# Succession of ruminal bacterial species and fermentation characteristics in preweaned Brangus calves[1]

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## INTRODUCTION

Historically, rumen development has been defined by anatomical change, fermentation end products, and cultured media for identification of bacterial composition (Tamate et al., 1962; Fonty et al., 1987). The rumen ecosystem from 1 d to 2 yr of age declines in aerobic and facultative anaerobic taxa, while increasing anaerobic taxa (Jami et al., 2013). Li et al (2012) reported *Bacteroidetes* as the prominent phylum in 42-d-old calves, different from 14-d-old calves. This research has focused on dairy calves, which wean earlier, live in different environments, and likely has a different rumen developmental timeline in comparison to beef calves.

The energetic efficiency of a cow converting grass to milk and the calf converting milk to retained energy is less efficient than directly converting forage to retained energy (Freetly et al., 2006). Intake of solid food increases ruminal microbial activity and higher concentration of total VFAs produced that can be utilized for retained energy (Coverdale et al., 2004). Butyrate is especially important in rumen development and has been linked to papillae development in the rumen (Coverdale et al., 2004). Ruminal ammonia is vital for adequate microbial growth, therefore important for microbial and fermentation development (Satter and Slyter, 1974). Thus, the objectives of this study are to assess the composition of the ruminal bacteria and the production of fermentation end products in preweaned Brangus calves as they age. We hypothesize that ruminal VFA, ammonia, and bacterial populations will increase as calves age.

## MATERIALS AND METHODS

All experimental procedures were approved by New Mexico State University Institutional Animal Care and Use Committee (Protocol 2017-001). Eleven Brangus calves born in February and March of 2015 were sampled at the Chihuahuan Desert Rangeland Research Center (4.1% CP, 72.5% NDF) in Las Cruces, NM. Cow–calf pairs were housed on native rangeland with grasses including Black grama (*Bouteloua eriopoda*), three awns (*Aristida*), dropseeds (*Sporobolus*), burrograss (*Scleropogon brevifolius*), and tobosa (*Pleuraphis mutica*). Ruminal samples were collected via oral lavage (Lodge-Ivey et al., 2009) for microbiota, VFA, and ammonia analysis on 7, 35,

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63, 91, 119, 147, 175 ± 5 d of age. Samples were immediately flash frozen in liquid Nitrogen and stored at −80 °C.

Ruminal ammonia was analyzed using the phenol-hypochlorite procedure adapted to a microtiter plate (Broderick and Kang-Meznarich, 1980). Volatile fatty acid concentration was determined by gas chromatography utilizing methods of May and Galyean (1996). The DNA was extracted from samples using a repeated beating plus column (**RBB + C**) method (Yu and Morrison, 2004) followed by 1 mL of lysis buffer using the QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA), and quality and quantity of DNA were determined using a Nanodrop (Thermo Scientific, Marietta, OH).

The V1–V3 region of 16S rRNA gene was amplified, using modified universal primers 27F (5′ Adapter/Index/AGAGTTTGAGCCG GGCGCAG) and 519R (5′ Adapter/Index/ GTATTACCGCGGCTGCGA) including TruSeq adapter sequences and indices. The PCR amplification was performed on Bio-Rad Dyad Thermal Cycler (Bio-Rad). Following purification, PCR products were diluted to approximately 4 nM, and PCR amplicons libraries were paired-end sequenced using the Illumina MiSeq. Sequence data were processed using MICCA pipeline for metagenomic analysis. Operational taxonomic units (**OTUs**) were generated after removing <150 bp sequences. Final OTUs were clustered by 97% similarity and classified using BLASTn against a curated database derived from GreenGenes.

All data were analyzed as a completely random design using the MIXED procedure of SAS (version 9.4; SAS Inst. Inc., Cary, NC) with repeated measures for all data. Animal was the experimental unit, and the treatment was day of age. Using Akaike's information criterion, compound symmetry was the covariance structure. Means were calculated using LSMEANS. Day effects were considered significant at a $P \leq 0.05$, and mean separations were performed using PDIFF.

