at the East Tennessee Research and Education Center, with an average of 80 ± 2.6 days postpartum (**DPP**) and 4.6 ± 0.57 yr old at TAI and not showing signs of any clinical diseases, were used for the study. Twenty-one days before TAI (day −21; average of 59 DPP) prostaglandin F2α (Lutalyse, 25 mg as 5 mL) IM was administered as a pre-synchronization step. An industry standard 7 Day Co-Synch Protocol was implemented 9 d before TAI (day −9; average of 71 DPP) using gonadotropin-releasing hormone (GnRH; Factrel, 100 μg as 2 mL IM injection). Two days before TAI (day −2; average of 78 DPP) an injection of prostaglandin F2α (Lutalyse, 25 mg as 5 mL) IM was administered. Controlled internal drug release (CIDR) were not used in this study due to collection methods of uterine and vaginal flush samples and to prevent bacterial growth on the device or within the vagina influencing results. Cows were administered 100 μg of GnRH IM on day 0 followed by TAI using a single sire and technician to minimize variation. Pregnancy diagnosis by transrectal ultrasound occurred on day 30 following TAI by observation of embryonic heartbeat. During the synchronization protocol, uterine and vaginal flush samples were collected at day −21, day −9, and day −2. Prior to flushing, the perineal area was cleaned and disinfected to prevent contamination. For vaginal flush collection, 60 mL of 0.9% sterile saline (Vetivex; pH = 5.6) was expelled into the vagina using a sterile syringe and recovered via vaginal lavage. For uterine flush collection, a sterile Foley catheter was placed in the body of uterus to prevent vaginal contamination of the sample. When placing the Foley catheter, a large speculum was used as well as sterile chemise to prevent contamination while placing the catheter in the uterus. Sterile saline (180 mL) was flushed through the catheter into the uterus and fluid was collected using industry standard approaches for embryo flushing. The pH of the uterine and vaginal flushes was measured by UltraBasic pH meter (Denver Instruments, Arvada, CO) and recorded immediately following collection. Samples were flash-frozen in liquid nitrogen and stored at −80 °C until analysis. Blood samples were collected in BD Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) at each time point of the synchronization protocol to determine progesterone concentration. Additionally, transrectal ultrasound was conducted at each time point to map ovarian structures. Twenty (10 pregnant and 10 nonpregnant) cows meeting all of the following criteria for the study were submitted for sequencing and further analysis. The criteria for inclusion in the study included: 1) corpus luteum (**CL**) present on day −21, day −9 and having P4 greater than 1 ng/mL, 2) response to GnRH on day −9 as assumed by the presence of a CL on day −2, 3) ovulatory follicle present on day 0 (TAI).

DNA Extraction and Sequencing

A total of 120 samples (10 pregnant and 10 nonpregnant cows, 3 time points, 2 reproductive tract locations) were collected from the uterine and vaginal flushes of resulting pregnant and nonpregnant cows by TAI. Sequencing was performed with extracted DNA as previously described by Clemmons et al. (2017). Once thawed at room temperature (22 °C), samples were vortexed and aliquots of 5 mL were removed, placed in 15 mL tubes and centrifuged (4,696 × g) at 4 °C. The resulting pellet was resuspended in 180 μL of sterile saline. The Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) was used according to manufacturer protocol for DNA extraction. Samples were stored at −20 °C until amplification. Polymerase chain reaction (PCR) was used for both DNA amplification and library construction. The hypervariable regions V1 to V3 of the 16S rRNA bacterial gene were targeted for bacterial identification. Sequencing libraries were produced using modified universal primers 27F (5′-Adapter/Index/AGAGTTTGATCCTGGCTCAG) and 519R (5′-Adapter/Index/GTATTACCGCGGCTGCTG) which included TruSeq indices and adapters and was performed using Accuprime Taq high-fidelity DNA Polymerase (LifeTechnologies, Carlsbad, CA). The PCR annealing temperature was 58 °C for 30 cycles. Libraries were quality-checked by gel electrophoresis. Products were purified and quantified with AmPure beads and Nanodrop 1000 spectrophotometer (Agencourt, Beverly, MA and ThermoScientific, Wilmington, DE) as well as real-time PCR on LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). Sequencing of the PCR libraries was performed at the United States Meat Animal Research Center (USDA-ARS-USMARC, Clay Center, NE) using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA) with the 2 × 300, v3 600 cycle kit.

