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FEED EFFICIENCY AND THE MICROBIOTA OF THE ALIMENTARY TRACT

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Introduction

There is considerable variation in the efficiency that cattle convert feed for maintenance and product (body weight gain, milk, and conceptus). Both intake and gain are polygenic traits and to better understand factors that contribute to variation in feed efficiency more defined phenotypes are needed. Several studies have associated differences in the microbiota of the alimentary tract between obese and non-obese rodents (Turnbaugh et al., 2006), obese and non-obese humans (Ley et al., 2006), and energy metabolism in birds (Torok et al., 2008 and Stanley et al., 2013). These finding suggest that there is a potential relationship between the microbiota of the alimentary tract and feed efficiency in beef cattle. Considerable research has been conducted on the rumen microbiota, but less consideration has been given to the rest of the alimentary tract.

Changes in the microbial community of the rumen-reticulum complex with changes in diet as well as across species of ruminants have been documented. The observed diversity in the microbiota has been limited to those organisms that can be cultured. The rumen is an anaerobic environment and many of the strict anaerobes are difficult to culture in vitro. The concern has been that relative differences in microbial species may have been a function of the ease of culturing some species in vitro and that the ability to grow in vitro is not indicative of their relative proportion of the rumen-reticulum microbiota. Using next-generation sequencing provides a tool to estimate the makeup of the microbiota by identifying bacteria by their DNA sequence rather than enumerating them through culture techniques.

Two studies were conducted as part of the Agriculture and Food Research Initiative Competitive Grant 2011-68004-30214 from the USDA National Institute of Food and Agriculture to determine the relationships between the microbiota and feed efficiency. The results of this research have been reported by Myer et al. (2015a, 2015b, 2015c, and 2016) and Freetly et al. (2015).

Study 1 – Microbial community profiles of the rumen-reticulum, jejunum, cecum, and colon of steers differing in feed efficiency.

Steers for this study were selected from two contemporary groups that individual feed intake and body weight were measured for a 63-d period. Group 1 (n = 148) was comprised of spring-born calves that were 371 ± 1 d of age and weighed 522 ± 4 kg at the start of the feed intake measurements. Group 2 (n = 197) was comprised of fall-born calves that were 343 ± 1 d of age and weighed 448 ± 4 kg at the start of the feed intake measurements. Steers were fed a ration that on a dry matter basis consisted of 57.35% dry-rolled corn, 30% wet distillers grain with solubles, 8% alfalfa hay, 4.25% supplement (containing 772 mg monensin/kg), and 0.4% urea. At the end of each feeding period, steers were ranked based their standardized distance

from the bivariate mean for average daily gain (**ADG**) and average daily feed intake (**ADFI**) assuming a bivariate normal distribution with a calculated correlation between ADG and ADFI . Within each contemporary group, four steers with the greatest deviation within each Cartesian quadrant were sampled (n = 16 steers/contemporary group). The resulting design was a 2 X 2 factorial consisting of greater and less ADG and greater and less ADFI.

At the end of the feeding period, selected steers were slaughtered and digesta was collected from the rumen-reticulum, jejunum, cecum, and colon. Samples were buffered in peptone water (pH 7.0) + 15% glycerol and stored at -70°C. Deoxyribonucleic acid (**DNA**) was extracted from the samples. Amplicon library preparations were performed by PCR amplification of the V1 through V3 hypervariable region of the 16S rRNA gene. The PCR amplicon libraries were sequenced using the 2 X 300, v3 600-cycle kit and the Illumina MiSeq sequencing platform (Illumina, Inc., San Diego, CA).

Sequences were processed using the QIIME 1.8.0 software package (Caporaso et al., 2010). Paired reads were joined using fastq-join (Aronesty, 2011) and filtered for quality using the Galaxy server (Blankenberg et al., 2010). Chimeric sequences were checked using ChimeraSlayer (Haas et al., 2011). All cleaned sequences were classified into taxa using Greengenes 16S rRNA Gene Database (DeSantis et al., 2006). Operational taxonomic units (**OTU**) were calculated using the uclust program (0.03 dissimilarity; Edgar, 2010). After calculating richness for each quadrant, singletons were removed from further diversity analyses. Based on rarefaction curves, the number of OTU was normalized via subsampling 25,000 sequences from each sample. A phylogenic tree was built with FastTree (Price et al., 2010) to determine α- and ß-diversity metrics.