## RESULTS

The effect of calf age at the phylum level is summarized in Table 1. *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *SRI*, *TM7*, *Tenericutes*, *Verrucomicrobia*, and Unclassified phyla differed based on age of calf ($P < 0.02$). *Bacteroidetes* was most abundant at 7 (40.3%), 147 (38.2%), and 175 (52.8%) d of age ($P < 0.01$). The lowest Shannon Wiener Index (**SWI**) and richness occurred at 7 d of age and increased as the calves aged ($P < 0.05$).

Table 2 summarizes the effect of calf age at the genera level. Richness data identified 214 genera however; not all OTUs are represented in the database and therefore were not able to be classified at the genera level. Unidentified genera are presented as the lowest taxonomic unit identified. *Prevotella* increased throughout the study and was most abundant at 175 d of age ($P < 0.01$). Family BS11, *BF311*, *Prevotella*, *RF16*, *Fibrobacter*, family Lachnospiraceae, family Veillonellaceae, unclassified *RS1*, order LD1-PB3, and unclassified genera differed based on age of calf ($P < 0.02$). Order Bacteroidales was not affected by age of calf ($P = 0.16$). Genera richness increased ($P < 0.01$)

**Table 1.** Effect of age of Brangus calf on phyla composition, Shannon Weiner Index, and richness

| | Days of age | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Phylum†, % | 7 | 35 | 63 | 91 | 119 | 147 | 175 | SEM‡ | *P* value |
| *Bacteroidetes* | 40.3$^{a}$ | 48.3$^{a}$ | 37.6$^{b}$ | 40.0$^{b}$ | 37.8$^{b}$ | 38.2$^{b}$ | 52.8$^{a}$ | 3.2 | <0.01 |
| *Firmicutes* | 26.5$^{a,c}$ | 27.4$^{a,c}$ | 31.6$^{a,b}$ | 35.2$^{b}$ | 33.9$^{a,b}$ | 32.3$^{a,b}$ | 23.5$^{c}$ | 2.95 | <0.01 |
| *Proteobacteria* | 3.9$^{a}$ | 2.2$^{b}$ | 2.3$^{b}$ | 2.8$^{b}$ | 2.8$^{b}$ | 2.9$^{b}$ | 2.5$^{b}$ | 0.42 | <0.01 |
| *SR1* | 1.2$^{a,c}$ | 8.3$^{b}$ | 7.4$^{b,d}$ | 2.1$^{c}$ | 2.5$^{c}$ | 6.1$^{b,d}$ | 5.1$^{d}$ | 1.28 | <0.01 |
| *TM7* | 0.9$^{a}$ | 1.9$^{b}$ | 1.3$^{a}$ | 2.3$^{b}$ | 2.7$^{b}$ | 2.4$^{b}$ | 1.0$^{a}$ | 0.40 | <0.01 |
| *Tenericutes* | 4.3$^{a}$ | 3.9$^{a}$ | 4.9$^{a}$ | 4.2$^{a}$ | 4.9$^{a}$ | 5.3$^{a}$ | 2.3$^{a}$ | 0.83 | 0.01 |
| *Verrucomicrobia* | 4.9$^{a}$ | 1.0$^{b}$ | 4.7$^{a}$ | 5.8$^{a}$ | 6.6$^{a}$ | 5.7$^{a}$ | 3.8$^{a,b}$ | 1.26 | 0.02 |
| Unclassified | 10.0$^{a}$ | 4.8$^{b,c}$ | 6.9$^{b}$ | 5.4$^{b,c}$ | 6.0$^{b,c}$ | 4.2$^{c}$ | 4.1$^{b,c}$ | 1.12 | <0.01 |
| Shannon Weiner Index | 1.9$^{a}$ | 2.5$^{b}$ | 2.6$^{b,c}$ | 2.5$^{b}$ | 2.6$^{c,d}$ | 2.8$^{d}$ | 3.0$^{d}$ | 0.08 | <0.01 |
| Richness | 10.6$^{a}$ | 13.5$^{b}$ | 14.9$^{c}$ | 13.7$^{b,c,d}$ | 15.5$^{d,e}$ | 15.5$^{d,e}$ | 16.5$^{e}$ | 0.49 | <0.01 |

†Twenty-three phyla were identified. The most abundant phyla are listed in the above table.

‡$n = 11$.

$^{a-e}$Values within rows with differing superscripts differ, $P \leq 0.05$, between days of age.