PhiX was used as a positive control and sterile saline used for collecting uterine and vaginal flushes as a negative control.

Sequence Reading and Analysis

Resulting sequence reads were processed using the Quantitative Insights Into Microbial Ecology (**QIIME**) version 1.9.1 (Caporaso et al., 2010) and Mothur version 1.36.1 (Schloss et al., 2009) programs. The Galaxy server was used for quality filtering to retain all sequences with quality scores ≥ Q 25. Adapter and index sequences were trimmed and sequences <300 bp were removed. Chimeric sequences were identified and removed by the usearch61 command (Edgar, 2010). The sequences which classified as chloroplasts and mitochondria were removed from analyses. Each sample was then subsampled to 30,000 sequences to remove sequence depth bias. Operational taxonomic unit (**OTU**) picking occurred with a pairwise identity threshold of 97% using UCLUST in QIIME with taxonomy assignment by UCLUST and the Greengenes v13_8 16S rRNA database (Caporaso et al., 2010). Phylogenetic trees were built with FastTree (Price et al., 2010) to determine α- and β-diversity. Alpha diversity, the measurement of bacterial species diversity within a sample, was analyzed using observed OTU, Faith's phylogenetic diversity, Shannon's diversity index, and Chao1 richness indices. Observed OTU indicates the number of species that are present from the subsampled sequences while Chao1 estimates the total number of species that are present. Shannon's diversity index uses the number of species present accounting for their abundance and evenness of distribution. Faith's phylogenetic diversity measures the diversity within a sample by using the total branch lengths of all bacteria detected. Beta diversity analyses, comparing the bacterial species diversity between samples using phylogenetics, were conducted using unweighted UniFrac distance matrices to generate principal coordinate analyses (**PCoA**) utilizing QIIME (Lozupone and Knight, 2005).

Progesterone Assay

Progesterone was measured to determine the physiological response to the protocol. Progesterone RIA was performed according to the previously described protocol (Pohler et al., 2016) using a double-antibody RIA kit (MP Biomedicals, Santa Ana, CA). A standard curve (ranging from 0 to 50 ng/mL) was used to calculate sample concentrations and in-house controls for quality control. Inter- and intra-assay CV were less than 10%.

Statistical Analyses

Uterine and vaginal data were separated for all analyses. Samples with OTU or chao1 < 10 and Shannon's diversity index or Faith's phylogenetic diversity values < 1.0 were removed from analysis. Uterine and vaginal alpha diversity metrics were confirmed to have normal distribution by PROC UNIVARIATE in SAS 9.4. Repeated measures analysis was performed on all alpha diversity metrics, pH, and progesterone data using PROC GLIMMIX in SAS 9.4 with fixed effects of day and status and their interaction, and random effects of cow within day. If no interaction was observed, overall effects of day and status were evaluated. Days postpartum and age were determined to have no significant effect on pregnancy status and were removed from analysis. Beta diversity differences between day of sample and pregnancy status by environment were analyzed using QIIME analysis of similarity (ANOSIM) with 9,999 permutations. Significance level for all analyses was set at $P \leq 0.05$.

Results

Sequence Information

After quality control and chimera removal, 10,448,316 total clean sequences remained among all samples, with an average of 92,463 ± 2,976 per sample. The total number of sequences ranged from 42 to 232,160 among individual samples. Table 1 indicates the average number of clean sequences and standard error of the mean (SEM) for each similar sample day, sample type, and pregnancy status group.

Alpha Diversity

A total of 112,953 OTU, indicating the number of observed taxa, were detected among all samples.