The mean abundance of data metrics and each taxon were compared among feed efficiency groups with contemporary group and Cartesian quadrant as fixed effects. Differences were determined at $P < 0.05$ with Benjamini-Hochberg method used for multiple-testing corrections (Benjamini and Hochberg, 1995) Multiple-testing corrections were made for the number of phyla, the number of OTU groups, and other classified taxa groups. Linear contrasts were applied to quadrants to separate whether microbial populations varied by ADG, ADFI or the interactions. Principal coordinate analysis was performed using weighted and unweighted UniFrac analyses (Lozupone and Knight, 2005).

In the study, the rumen content yielded an average of 1,098 ± 382 OTU, of which were classified into 24 phyla, 48 classes, 89 orders, 173 families, and 317 genera. The bacterial diversity was reflected by the diversity index of 6.85 ± 0.36 (Shannon). Although the rumen has a great abundance of microorganisms, their functionality is more specialized, which may result in a lower diversity index than that of the large intestine. Indeed, the most abundant phyla in the rumen were Bacteroidetes and Firmicutes, present at abundances of 53 to 63% and 23 to 33%, respectively. However, over 90% of the Bacteriodetes phylum was composed of the genus *Prevotella*, exemplifying this specialization. Reports have indicated that *Prevotella* is the most

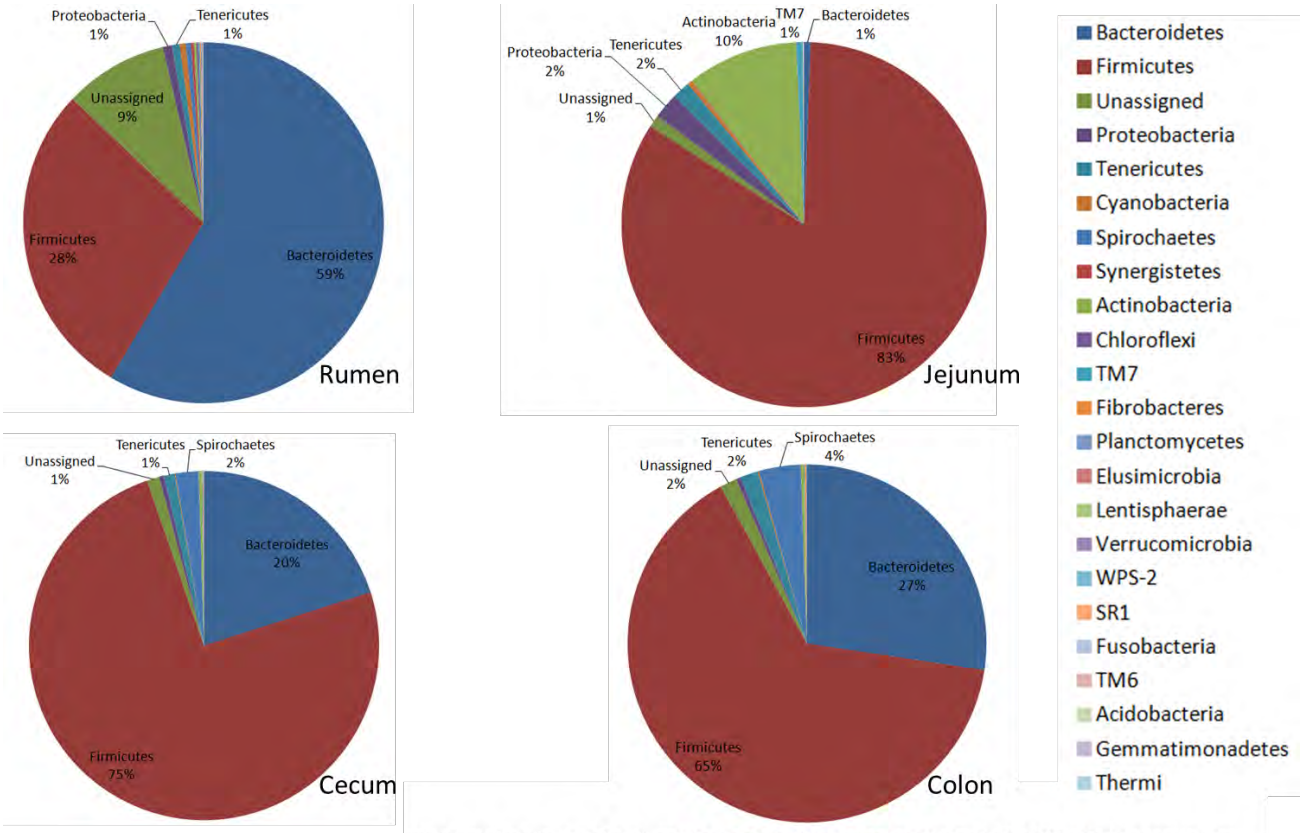
abundant ruminal genus (Stevenson and Weimer, 2007), and these data overwhelming supported those findings. Along with the *Prevotella* abundances (45 to 57%), the bacterial genera in the rumen representing ≥1% of the total sequences included *Dialister* (2.6 to 4.1%), *Succiniclasticum* (2.0 to 4.0%), *Ruminococcus* (1.0 to 2.5%), *Butyrvibrio* (0.5 to 1.1%), and *Mitsuokella* (0.6 to 1.2%).