**Table 2.** Effect of age of Brangus calf on genera composition, Shannon Weiner Index, and richness

| | Days of age | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Genera[†], % | 7 | 35 | 63 | 91 | 119 | 147 | 175 | SEM[‡] | *P* value |
| Family BS11 | 1.0$^{a,b}$ | 1.7$^{a,b}$ | 2.7$^{a,b}$ | 1.9$^{a,b}$ | 3.6$^{a}$ | 3.1$^{a}$ | 8.5$^{b}$ | 1.51 | <0.01 |
| *BF311* | 1.5 | 0.9 | 0.8 | 0.5 | 0.5 | 0.7 | 0.9 | 0.28 | 0.16 |
| *Prevotella* | 13.4$^{a}$ | 14.1$^{a}$ | 14.9$^{a}$ | 13.9$^{a}$ | 14.8$^{a}$ | 13.9$^{a}$ | 27.1$^{b}$ | 2.6 | <0.01 |
| Family RF16 | 0.9$^{a,b}$ | 0.8$^{a}$ | 1.4$^{a,b}$ | 2.3$^{a,b}$ | 2.2$^{a,b}$ | 1.4$^{a,b}$ | 2.6$^{b}$ | 0.72 | <0.01 |
| Family S24-7 | 0.8 | 1.8 | 2.6 | 1.1 | 0.9 | 2.0 | 2.3 | 0.69 | 0.35 |
| Order Bacteroidales | 12.9 | 16.1 | 9.1 | 10.2 | 10.2 | 14.5 | 8.7 | 2.34 | 0.16 |
| *CF231* | 6.7 | 5.3 | 3.1 | 4.6 | 4.0 | 3.0 | 1.5 | 1.06 | 0.47 |
| *YRC22* | 0.1 | 0.2 | 0.3 | 0.4 | 03 | 0.4 | 0.8 | 0.35 | 0.08 |
| *Fibrobacter* | 0.5 | 0.2 | 0.9 | 0.5 | 0.4 | 0.6 | 0.8 | 0.28 | 0.10 |
| Family Lachnospiracea | 3.3$^{a}$ | 3.7$^{a}$ | 3.2$^{a}$ | 5.2$^{b}$ | 5.0$^{b}$ | 4.2$^{a,b}$ | 3.0$^{a}$ | 0.56 | <0.01 |
| Family Veillonellaceae | 0.0 | 0.2 | 1.3 | 1.0 | 1.0 | 0.8 | 1.2 | 0.59 | 0.04 |
| *RFN20* | 1.5$^{a}$ | 0.7$^{a,b}$ | 1.0$^{a,b}$ | 0.6$^{a,b}$ | 1.0$^{a,b}$ | 1.2$^{a,b}$ | 0.6$^{b}$ | 0.36 | <0.01 |
| Order RF32 | 0.1 | 0.3 | 0.5 | 0.3 | 0.6 | 0.3 | 0.4 | 0.15 | 0.43 |
| Unclassified *RS1* | 1.2$^{a}$ | 8.3$^{b}$ | 7.4$^{b}$ | 1.9$^{a}$ | 2.5$^{a}$ | 5.8$^{a}$ | 3.9$^{a}$ | 1.32 | <0.02 |
| Order RF39 | 3.5 | 3.6 | 4.2 | 3.6 | 4.1 | 3.9 | 1.9 | 0.84 | 0.24 |
| Order LD1-PB3 | 0.6$^{a}$ | 0.1$^{a,c}$ | 3.8$^{b,c}$ | 4.4$^{b}$ | 5.8$^{b}$ | 4.8$^{b}$ | 1.8$^{b}$ | 1.03 | <0.01 |
| Family RFP12 | 2.2 | 0.9 | 0.7 | 0.8 | 0.6 | 0.7 | 1.5 | 0.54 | 0.38 |
| Unclassified | 10.0$^{a}$ | 4.8$^{b,c}$ | 6.8$^{b}$ | 5.5$^{b,c}$ | 6.0$^{b,c}$ | 3.9$^{c}$ | 4.1$^{c}$ | 1.10 | <0.01 |
| Shannon Weiner Index | 4.0$^{a}$ | 4.6$^{b}$ | 4.7$^{b,d}$ | 5.3$^{c}$ | 4.9$^{b,c}$ | 5.0$^{c,d}$ | 5.1$^{c,d}$ | 0.14 | <0.01 |
| Richness | 40.6$^{a}$ | 44.1$^{a,b}$ | 48.5$^{b,c}$ | 50.7$^{c}$ | 51.7$^{c}$ | 52.4$^{c}$ | 48.4$^{b,c}$ | 1.79 | <0.01 |

[†]Two hundred and fourteen genera were identified. The most abundant genera are listed in the above table.

