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Pregnancy Influences on The Rumen Environment of Angus Heifers Differing in Feed Efficiency

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Pregnancy Influences on The Rumen Environment of Angus Heifers Differing in Feed Efficiency

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ABSTRACT

With an expected increase in population by 2050, the demand for more animal protein will increase. To improve the sustainability of US cattle production, producers and researchers have historically focused on improving the feed efficiency of steers through improvements in breeding, genetics, nutrition, and microbiome management, producing a more marketable beef product. However, without a successful pregnancy, there would be no marketable animal to feed. The objective of this study was to examine the impact of pregnancy on the rumen environment, microbial communities, and the correlation to feed efficiency status in Angus heifers. Utilizing 17 cannulated Angus heifers, feed efficiency status was determined utilizing the GrowSafe to monitor feed intake for a 70d trial period to calculate residual feed intake. Following the trial, heifers were bred, and 40mL rumen fluid and content samples were collected every two weeks during gestation from initial AI date. Metagenomic DNA was extracted from the rumen samples, sequencing libraries were prepared targeting the bacterial 16S rRNA gene and was sequenced using an Illumina MiSeq. All microbial analyses were analyzed in the R environment where alpha diversity, beta diversity, and relative abundances were determined. Metabolites were determined using ultra high-performance liquid chromatography high resolution mass spectrometry, and Metabolomic Analysis and Visualization Engine (MAVEN) was used for metabolite identification. Using the "ANCOMBC" package, with a focus on the global results, downstream analyses indicated 10 ASVs, seven Prevotella and three Succiniclasticum at the genus level, with significant changes across time points ($P<0.05$). Based on a one-way ANOVA, 90 metabolites were determined significant throughout pregnancy ($P<0.05$). The 10 metabolites with the highest variable importance of projection (VIP) scores from a partial least squares discriminant analysis were used for correlation calculations. Of the 10 ASVs and 10 metabolites, significant correlations with feed efficiency status were found at various time points ($P<0.05$). The rumen microbiome and its fermentative profile were different at various time points during pregnancy with several correlations to feed efficiency status. Further research examining pregnancy impacts on the rumen microbiome and feed efficiency will continue to improve the cow-calf enterprise.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

As the world's population is expected to reach 10 billion by the year 2050, the demand for a high-quality protein source is expected to increase. The increase in population growth comes with a cost of decreased resources such as land, feed and grain commodities, and labor. A shift in production practices towards increasing crop and carcass yields, environmentally conscious management practices, and maintaining feed efficient beef cattle will help to improve sustainable practices in agriculture.

Feed efficiency in ruminant animals considers their ability to convert inputs into profitable outputs. In beef cattle, those inputs are low quality feed options for humans into high-quality end products like milk, meat, and to produce feed efficient calves. More specifically, the rumen microbiota, consisting of bacteria, protozoa, fungi, and archaea, are largely responsible for this conversion process in supplying usable nutrient sources to the animal and roughly 70% of a ruminant animal's energy requirements (1, 2). Research has proven variety of relationships between the host feed efficiency status and the rumen microbiome populations (3, 4). In addition to feed conversion and processing, the rumen microbiome is also linked with the host's health and disease state (5) and methane production (6). The composition of the rumen microbiome is influenced by a variety of different factors including age (7), diet (8), environment (9), and host physiology (10, 11).

Different production sectors within the beef cattle industry have a unique definition of what is considered a quality end product, as different management practices are implemented to ensure that every opportunity to meet end point goals defined by the operation are offered. Feed lots are focused on cattle growing and increasing red muscle tissue and fat deposition. Highly concentrated diets are commonly offered as an efficient way to meet an animal's nutritional requirements. Cow-calf producers are more focused on maintaining cow health and body condition, encouraging fetal growth and health during pregnancy, and raising a healthy, profitable calf. In this setting, producers often rely on forage-based diets that are fed on an inclining plane of nutrition with the intent to develop future generations of efficient cattle.