Compared to the rumen, changes in the bacterial populations of the jejunum were anticipated, as the role of this alimentary tract segment transitions more towards host digestion of nutrients and absorption. The bacterial population of the jejunum was greatly reduced in species abundance and diversity compared with that of the rumen, with an average of 499 ± 159 OTU and a diversity index of 3.91 ± 0.48. These OTU were classified into 21 phyla, 51 classes, 94 orders, 198 families, and 397 genera. The differences in function between the rumen and the jejunum were reflected in the bacterial abundances, as the phylum Firmicutes accounted for up to 90% of the populations in the jejunum, and Bacteroidetes was greatly reduced to 0.4 to 1.1%. Actinobacteria (6 to 13%) made up the greatest abundance of the remaining phyla, followed by Proteobacteria (0.8 to 5.8%), and Tenericutes (0.4 to 4%). Genera and OTU representing ≥1% of the total sequences included *Ruminococcus* (12.2 to 19.6%), *Butyrivibrio* (2.6 to 7.7%), *Lactobacillus* (2.8 to 4.2%), *Bulleidia* (0.8 to 1.9%), *Mogibacterium* (1.1 to 1.7%), *Mitsuokella* (0.05 to 1.27%), and *Propionibacterium* (0.07 to 7%).

The role of postruminal degradation of cellulose and starch as well as the importance of microbial interaction and the maturation of the mucosal immune system highlights the changes in abundance and diversity of bacterial populations observed in the cecum. The cecum averaged 5,572 ± 1,428 OTU with a bacterial diversity index of 7.89 ± 0.47 (Shannon). These data were far greater than that of the rumen and jejunum. The cecal OTU were classified to 18 phyla, 40 classes, 75 orders, 148 families, and 225 genera. Similar to the jejunum, Firmicutes was the most abundant phylum at 68 to 81% of the total sequences. However, Bacteroidetes was greater in abundance compared to the small intestinal segment, at 18 to 26%, followed by the phyla Spirochaetes (1.4 to 3.19%), Tenericutes (0.7 to 1.2%), and Actinobacteria (0.2 to 0.4%). The remaining phyla accounted for less than 0.1% of the sequences. Abundant genera consisted of *Prevotella* (2.1 to 7.3%), *Turicibacter* (4.6 to 6.7%), *Coprococcus* (1.2 to 2.8%), *Ruminococcus* (1.5 to 2.7%), *Dorea* (2.2 to 3.3%), *Blautia* (0.5 to 2.0%), *Clostridium* (1.0 to 1.2%), and *Oscillospira* (1.1 to 1.6%).

