

# Potential response of the rumen microbiome to mode of delivery from birth through weaning<sup>1,2</sup>

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Transl. Anim. Sci. 2018.2:S35–S38  
doi: 10.1093/tas/txy029

## INTRODUCTION

Increasing evidence in mice (Ley et al., 2005), humans (Biasucci et al., 2008), and ruminants (Cannon et al., 2010) suggests maternal influences and preparturition environment may affect infant gastrointestinal tract (GIT) microbiome. Early GIT colonization is critical to the development of the GIT and the immune system (Suárez et al., 2006; Malmuthuge et al., 2012). In addition, the colonization phase may be suitable for intervention strategies for improved host performance (Yañez-Ruiz et al., 2015).

In humans, research focuses on increased autoimmune disorders in children born via cesarean section (Neu and Rushing, 2011). Dominguez-Bello et al. (2010) reported that the

gut microbiome of infants born via cesarean more closely resembles the microbiome of the mother's skin rather than the vaginal microbiome. This research suggests that mode of delivery can alter the gut microbiome with potential long-term impacts for the host. In ruminants, the frequency of cesarean delivery is not of concern; however, understanding the potential influence of mode of delivery on the calf microbiome may bring to light new intervention strategies to optimize the rumen microbiome.

Although the rumen is not functional until nearly 4 to 6 wk of age (Church, 1988), and the rumen microbiome shifts rapidly during this period, the early microbiome is responsible for production of volatile fatty acids that affect rumen development (Flatt, 1958; Suárez et al., 2006) and ensures proper absorptive capacity for the mature ruminant. Thus, we hypothesized that the rumen microbiome of calves would be altered by mode of delivery, and these changes would persist through weaning. Our objective was to determine whether cesarean section delivery would affect the early calf microbiome compared with vaginal delivery and whether these differences would be evident through weaning.

## MATERIALS AND METHODS

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee.

<sup>1</sup>This project was supported by Agriculture and Food Research Initiative Competitive Grant no 2016-67016-24903 from the USDA National Institute of Food and Agriculture.

<sup>2</sup>The authors acknowledge the work of University of Wyoming undergraduate students (Jaelyn Whaley, Hailey Barton, and McKenna Brinton), graduate student (Courtney Sutton), and Laramie Research and Extension Center (Dr. Doug Zalesky and Travis and Kaci Smith) for their contributions to this project including animal procurement, management, and sample collections.

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Received March 16, 2018.

Accepted April 14, 2018.

### ***Cow Management and Diet***

Mature Charolais ( $n = 24$ ) cows from the University of Wyoming (UW) beef herd were used in this study. Cows were bred via natural service, and their expected calving date was calculated as 250 d after the date the bull was introduced. Cows were fed ad libitum grass hay (6.8% CP, 40.2% ADF, 56.8% TDN, 1.2 NE<sub>m</sub> MCal kg<sup>-1</sup>, 0.64 NE<sub>g</sub> MCal kg<sup>-1</sup>) and 2 lb d<sup>-1</sup> dried distillers grains (29.9% CP, 12.3% ADF, 75.0 TDN%, 1.79 NE<sub>m</sub> MCal kg<sup>-1</sup>, 1.16 NE<sub>g</sub> MCal kg<sup>-1</sup>). Prior to parturition, cows were moved into pens and monitored closely for signs of parturition. Cows were randomly assigned to either the control group (CON;  $n = 12$ ) or cesarean section group (CSECT;  $n = 12$ ). The CON cows were allowed to calve naturally with no intervention. The CSECT group was monitored closely for signs of parturition, and a veterinarian was summoned to perform the cesarean section using standard protocol including pain management and postsurgical care. Cows in both CON and CSECT groups reared their respective calf until weaning at 180 d; each treatment group was housed in separate pens.