Between resulting pregnant and nonpregnant cows

In the uterus, no day × status or overall effect of pregnancy status occurred for any alpha diversity metrics ($P > 0.05$; Table 1). In the

Table 1. Sequence and alpha diversity statistics between pregnant and nonpregnant cows

Day	Type	Status	Cleaned sequences	Observed OTUs[1]	Chao1[2]	Shannon's diversity index[3]	Faith's phylogenetic diversity[4]
–21	Uterine	Nonpregnant	88,494 ± 12,926	1,150 ± 140.28[a]	1,550 ± 110.36[a]	7.8 ± 0.28[a]	106.5 ± 8.62[a]
		Pregnant	101,430 ± 6,947	1,210 ± 133.08[a]	1,552 ± 104.70[a]	8.4 ± 0.27[a]	109.5 ± 8.17[a]
	Vaginal	Nonpregnant	98,085 ± 10,238	1,062 ± 69.65[a]	1,551 ± 53.74[a]	8.7 ± 0.41[a]	97.8 ± 4.44[a]
		Pregnant	94,675 ± 7,847	1,260 ± 62.48[b]	1,638 ± 48.14[a]	8.2 ± 0.36[a]	111.3 ± 4.00[b]
–9	Uterine	Nonpregnant	88,024 ± 7,185	1,215 ± 61.60[a]	1,564 ± 42.29[a]	8.4 ± 0.22[a]	107.6 ± 3.22[a]
		Pregnant	89,521 ± 4,474	1,135 ± 64.93[a]	1,564 ± 44.58[a]	8.2 ± 0.23[a]	105.2 ± 3.39[a]
	Vaginal	Nonpregnant	93,705 ± 6,077	1,106 ± 53.88[a]	1,459 ± 51.42[a]	7.8 ± 0.12[a]	100.3 ± 3.76[a]
		Pregnant	94,353 ± 4,624	1,018 ± 50.84[a]	1,375 ± 48.49[a]	7.8 ± 0.10[a]	94.6 ± 3.55[a]
–2	Uterine	Nonpregnant	114,937 ± 5,304	436 ± 130.72[a]	696 ± 139.29[a]	5.6 ± 0.73[a]	55.0 ± 11.56[a]
		Pregnant	82,814 ± 22,483	417 ± 148.22[a]	670 ± 157.94[a]	5.6 ± 0.83[a]	49 ± 13.11[a]
	Vaginal	Nonpregnant	93,118 ± 6,730	1,166 ± 129.44[a]	1,455 ± 130.51[a]	7.6 ± 0.35[a]	108.3 ± 9.81[a]
		Pregnant	71,270 ± 10,776	987 ± 129.44[a]	1,273 ± 130.51[a]	7.5 ± 0.35[a]	93.2 ± 9.81[a]

[1]Observed operational taxonomic units (OTU): number of observed species.
[2]Chao1: estimate of total number of species present.
[3]Shannon's diversity index: measurement of species abundance and evenness of distribution.
[4]Faith's phylogenetic diversity: phylogenetic diversity of species measured by total branch lengths.
[ab]Between each day of each column indicates $P \leq 0.05$.