Although the colon microbial communities are likely to be similar to those of the cecum, as digesta travels to the more distal regions of the gastro-intestinal tract (**GIT**) functionality as well as environmental conditions shift. Appropriately, the bacterial species abundance and diversity of the colon was greater than the cecum, and expectedly more similar than the jejunum or rumen, averaging 6,025 ± 1,225 OTU and an average diversity index of 8.05 ± 0.20 (Shannon). The OTU were classified to 20 phyla, 46 classes, 83 orders, 152 families, and 231 genera. Most similar to the cecum, but continuing the trend in the lower intestinal tract, the colon bacterial population predominantly consisted of Firmicutes (60-70%). As digesta moves

distally, a trend of greater Bacteroidetes populations was observed, compared to the jejunum and cecum, where this phylum was present in the colon at abundances of 21–33%. Abundances of remaining phyla greater than 0.1% of the sequences consisted of Spirochaetes (2.5–4.5%), Tenericutes (1.2–1.9%), Proteobacteria (0.3–0.5%), Actinobacteria (0.23–0.33%), and Fibrobacteres (0.02–0.29%). The bacterial genera present in greatest abundance within the colon were *Prevotella* (3.0–11.1%), *Ruminococcus* (1.7–2.9%), *Coprococcus* (1.0–2.9%), *Dorea* (1.7–2.2%), *Turicibacter* (1.9–4.4%), *Blautia* (0.3–1.3%), *Oscillospira* (1.1–1.6%), and *Parabacteroides* (0.4–1.4%).



The taxonomic profile for the relative phylum-level abundance of each section classified by representation at ≥0.001% of total sequences. Taxonomic composition of the GIT microbiota was compared based on the relative abundance (reads of a taxon/total reads in a sample).

Within the rumen, although bacterial abundances were consistent with other research (Jami et al., 2014), significantly increased populations of Firmicutes were observed within the $ADG_{Greater} - ADFI_{Less}$ group ($P = 0.0364$), representing the feed efficient group. This association

was of note, for changes in Firmicutes-to-Bacteroidetes ratio is often implicated in obesity research (Ley et al., 2006), and has been correlated with energy harvesting and fat increases in dairy cattle (Jami et al., 2014). Other taxa and OTU abundances were associated with varying degrees of efficiency based on the experimental design, which may play important roles in the fermentative and cellulolytic capacity of the rumen based upon their putative functions. Those abundances associated with the $ADG_{Greater}$-$ADFI_{Greater}$ group included increases in *Prevotella* ($P = 0.0154$), *Lactobacillus* ($P = 0.0419$), and *Dialister* ($P = 0.0062$) populations, whereas *Anaerovibrio* ($P = 0.0291$) was least prevalent. The $ADG_{Greater}$-$ADFI_{Less}$ group included increases in *Butyrivibrio* ($P = 0.0391$) and *Leucobacter* ($P = 0.0215$). The ADG_{Less}-$ADFI_{Less}$ group included increases in *Ruminococcus* ($P = 0.0255$) abundances, but decreases in *Acidaminococcus* ($P = 0.0306$). Finally, the ADG_{Less}-$ADFI_{Greater}$ group saw increases in *Lysobacter* ($P = 0.0462$), *Janibacter* ($P = 0.0161$), and *Succiniclasticum* ($P = 0.0276$) populations. Additionally, the data were analyzed to determine whether microbial populations differed by less vs. greater ADG, less vs. greater ADFI, or their interaction. Within the rumen, the significant changes in microbial abundances were primarily associated with ADG.

Jejunal microbial population analyses significantly associated the genus *Butyrivibrio*, and its family Lachnospiraceae, with the $ADG_{Greater}$–$ADFI_{Less}$ group; the feed efficient group. The *Butyrivibrio* species of greatest abundance that was significant for this association ranged from 1.9 to 6.0% ($P = 0.041$). This may be important functionally, as the hemicellulolytic *Butyrivibrio* can ferment a wide range of sugars, as well as influence the energy pool to enterocytes via butyrate production. The phylum Proteobacteria ($P = 0.030$) was also associated with the $ADG_{Greater}$–$ADFI_{Less}$ group, and has been demonstrated to negatively correlate with Firmicutes populations, as well as positively correlate with feed conversion ratio (Cook et al., 1994; Jami et al., 2014). Several other taxa were significantly associated with variation among feed efficiency groups, such as increases in the AA-fermenting genus *Acidaminococcus* ($P = 0.018$; $ADG_{Greater}$-$ADFI_{Less}$) and the obligately oxalotrophic, ammonium-dependent, aerobic genus *Ammoniphilus* ($P = 0.022$; ADG_{Less}-$ADFI_{Greater}$). All significant taxa and OTU were associated with ADG or the interaction of ADG and ADFI. Interestingly, *Butyrivibrio* was the only assignment that was solely associated with intake.

