INTRODUCTION

Although the existence of a reproductive microbiome is evidenced in cattle (Ault et al., 2019; Hummel et al., 2020), the methods by which microbes colonize the placenta remain poorly understood. Mechanisms facilitating microbial transfer from the maternal gut have been investigated in cattle, which include passage through the circulatory system to the placental environment (Jeon et al., 2017). Contributions of the fecal microbiome to the vaginal flora, which have themselves been suggested to ascend to and colonize the placental microbiome (Goldenburg et al., 2008), have also been considered (Jeon et al., 2017). Therefore, it is necessary to evaluate the maternal gut as a potential niche for microbes inhabiting the reproductive tract and placenta in late gestation.

Changes to feed intake are capable of altering the maternal gut microbiome in ruminants (Hu et al., 2018), as well as the placental microbiome of humans (Antony et al., 2015). We hypothesize that feed intake restriction will result in a less diverse rumen microbiome in the gravid cow, which will in turn result in decreased placental microbial diversity. We also hypothesized that the vaginal microbiome, which remains stable throughout pregnancy (Romero et al., 2014; Ault et al., 2019), will be unaffected. Our objectives were to isolate and identify bacterial and archaeal species in the rumen fluid (RF) of both control (CON) and feed restricted (FR) cows in late gestation, and compare these microbes to microbial populations within various structures of the placenta, including the cotyledon (COT), intercotyledonary membrane (ICM), and allantois (AL). Swabs of vaginal epithelium (VAG) were also collected and compared to both the maternal gut and placenta in order to evaluate the effects of feed restriction on this reproductive microbiome.

MATERIALS AND METHODS

Experimental protocols were approved by the University of Wyoming International Care and Use Committee.

Animals and Study Design

Multiparous Angus cross-bred cows (n = 16) were stratified by initial body weight and BCS to either a control (CON; n = 8) group with ad libitum access to grass hay and alfalfa, or a 70% feed intake-restricted group (FR; n = 7), although one cow of the FR group calved prior to sampling. Treatment group sorting occurred 3 mo prior to each cow's expected calving date, calculated from the date of AI.

Sample Collection

Ten days prior to their expected calving date, RF was collected from each cow by orally passing a flexible stomach tube to the rumen and applying
suction via syringe until 20–30 mL RF were aspirated (Zhou et al., 2009). Swabs of vaginal epithelium were also collected at this time using a sterile double-sheathed equine uterine culture swab (Jorgenson Labs, Loveland, CO) following previously described methods (Hummel et al., 2020). Naturally delivered placentae were collected and COT dissected from the middle of the gravid horn. Excess tissue was trimmed, and COT were sectioned to fit within a 2-mL tube. Sections of the chorioallantois were separated by blunt dissection, and samples of the ICM and AL, no greater than 2 in², were collected no more than 3 in from the umbilical insertion site. All samples were flash frozen at −80 °C until further analysis.

**Microbial DNA Isolation and Sequencing**

Bead tubes containing sterilized silicon (0.1 g of 0.5-mm beads) and zirconia (0.3 g of 0.1-mm beads) were prepared for microbial DNA extraction of RF (0.25 g). Whole swab heads of VAG were steriley cut and placed in their respective bead tubes. Lysis buffer (1 mL; 500 mM NaCl, 400 mM Tris-HCL, 50 mM EDTA, 4% SDS; Zhao et al., 2015) was added to each bead tube and chemical and mechanical lysis was performed utilizing the Precellys Evolution (Bertin Instruments, Rockville, MD). Placental tissue underwent the same lysis step with bead tubes and lysis buffer provided with the QIAamp PowerFecal kit (Qiagen, Germantown, MD), and all samples subsequently underwent a 70 °C incubation for 10 min. All samples repeated the lysis step with 300-µL lysis buffer and centrifugation to precipitate (Yu and Morrison, 2004). The QIAamp PowerSoil Minikit (Qiagen) was used to further purify microbial DNA from RF and VAG. Quality and concentration of DNA was assessed with the Nanodrop spectrophotometer. Following the Illumina MiSeq System library preparation protocol, each sample was prepared as 30-ng/µL aliquots and pooled for sequencing of the V4 region of the 16S rRNA gene. Samples were sequenced at the Colorado State University MIP NGS Illumina Core.

**Bioinformatic Analysis**

Sequence analysis was conducted in QIIME2 v. 2020.8 (Bolyen et al., 2019) and quality filtering, pairing, and denoising were accomplished through the DADA2 plugin (Callahan et al., 2016) on the University of Wyoming Advanced Research Computing Center Teton computing environment (Advanced Research Computing Center, 2018). Alpha-diversity metrics, including Shannon index and Faith’s Phylodiversity, were generated in QIIME2. Pairwise comparisons were made for sample type by treatment using Kruskal–Wallis permutational multivariate analysis of variance (PERMANOVA). Similar pairwise comparisons were made for phylogenetic beta-diversity indices, including weighted and unweighted UniFrac distances. Statistical significance was considered when $q \leq 0.05$, and a tendency when $0.05 < q \leq 0.10$, with $q$ representing each $P$-value adjusted for false discovery rate under the Benjamini–Hochberg method.