Table 2. Alpha diversity statistics among days of the TAI protocol

Type	Status	Day	Observed OTU[1]	Chao1[2]	Shannon's diversity index[3]	Faith's phylogenetic diversity[4]
Uterine	Pregnant	–21	1,210 ± 133.08[a]	1,552 ± 104.70[a]	8.4 ± 0.27	109.5 ± 8.17[a]
		–9	1,135 ± 64.93[a]	1,564 ± 44.58[a]	8.2 ± 0.23	105.2 ± 3.39[a]
		–2	417 ± 148.22[b]	670 ± 157.94[b]	5.6 ± 0.83	49 ± 13.11[b]
	Nonpregnant	–21	1,150 ± 140.28[a]	1,550 ± 110.36[a]	7.8 ± 0.28	106.5 ± 8.62[a]
		–9	1,215 ± 61.60[a]	1,564 ± 42.29[a]	8.4 ± 0.22	107.6 ± 3.22[a]
		–2	436 ± 130.72[b]	696 ± 139.29[b]	5.6 ± 0.73	55.0 ± 11.56[b]
Vaginal	Pregnant	–21	1,260 ± 62.48[a]	1,638 ± 48.14[a]	8.2 ± 0.36	111.3 ± 4.00[a]
		–9	1,018 ± 50.84[b]	1,375 ± 48.49[b]	7.8 ± 0.10	94.6 ± 3.55[b]
		–2	987 ± 129.44[ab]	1,273 ± 130.51[b]	7.5 ± 0.35	93.2 ± 9.81[ab]
	Nonpregnant	–21	1,062 ± 69.65[a]	1,551 ± 53.74[a]	8.7 ± 0.41	97.8 ± 4.44[a]
		–9	1,106 ± 53.88[a]	1,459 ± 51.42[a]	7.8 ± 0.12	100.3 ± 3.76[a]
		–2	1,166 ± 129.44[a]	1,455 ± 130.51[a]	7.6 ± 0.35	108.3 ± 9.81[a]

[1]Observed operational taxonomic units (OTU): number of observed species.
[2]Chao1: estimate of total number of species present.
[3]Shannon's diversity index: measurement of species abundance and evenness of distribution.
[4]Faith's phylogenetic diversity: phylogenetic diversity of species measured by total branch lengths.
[ab]Between each day of each column indicates $P \leq 0.05$.

vagina, a day × status occurred ($P = 0.03$) with nonpregnant cows having significantly less observed OTU than pregnant animals at day –21 ($P = 0.04$) with no difference at day –9 or day –2 ($P > 0.05$; Table 1). Faith's phylogenetic diversity also indicated an interaction of day × status ($P = 0.02$) with greater diversity in pregnant cows at day –21 in the vagina ($P = 0.03$; Table 1). Chao1, however, indicates there was no difference in the predicted number of species in the vagina between resulting pregnant and nonpregnant cows at all days ($P > 0.05$; Table 1).

Days of the TAI protocol

Observed OTU significantly decrease in the uterus over time ($P < 0.0001$), independent of pregnancy status, between day –21 to day –2 ($P < 0.0001$) and day –9 to day –2 ($P < 0.0001$) with no significant difference between day –21 and day –9 ($P > 0.05$; Table 2). Similarly, Chao1, Faith's phylogenetic diversity, and Shannon's diversity index significantly decreased in the uterus from day –21 and day –9 to day –2 ($P < 0.05$; Table 2). In the vagina, observed OTU and Faith's phylogenetic diversity did not differ over time in nonpregnant cows. Although the least number of OTU occurred in the uterus at day –2, the greatest number OTU occurred in the vagina at day –2 of resulting nonpregnant cows. In resulting pregnant cows, however, OTU decreased over time with day –2 having the least number of observed OTU, similar to the uterus. Contrasting the observed OTU, Chao1 indicated a significantly greater predicted species richness in the vagina at day –21 compared to day –9 ($P = 0.0007$) and day –2 ($P = 0.03$; Table 2) in resulting pregnant cows. Similar to Chao1, Shannon's diversity index significantly changed over time in the vagina of resulting pregnant cows ($P = 0.04$) with day –21 significantly greater than day –9 ($P = 0.02$) and day –2 ($P = 0.02$), and no difference between day –9 and day –2 ($P > 0.05$; Table 2).

Beta Diversity

Principal coordinate analyses using UniFrac unweighted metrics were generated to analyze and visualize beta diversity to determine the phylogenetic relationship between samples in the uterus and vagina.