The evaluation of the cecal bacterial communities revealed no significant differences among the feed efficiency groups at phylum-level abundances. However, among OTU and genus level abundances, *Prevotella* ($P = 0.042$), *Blautia* ($P = 0.042$), *Coprobacillus* ($P = 0.004$), *Dorea* ($P = 0.042$), *Clostridium* ($P = 0.044$), and *Parabacteroides* ($P = 0.027$) were detected with greatest abundance within the $ADG_{Greater}$-$ADFI_{Greater}$ group, while *Ruminococcus* ($P = 0.040$) and *Oscillospira* ($P = 0.041$) were least abundant within the group. The species *Lactobacillus ruminis* ($P = 0.047$) was also least abundant in the ADG_{Less}-$ADFI_{Greater}$ group. The genus *Blautia* is of recent research interest due to its ubiquitous presence among humans and other mammals. These bacteria exist at low abundances, but are thought to contribute to the

metabolic capacity of the host by providing energy from polysaccharides that other gut commensals cannot degrade (Eren et al., 2015).

Similar to the cecum, no significant differences among the feed efficiency groups were observed within the bacterial community abundances of the colon. Differences among the groups did exist at lower levels of classification and at the OTU-level. Among taxa, these organisms included *Anaeroplasma* ($P = 0.0222$), *Paludibacter* ($P = 0.0226$), *Faecalibacterium* ($P = 0.0361$), *Succinivibrio* ($P = 0.0412$), and *Pseudobutyrivibrio* ($P = 0.0479$). *Anaeroplasma* and *Faecalibacterium* were in greatest abundance within the $ADG_{Greater}$–$ADFI_{Greater}$ group, *Paludibacter* was in greatest abundance within the ADG_{Less}– $ADFI_{Less}$ group, and *Succinivibrio* and *Pseudobutyrivibrio* were least abundant within the $ADG_{Greater}$–$ADFI_{Less}$ and ADG_{Less}–$ADFI_{Greater}$ groups, respectively. *Coprococcus* ($P = 0.0323$) and *Clostridium* ($P = 0.0446$) were in greatest abundance within the $ADG_{Greater}$–$ADFI_{Greater}$ group, while *Dorea* ($P = 0.0225$) was least abundant within this group. *Butyrivibrio* ($P = 0.0240$) and *Prevotella* ($P = 0.0435$) were greatest in the ADG_{Less}–$ADFI_{Greater}$ group; the least efficient group, while the abundance of *Oscillospira* ($P = 0.0456$) was greatest within the ADG_{Less}– $ADFI_{Less}$ group. The butyrate producing and strong xylan-degrading activities of *Butyrivibrio* and *Pseudobutyrivibrio* species may lend further insight to their association with feed efficiency in the colon (Morgavi et al., 2013). The bacterial populations within both the cecum and colon were solely associated with intake and/or the interaction of gain and intake, but not gain alone, which may be expected as digesta travels further from the fermentative and digestive sections of the GIT.

Determination of the microbial population associations with feed efficiency, ADG, and ADFI throughout the alimentary tract provides great insight as to the interactions that may occur at both the host and microbial levels based on the putative functions of the organisms involved. As these tissues are distinct in function and environment, so are their microbial populations and effect on the host. The varying microbial phylogenetic diversity and abundance along the tract likely also plays a role, and is a function of, the degree of specialization. However, it is not clear whether changes in the microbiome are contributing to differences in feed efficiency or host factors are driving changes in the microbiome.

Study 2 – Methane production and methanogen levels in steers that differ in residual gain.

Individual feed intake and body weight were measured on 132 fall-born steers for a 70-d period. At the start of the study, steers were 348 ± 1 d of age and weighed 444 ± 0.4 kg. Steers had ad libitum access to a ration that as a percentage of dry matter consisted of 82.75% dry-rolled corn, 12.75 corn silage, 4.5% supplement. The supplement contained 0.95% Rumensin-80. Seven steers with extreme positive residual gain (**RG**), and seven steers with extreme negative RG whose dry matter intake was within 0.32 standard deviations of the means were selected for subsequent measurements.

In vivo methane production was measured over a 6-h period using a headbox respiration chamber. Steers were subsequently slaughtered and rumen and cecum contents were sampled to determine *in vitro* methane production. *In vitro* methane was determined in vials gassed with hydrogen over 2 through 8 h of incubation.