### ***Calf Management and Calf Rumen Fluid Sample Collection***

At parturition, calves were monitored to ensure survivability. Calves were allowed ad libitum access to their dam's colostrum and hay. At approximately 1.5 mo of age, calves were fed Purina Stocker Grower (Purina Mills/Land O'Lakes, Inc.) at the rate of 2 lb h<sup>-1</sup> d<sup>-1</sup> through weaning (180 d of age). At 24 ± 4 h, rumen fluid was collected from calves via oral-lavage using methods described by Lodge-Ivey et al. (2009). Briefly, a 0.5-cm-interdiameter, flexible vinyl tube, 3 feet in length, was lubricated and passed through the mouth into the rumen; suction via an attached syringe was used to collect the rumen fluid. Samples were aliquoted, flash frozen, and stored at -80 °C for subsequent analysis. These samples were collected again on day 3, day 28, and at weaning.

### ***Rumen Microbial DNA Extraction***

Rumen fluid samples were used for shotgun metagenomic sequencing. First, DNA was isolated from 8 calves per treatment group using methods described by Yu and Morrison (2004). Briefly, a 0.25-g sample of rumen fluid (thawed immediately prior) was added to sterilized zirconia (0.3 g of 0.1 mm) and silicon (0.1 g of 0.5 mm) beads along

with 1 mL of lysis buffer (500 mM NaCl, 400 mM Tris-HCl, 50 mM EDTA, 4% SDS). Tubes were then homogenized using a Mini-Beadbeater-8 at maximum speed for 3 min, incubated at 70 °C for 15 min with gentle mixing, and centrifuged at 4 °C for 5 min. Supernatant (~1 mL) was transferred to a new 2-mL flat cap tube, and 300-μL fresh lysis buffer was added to the pelleted beads. The homogenization, incubation, and centrifugation steps described previously were repeated, and the supernatant was pooled. Precipitated DNA was purified further using the QIAamp DNA Stool Mini Kit (Qiagen, Santa Clarita, CA), and the manufacturer's protocol except that buffer EB was used for elution of purified DNA. The DNA was precipitated in ethanol and resuspended to 80 ng μL<sup>-1</sup> (2-μg aliquots) and shipped to the University of Missouri DNA Core Facility, Columbia, MO, for sequencing.

### ***Library Preparation and Metagenomic Sequencing***

Libraries were constructed using manufacturer's (Illumina) protocol with reagents supplied in Illumina's TruSeq DNA PCR-Free sample preparation kit. Briefly, 1 μg of genomic DNA was sheared using standard Covaris methods to generate an average insert size of 350 bp. The 3' and 5' overhangs were converted to blunt ends by an end repair reaction utilizing 3' to 5' exonuclease/polymerase activity. Using purification beads (AMPure XP), the desired size fragment was selected. Then, a single adenosine nucleotide was attached to the 3' ends of the blunt fragments followed by ligation of Illumina indexed paired-end adapters. The library was purified twice using sample purification beads. This purified library was then quantified with a Qubit assay, and library fragment size was confirmed by the Fragment Analyzer (Advanced Analytical Technologies, Inc.). The library was then diluted and sequenced according to Illumina's standard sequencing protocol for HiSeq.

### ***Metagenomic Sequencing Analysis and Identification of 16S rDNA Genes***

Metagenomic sequences were quality filtered before 16S rDNA genes were identified using Metaxa2. Briefly, hidden Markov models using HMMER identified the conserved regions of the small subunit by aligning to the SILVA database and then were subjected to a BLAST search. Taxonomic classification occurred by taking each rRNA entry and comparing the top 5 BLAST matches until a reliability score of 80 was achieved; this resulted

in accurate taxonomic classification but may not allow for specific classification (Bengtsson-Palme et al., 2015). These taxonomic profiles were further analyzed to assess diversity among and between samples using QIIME 1 (Caporaso et al., 2010).