Between resulting pregnant and nonpregnant cows

Significant clustering between resulting pregnant and nonpregnant cows occurred in the uterus at day –2 ($R = 0.28$, $P = 0.005$; Fig. 1a). Samples from nonpregnant cows clustered tightly while pregnant cows were less clustered, but distinctly separate from the nonpregnant cow samples. In the vagina, significant clustering was observed at day –21 between pregnant and nonpregnant cow samples with slight overlap ($R = 0.24$, $P = 0.002$; Supplementary Fig. 1a). No significant clustering by pregnancy status was observed in the uterus at day –21 ($R = –0.007$, $P = 0.46$; Supplementary Fig. 1b) or day –9 ($R = –0.024$, $P = 0.64$; Supplementary Fig. 1c), or in the vagina at day –9 ($R = –0.004$, $P = 0.43$; Supplementary Fig. 1d) or day –2 ($R = –0.004$, $P = 0.43$; Fig. 1b).

Days of the TAI protocol

Significant clustering by day was observed in both uterine and vaginal samples. Uterine samples clustered by day as the day –21 and day –9 samples were clustered together and separate from day –2 ($R = 0.23$, $P = 0.0001$; Fig. 2a). In the vaginal samples, contrasting the uterine samples, there was tight clustering of day –21 and day –9 samples with a separation of day –2 samples ($R = 0.27$, $P = 0.0001$, Fig. 2b).

Circulating Progesterone Concentrations

Circulating progesterone concentrations were similar in resulting pregnant and nonpregnant cows as expected at day –21 (2.34 ± 0.62 ng/mL vs. 2.2 ± 0.74 ng/mL), day –2 (6.11 ± 1.81 ng/mL vs. 4.76 ± 1.77 ng/mL), and day 0 (0.83 ± 0.31 ng/mL vs. 0.31 ± 0.09 ng/mL). Progesterone was greater in nonpregnant cows than pregnant cows at day –9 ($P = 0.01$; 7.58 ± 1.30 ng/mL vs. 3.83 ± 0.68 ng/mL); however, no day × status was observed.

Discussion

Although bacterial communities have many beneficial contributions to the overall physiological system, dysregulation of these communities concurrent with a change in abundances may have negative consequences (Macklaim et al., 2013; Belizário and Napolitano, 2015; Green et al., 2015). Our study suggests that shifts in the bacterial communities' diversity occurs in the uterus over time, potentially in response to changes through the estrous cycle to prepare for pregnancy. According to beta diversity analyses, the significant clustering of uterine samples between resulting pregnant and nonpregnant cows