Digesta samples were collected from the rumen, cecum, and rectum at slaughter for the determination of methanogen 16S rRNA. Deoxyribonucleic acid was extracted from the digesta samples. Primer sets that targeted conserved regions of the 16S rRNA gene were used in real-time quantitative PCR using a LightCycler 480 (Roche, Indianapolis, IN) to measure total bacteria, total methanogens, Methanomicrobiales, Methanobacteriales, Methanosarcina, Methanobacterium, Methanobrevibacter Group 1 (*Mbb. ruminantium* + *Mbb. cutcularis*), and Methanobrevibacter Group 2 (*Mbb.. smithii* + *Mbb. wolinii* + *Mbb. thaueri* + *Mbb. gottschalkii* + *Mbb. woesi*). The experimental design for the level of methanogens was a 2 × 3 factorial. The main effects were RG and alimentary tract location.

In vivo enteric methane production did not differ ($P = 0.11$) between the positive RG (112 ± 13 L/d) and the negative RG (74 ± 13 L/d). *In vitro* rumen methane production did not differ between positive RG ($64.26 \times 10^{-5} \pm 10.85 \times 10^{-5}$ mmol·DM g^{-1}·min^{-1}) and negative RG ($61.49 \times 10^{-5} \pm 10.85 \times 10^{-5}$ mmol·DM g^{-1}·min^{-1}; $P = 0.86$). *In vitro* cecum methane production did not differ between positive RG ($4.24 \times 10^{-5} \pm 1.90 \times 10^{-5}$ mmol·DM g^{-1}·min^{-1}) and negative RG ($4.35 \times 10^{-5} \pm 1.90 \times 10^{-5}$ mmol·DM g^{-1}·min^{-1}; $P = 0.97$).

Methanogen 16S rRNA as a percent of the total 16S rRNA bacteria did not differ between RG groups ($P = 0.18$). The methanogen 16S rRNA as a percentage of rumen fluid total bacteria 16S rRNA (5.3 ± 3.1%) did not differ from the methanogen 16S rRNA as a percentage of cecum content total bacteria 16S rRNA (11.8 ± 3.1%; $P = 0.14$). The methanogen 16S rRNA as a percentage of the rectum content total bacteria 16S rRNA (0.7 ± 3.1%) was not different than the rumen content ($P = 0.29$), but was less than the cecum content ($P = 0.01$). Methanomicrobiales 16S rRNA as a percentage of total methanogen 16S rRNA did not differ across sample sites ($P = 0.81$); however, steers with positive RG (10.5 ± 1.6%) was greater compared to steers with negative RG (5.1 ± 1.6%; $P = 0.02$). As a percent of the total methanogen 16S rRNA, 16S rRNA of Methanobacteriales ($P = 0.23$), *Methanobacterium* ($P = 0.60$), and the Methanobrevibacter Group 2 group ($P = 0.41$) did not differ between RG groups. Methanobacteriales and the Methanobrevibacter Group 2 had a greater percentage of the total methanogen 16S rRNA in the rumen compared to the cecum and rectum which did not differ from each other. *Methanobacterium* was a lesser percentage of the total methanogen gene 16S rRNA in the rumen compared to the cecum and rectum which did not differ from each other. Methanobrevibacter Group 1 did not differ between RG group ($P = 0.25$) and tended to differ with collection site. The percentage of 16S rRNA of *Methanosarcina* did not differ with RG group ($P = 0.42$) or collection site.

In our study, total methanogens represent approximately 5% of the total bacterial 16S rRNA in the rumen which is similar to what Frey et al. (2010) observed in the dairy cow and there was not a difference between the RG groups. These findings are consistent with the lack of a difference in rumen *in vitro* methane production. In our study and the study of Popova et al. (2013) in sheep, the cecum had a lower *in vitro* methane production than the rumen. Similar to the rumen, there were no differences in methane production between the RG groups.

The relative contribution of the different methanogens differed across sites in the alimentary tract; however, with the exception of Methanomicrobiales 16S rRNA did not differ between RG groups. This shift in Methanomicrobiales 16S rRNA was not reflected in the Total Methanogen 16S rRNA. The lack of difference in Total Methanogen 16S rRNA is consistent with the lack of difference in methane production. Our findings do not support the hypothesis that differences in RG at an average intake are a consequence of differences in methane production or methanogen populations in the alimentary tract.

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