**RESULTS**

**The Microbiomes of the Maternal Gut and Vagina Under Feed Restriction**

Shannon’s diversity index, a measure of abundance and evenness in microbiome alpha-diversity, revealed that the CON RF microbiome was significantly greater in richness (Figure 1; $q = 0.03$) than FR RF. Faith’s phylodiversity, a measure of phylogenetic diversity between microbial species, tended to be greater in CON RF ($q = 0.09$). In addition, FR RF tended toward decreased phylosdiversity among those more dominant microbial taxa, represented by weighted UniFrac ($q = 0.06$). The relatedness and diversity of less dominant microbial taxa was significantly decreased in FR RF, represented by unweighted UniFrac ($q = 0.05$).

The VAG microbiome did not differ between treatments in any alpha- or beta-diversity metric ($q \geq 0.11$).

**Relationship Between the Maternal Microbiome and Placenta**

The maternal gut of CON cows was richer and more diverse than the CON AL ($q \leq 0.03$), but these FR microbiomes did not differ in alpha-diversity ($q \geq 0.26$). The RF of both CON and FR cows was similar in richness ($q = 0.11$) and tended to differ in phylosdiversity ($q = 0.09$) from the ICM. Although CON RF was similar to the COT in both richness and phylosdiversity ($q \geq 0.11$), FR RF tended toward dissimilarity with the COT in both alpha-diversity metrics ($q = 0.06$).

Unlike the maternal gut, the VAG of FR cows was similar to the AL in richness and phylosdiversity ($q \geq 0.43$), although the CON VAG tended to be richer than the AL ($q = 0.06$). Like the maternal gut, the VAG of both CON and FR cows was similar in richness ($q \geq 0.11$) and the CON VAG tended to
differ in Faith’s phylodiversity ($q = 0.10$) from the ICM where the FR VAG did not ($q = 0.55$). Finally, the VAG and COT of both treatment groups were similar in richness and phylodiversity ($q \geq 0.11$).

The RF and VAG of CON tended to share both dominant and nondominant microbial taxa with the COT and ICM, represented by weighted and unweighted UniFrac ($q = 0.07$). Where FR RF differed in dominant and non-dominant taxa with the COT and ICM ($q \leq 0.03$), it tended to be similar to the AL under both UniFrac metrics (Figure 2; $q = 0.07$), as did the FR VAG ($q = 0.09$). Both the CON VAG and RF remained different from the AL in both dominant and non-dominant microbial
taxa (q ≤ 0.02). The FR VAG also differed in both UniFrac values from the FR COT and ICM (q ≤ 0.03), although the FR VAG and ICM only tended toward dissimilarity regarding dominant microbial taxa (q = 0.06).

DISCUSSION

Consistent with previous findings in feed restricted ewes (Hu et al., 2018), the mature bovine rumen is influenced by feed intake restriction, where the FR microbiome is less rich and less diverse than that of CON cows. The species counts of phyla that are present in a low relative abundance in the mature rumen were most impacted, which may exert changes on VFA production, microbial lipid metabolism, and overall ruminal pH (Hu et al., 2018) and potentially altering metabolites and microbes available to the gestating fetus.

Consistent with our hypothesis, these differences did not carry to the VAG microbiome, which is resistant to change throughout gestation (Romero et al., 2014; Ault et al., 2019). However, the FR VAG displayed similarities in alpha-diversity with the maternal gut that the CON treatment did not, potentially validating a relationship between these microbial sites (Jeon et al., 2017). It remains possible that a prolonged period of feed intake restriction may be capable of altering the vaginal microbiome, similar to the effects of dietary changes in pregnant women (Ravel et al., 2011).

Importantly, the maternal gut shared a greater degree of microbial characteristics with the COT within the CON treatment. This may indicate that the COT plays a role in microbial transmission to the placenta, as the COT is responsible for perfusing the placental organ with blood (Wooding and Burton, 2008), a hypothesized mode of bacterial transmission in cattle (Jeon et al., 2017). Additionally, the maternal rumen of FR pregnancies shared alpha-diversity characteristics with the AL, implicating decreased microbial diversity within the FR placenta as a whole, and potentially indicating decreased microbiome diversity within the fetal gut, which may be contributing a microbiome to the AL through the elimination of waste (Schlafer et al., 2000).

IMPLICATIONS

These data indicate a critical influence of the maternal gut upon the placental microbiome, as both sites were altered by feed intake restriction. Feed intake restriction shaped a less diverse, less robust maternal rumen microbiome, which was reflected within the placental microbiome and may further influence inoculation of the fetal gut. A greater understanding of the maternal gut’s influence on the reproductive microbiome throughout gestation is required in order to guide nutritional decisions and management practices in beef cows and to identify the optimal microbiome for reproductive performance.

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