[‡]$n$ = 11.

$^{a–d}$Values within rows with differing superscripts differ, $P \leq 0.05$, between days of age.

**Table 3.** Age of Brangus calf effects on ruminal volatile fatty acids and ammonia

| | Days of age | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | 7 | 35 | 63 | 91 | 119 | 147 | 175 | SEM[†] | *P* value |
| Total VFA, mM | 45.1$^{a}$ | 61.9$^{a,b}$ | 76.6$^{b,c}$ | 79.0$^{b,c}$ | 82.8$^{c}$ | 85.5$^{c}$ | 49.3$^{a}$ | 6.69 | <0.01 |
| VFA, mol/100 mol | | | | | | | | | |
| Acetate | 79.3$^{a}$ | 75.7$^{b}$ | 75.9$^{b}$ | 76.4$^{b}$ | 75.0$^{b,c,d}$ | 73.4$^{c,d}$ | 71.9$^{d}$ | 0.83 | <0.01 |
| Propionate | 14.3$^{a,b}$ | 15.3$^{a}$ | 13.9$^{b}$ | 14.7$^{b}$ | 14.7$^{a,b}$ | 15.2$^{a}$ | 13.5$^{b}$ | 0.41 | <0.01 |
| Isobutyrate | 1.2$^{a}$ | 1.1$^{a}$ | 1.2$^{a}$ | 1.1$^{a}$ | 1.2$^{a}$ | 1.7$^{b}$ | 2.7$^{c}$ | 0.14 | <0.01 |
| Buytrate | 4.2$^{a}$ | 7.0$^{b}$ | 7.6$^{b,c}$ | 7.8$^{b,c}$ | 8.1$^{b,c}$ | 8.0$^{b,c}$ | 8.6$^{c}$ | 0.53 | <0.01 |
| Acetate:Propionate | 5.7$^{a}$ | 5.0$^{b}$ | 5.5$^{a,c}$ | 5.5$^{a,c}$ | 5.1$^{b,c}$ | 4.9$^{b}$ | 5.4$^{a,b,c}$ | 0.20 | <0.01 |
| Ammonia, mM | 10.2$^{a}$ | 6.6$^{b}$ | 5.4$^{b,d}$ | 2.9$^{c}$ | 2.7$^{c}$ | 3.0$^{c}$ | 4.8$^{d}$ | 0.68 | <0.01 |

[†]$n$ = 11.

$^{a–d}$Values within rows with differing superscripts differ, $P \leq 0.05$, between days of age.

from 7 to 147 d of age and with a slight decrease denoted at 175 d of age. The lowest SWI occurred at 7 d of age ($P < 0.01$) and the 91, 119, 147, and 175 d of age did not differ ($P \geq 0.05$).

Table 3 summarizes the effect of calf age on ruminal VFA and ammonia production. Total VFA production was similar for days 7, 35, and 175 and was lower than days 63, 91, 119, and 147 ($P < 0.01$). Acetate was greatest and butyrate was lowest at day 7 ($P < 0.01$). Propionate was greater on days 7, 35, 119, and 147 and was least on days 63, 91, and 175 ($P < 0.01$). Acetate:Propionate was greatest on days 7, 63, 91, and 175 ($P < 0.01$). Butyrate production increased as calves aged with the greatest butyrate concentration at day 175 ($P < 0.01$). Ruminal ammonia was greatest on day 7 and remained below 6.6 mM for the remainder of the study ($P < 0.01$).