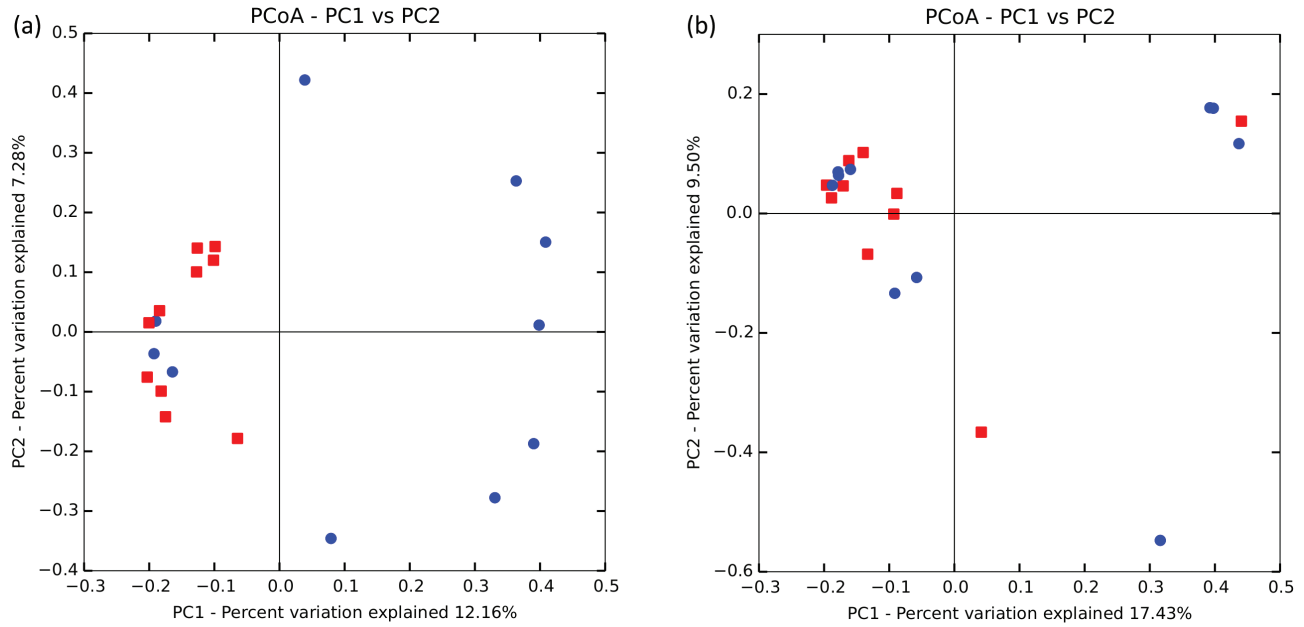

Figure 1. Principal coordinate analyses using UniFrac unweighted metrics depict clustering at day −2 in the uterus (a; $R = 0.28$, $P = 0.005$) and vagina (b; $R = -0.004$, $P = 0.43$) between nonpregnant and pregnant cows. Nonpregnant cows are indicated by red squares and pregnant cows by blue circles.

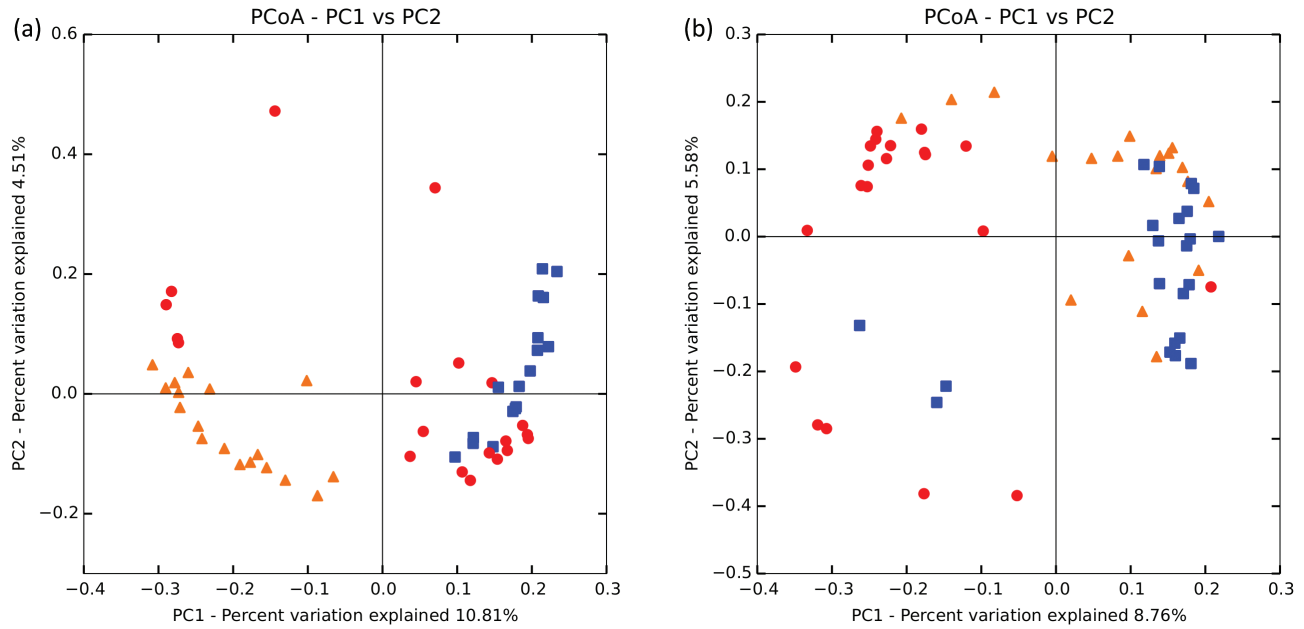

Figure 2. Principal coordinate analyses (PCoA) determined significant clustering of vaginal (a; $R = 0.27$, $P < 0.0001$) and uterine (b; $R = 0.23$, $P < 0.0001$) samples by day of the protocol using UniFrac unweighted metrics. Legend: day −21, orange triangles; day −9, blue squares; day −2, red circles.