## RESULTS

A total of 117 taxa were significantly different ( $P < 0.05$ ) in terms of abundance between CON and CSECT; 981 taxa differed by day ( $P < 0.05$ ), and 910 taxa were significantly different ( $P < 0.05$ ) by day within treatment. The microbial richness (Chao1) was unaffected ( $P = 0.97$ ) by treatment group when averaged across all collection day. Whereas days 1 and 3 had lower richness scores compared with day 28 ( $P = 0.006$ ), and day 28 had increased richness compared with weaning. Microbial richness was increased ( $P = 0.03$ ) for CSECT on day 28 compared with CSECT day 3, CON day 28 compared with CON day 1, and tended to be greater ( $P = 0.054$ ) for CSECT day 28 compared with CON day 3. No differences ( $P > 0.50$ ) in beta-diversity were detected between CON and CSECT. However, beta-diversity differences were detected ( $P < 0.05$ ) for each sampling day and several day within treatment where CSECT day 1 tended ( $P = 0.06$ ) to be different from CSECT day 3, CSECT day 28, and CON day 28 and was significantly ( $P = 0.03$ ) different from CON day 3. The CSECT day 3 was significantly ( $P < 0.05$ ) different from CSECT day 28, CON day 1, CON at weaning, and CON day 28. Significant differences ( $P < 0.05$ ) were also detected between CSECT day 28 and CSECT day 3, CON day 1, CON day 3, and CON at weaning. The CON day 1 differed ( $P < 0.05$ ) from CSECT at weaning, CSECT day 3, CON at weaning, and CON at day 28 and tended ( $P = 0.06$ ) to be different from CON day 3. Samples from CON day 3 tended ( $P = 0.06$ ) to be different from CSECT day 1 and was significantly ( $P < 0.05$ ) different than CSECT at weaning, CSECT day 28, CON day 1, CON at weaning, and CON day 28. Beta-diversity tended ( $P = 0.07$ ) to be different between CON day 28 and CSECT at weaning and CON at weaning and was significantly ( $P < 0.05$ ) different from CSECT day 1, CSECT day 3, CON day 1, and CON day 3.

## DISCUSSION

Overall, fewer differences in the rumen microbiome were associated with mode of delivery (CON vs. CSECT) compared to day; some distinct alpha- and beta-diversity differences were detected when comparing day within treatment group. This suggests an interaction of mode of delivery and stage

of maturity with the microbiome in terms of the richness and composition. Richness was improved with increased age and CSECT day 28 compared with CON day 3. The microbiome of human infants born via cesarean was less diverse compared with those delivered naturally (Biasucci et al., 2008). It is possible that our contradictory data are a result of differences in age (day 28 vs. day 3). A multitude of beta-diversity differences were detected with day and day within treatment suggesting compositional differences between mode of delivery and stage of development.

The most prominent effect on the microbiome in our data resulted from sampling day where day 28 samples had the greatest richness compared with other day and day within treatment group, even compared with samples at weaning. At day 28, calves are transitioning from a preruminant to a functioning ruminant, and the rumen grows 4 to 8 times in weight (Church, 1988). Calves are consuming more solid feed, including hay and grain in addition to milk from their dam. Richness at day 28 is greater than at weaning, suggesting that the microbiome stabilizes as the calf matures. This is in agreement with other data that report the microbial profile begins to stabilize at weaning (Benson et al., 2010) into maturity (Jami et al., 2013). The microbial profiles at days 1 and 3 were similar in terms of taxa, but differences in abundance were detected (Jami et al., 2013). In this study, although alpha-diversity was not different between days 1 and 3, several comparisons of day within treatment indicate differences in days 1 and 3. As the rumen begins to shift from microbes associated with aerobic and facultative fermentation to strictly anaerobic fermentation the microbial profiles shift as well, with distinct clustering according to stage of development (Bath et al., 2013; Jami et al., 2013).

Although distinct microbial profile differences were not evident between CSECT and CON when averaged across all days, several differences in both abundance and composition were highlighted at specific day and day within treatment group. Stage of development appeared to have the largest impact on microbial profiles, which is in agreement with other literature across multiple species. Thus, we can conclude that mode of delivery and stage of development affect the rumen microbial profiles and differences are more distinct in the preruminant phase.

## IMPLICATIONS

The rumen microbiome is critical to host performance. Understanding factors that contribute to

variation in the microbiome may be key to identifying opportunities for optimizing the microbiome to improve efficiency. Although cesarean sections are uncommon in livestock situations, these data provide insight into the influence that the birth canal has on colonization of the microbiome and whether detected differences persist through weaning. These data may allow for identification of critical stages and intervention strategies that may improve host performance later in life.

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