## DISCUSSION

Limited data exist on beef calf ruminal development in extensive landscapes. In this study, ruminal fluid was examined for bacterial composition and fermentation end products and generated conflicting results compared with dairy calf data. One area of agreement was bacterial composition at the phylum level. In this study, *Bacteroidetes* and *Firmicutes*

comprised approximately 72.2% of the rumen bacterial population, agreeing with previously reported data from Jami et al. (2013). Approximately 70% of the rumen bacterial population comprised *Bacteroidetes* and *Firmicutes* in 2-mo-old dairy calves (Jami et al., 2013). Rumen liquid fractions commonly show increased *Bacteroidetes* populations compared with *Firmicutes* (Pitta et al., 2010). In the current study, ruminal contents were collected via a suction strainer, and samples should be similar to the liquid fractions commonly reported in studies that strain the ruminal contents through cheesecloth (Pitta et al., 2010). Pitta et al. (2010) reported increased *Bacteroidetes* populations compared with *Firmicutes*, which is in agreement in with our study where *Bacteroidetes* population was 3.9% to 29.3% greater than *Firmicutes*.

In 42-d-old calves, *Firmicutes* comprised 12% of the population (Li et al., 2012), differing from the present study where *Firmicutes* ranged from 27.4% to 31.6% from day 35 to 63 of age. Calves fed milk replacer have decreased levels of *Firmicutes* compared with calves grazing on native rangeland (Li et al., 2012). Beef calves that are raised on rangelands may consume forage at earlier ages, leading to increased *Firmicutes* population. Bacterial genera associated with fiber degradation in the phylum *Firmicutes* include *Butyrivibrio*, *Ruminococcus*, and *Succiniclasticum* (Latham et al., 1978). In dairy calves ranging from 14 d old to 12 mo, *Ruminococcus* maintained a low abundance ranging from 0.3% to 3.6% of the population (Li et al., 2012). The low abundance of *Ruminococcus*, in the present study, is unexpected for calves on forage diet. It can be speculated that calves were selecting a higher quality diet that may have been low in cellulose and hemicellulose. Many unknown genera made up a substantial portion of the ruminal bacteria in the present study, warranting further research on what role they may play in a ruminal environment.

At 8 wk, calves on concentrate diets have demonstrated similar VFA concentrations of adult cattle (Suárez et al., 2006). Beef calves primarily receive nourishment from dams' milk and available forages. Forage inclusion in dairy calf rations showed increased rumen weight, body weight, and feed efficiency compared with nonforage fed calves (Tamate et al., 1962), while whole milk has shown improved small intestine development (Górka et al., 2011). Bartle et al. (1984) reported at 9 wk of lactation, milk production alone did not adequately support calf growth, and creep feeding was needed to meet nutrient requirements. Thus, beef calves raised in conventional systems have an opportunity to capitalize on body weight gain and feed efficiency due to consumption of both available forage and dams' milk.

Day 7 showed the lowest total VFA levels, and these are indicative of a low structural carbohydrate diet (Asai et al., 1973). However, by day 35, total VFA production increases to 61.9 mM only to decrease to 49.3 mM on day 175. Forage quality of New Mexico rangeland is greatest during July and August due to monsoon-type rainfall events. During 2015, less than average rainfall occurred during July and August; thus by September and October (day 175), forage quality declined, resulting in low total VFA production.

The greatest ruminal ammonia occurred on day 7 when calves were primarily consuming milk. Milk may enter the rumen causing increased ruminal ammonia (Jami et al., 2013). Ruminal ammonia concentration of 2 mM is needed for maximum microbial growth rate (Satter and Slyter, 1974). Despite low protein and high structural carbohydrates that are typical of rangelands, calf rumen function was not impaired for the duration of this experiment. Ammonia production is vital for developing calves' nutrient synchrony. Microbial, VFA, and ammonia data support the theory that calves are consuming available forage, and microbial population makes use of these feedstuffs. Hollingsworth-Jenkins et al. (1994) support this conclusion, where calves raised in Sandhills region of Nebraska consumed a diet primarily of forage prior to weaning. Calves consuming forage are potentially more efficient at feed conversion than milk (Freetly et al., 2006). By 35 d of age, calves in the present study had bacteria composition, VFA, and ammonia concentrations comparable to mature ruminants on native rangeland (Smith et al., 2017). According to dairy calf data, a functional rumen occurred at 8 wk of age. Based on present data, beef calves that consume native rangeland forages develop a functional rumen at 35 d of age.

## IMPLICATIONS

We hypothesized that calf age would relate to rumen maturity when considering fermentation and feed efficiency. Our data indicate that the rumen may become mature and functional at an earlier age than reported in previous literature. These data could lead to new management strategies in extensive landscapes such as genetic selection of cows for lower milk production and early weaning methodologies.

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