at day −2 indicates the differences in bacteria species present in the uterus at this time may be affecting the establishment of pregnancy. The majority of uterine samples from pregnant cows cluster separately from nonpregnant cows, but with much greater variation as the nonpregnant cow samples cluster very tightly. This large variation in the reproductive tract bacterial community diversity suggests there is no single microbiome potentially benefiting fertility in the cows that were able to conceive, supporting previous research of the high variation and diversity of bovine reproductive tract bacterial communities (Swartz et al., 2014; Laguardia-Nascimento et al., 2015). The tight clustering of samples from cows who failed to conceive suggests there is a group of closely related bacteria present potentially preventing or decreasing the establishment of pregnancy. Further characterization of this closely related group of bacteria may allow for identification and selection against cows containing an unfavorable reproductive microbiome potentially unable to support a pregnancy. Three samples, however, from resulting pregnant cows clustered with the resulting nonpregnant cows. The beta diversity analyses evaluates their phylogenetic relationship between samples and not the abundances of the bacteria, suggesting that although the resulting cows may have similar bacteria present their abundances may differ significantly.

Previous research studies agree that the human reproductive microbiome has a low diversity, with an increase in diversity leading to disease and fertility issues (Ravel et al., 2011; The Human Microbiome Project, 2012). Our study along with previous evidence, however, indicated the bacterial communities of the cow's reproductive tract are distinctly different than in humans with an increased number of bacterial species and greater phylogenetic diversity (Otero et al., 2000; Rodríguez et al., 2011; Swartz et al., 2014; Clemmons et al., 2017). The greatest average of OTU detected in the vagina occurred in pregnant cows on day –21 (1,260 ± 61.47). As stated by Clemmons et al. (2017), many assigned taxa in the vagina were associated with digestive tract bacterial species possibly due to its proximal location on the cow and opening to the external environment which may contribute to the great number of OTU observed in the vagina of cows. These same vaginally identified OTU were less abundant in the uterus which theoretically is protected by the cervix from the outside environment (Clemmons et al., 2017). Although the microbiome composition of the bovine reproductive tract has been found to be greatly diverse, our results indicated reduced alpha diversity of the uterus prior to TAI on day –2. In the uterus, observed OTU decreased in cows that became pregnant from an average of 1,210 ± 126.35 at day –21 to 354 ± 130.24 at day –2. This supports previous evidence that reproductive bacterial communities of bovine are highly dynamic and fluctuating with shifts in bacterial diversity over time (Santos and Bicalho, 2012; Jeon et al., 2015). Otero et al. (1999) used a culture-based method to evaluate the shift in the bovine vaginal microbiome, specifically *Lactobacillus* and *Enterococci* genera of the phylum Firmicutes, throughout the estrous cycle. From their results, they suggested hormonal shifts through phases of the estrous cycle affected the fluctuations of bacterial abundance with *Lactobacillus* and *Enterococci* decreasing during high progesterone phases (Otero et al., 1999). Our results did not indicate differences in progesterone concentration between resulting pregnant and nonpregnant animals at day –21, day –2, or day 0. As progesterone shifts throughout synchronization, however, it may result in shifts of bacterial diversity leading to changes in the overall bacterial community composition over time.

The current study demonstrates bacterial communities of the reproductive tract undergo changes in diversity during a TAI synchronization protocol. The uterine environment experiences significant decreases in species richness and phylogenetic diversity during this time. According to beta diversity analyses, the phylogenetic composition of the uterine bacterial community may affect fertility. Future research should evaluate the cause of variation in the reproductive tract microbiome such as environment, genetics, nutrition, or health management methods, as well as the factors leading to and the importance of the decrease in the bacterial diversity of the uterus prior to breeding.

Supplementary Data

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

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