If an animal is efficient in one production system, that same cow may not necessarily be feed efficient in the other operation. Shi (12) found that shifting growing heifer diets to include a greater percentage of grain allowed for the heifers to achieve a greater level of average daily gain and feed conversion ratio. When the amount of grain versus forage components in cattle diets is greater for grains, cattle are expected to be more efficient in converting that into more pounds of product (13). Grains are typically highly digestible and spend less time in the rumen being processed, whereas forages take longer to undergo the fermentation process once in the rumen (14). However, roughages, or physically active fiber, should be included in cattle diets at 5-10% of total dry matter intake to encourage the rumination process and keep the rumen healthy (15, 16). Ultimately, understanding feed efficiency in context of production type should aid in the progression of economics and outputs.

Historically, there has been a heavy focus on studying steers' rumen environment and feed efficiency through stages in production (3, 4, 17). These animals are managed to promote growth and muscle development in a cost-efficient way to protect profit margins. Previous studies have utilized new technology and different management strategies including a of variety feed rations and feed trials, ionophores, and growth promoting implants to determine their impact on rumen microbiome health and resultant performance and rumen microbiome differences (18-20). However, very few studies have aimed to investigate the effect of feed efficiency status on the female's rumen microbial environment throughout her stages of production, especially during pregnancy.

A productive cow is the most important asset to a profitable cattle producer's herd (21). Pregnancy in beef cattle is of the utmost importance to producers where it is optimal to have cows that can produce efficient calves and maintain a stable, efficient rumen throughout stages in production. Although there is a higher expectation of profit and value in a market animal, without a successful pregnancy on the brood cow's part, there would be no animal to market. Ideally, a cow is a long-term constituent of a herd and is a consistent contributor of genetic attributes for the next generation of cattle, and it is important to examine the rumen environment to improve the cow-calf enterprise. The cow is undergoing demanding physiological changes and the rumen

microbiome is essential to provide energy for herself and the resultant calf. Additionally, studies (22, 23) have shown that the rumen microbiome is moderately heritable from dam to offspring, and when the goal of production is to maintain feed efficient animals, understanding the feed efficiency status and rumen environment of current producers within the herd is necessary to achieve goals. Therefore, there is a critical need to examine the physiological changes that occur in heifers during pregnancy and their impact on the rumen environment and resulting feed efficiency. These data will aid in the development of management strategies to improve the sustainability of the cow-calf enterprise.

To achieve this goal, we must first determine the changes occurring within the rumen environment during pregnancy and understand the impact it has on the host.

Feed Efficiency, Diet, and the Rumen Microbiome of Fed Cattle Systems

The selection for greater feed efficient cattle has been a production focus for the past several decades (24). Roughly 70% of costs associated with beef cattle production are in feed alone (25). With rising costs in feed commodities in current years (26), it is imperative that cattle are efficient in utilizing feed to ensure profitability and continuation of production practices.

Feed efficiency can be calculated multiple ways. Feed conversion ratios have been utilized previously to calculate feed efficiency; however, these ratios rely on weight gain and growth to determine efficiency status. A larger number would represent better efficiency and conversion rates when using a gain:feed intake ratio (24). These methods are more geared toward younger, growing animals and do not consider energy requirements for other later stages of production.

Currently, residual feed intake (RFI) is the most practical and consistent feed efficiency measure. RFI is defined as the difference between the actual dry matter intake (DMI) of an animal and the expected DMI required for maintenance and growth estimated through a regression equation involving metabolic BW and average daily gain (ADG) (24). RFI does consider the energy requirements for the current stage of production to determine nutrient requirements. An animal with a low-RFI requires less feed than expected to meet production threshold standards resulting

in a lower input cost. An animal with a high-RFI consumes more feed than expected to meet production thresholds resulting in a higher input cost or loss in profit from poor performance (27).

Feed efficiency research has recently had an increased focused on the rumen microbiome due to its fermentative capacity and impact on the nutritional status of the animal (28, 29) Several studies have noted differences in rumen microbiome in animals differing in feed efficiency status across multiple species of ruminant animal (30-32). In steers, differences in the relative abundances of higher orders of taxa, low-abundance bacteria, alpha diversity, and beta diversity of microbiomes have been identified in greater feed efficient animals Zhang (33) demonstrated low-RFI steers tended to have more eukaryotic richness in the rumen when compared to high-RFI steers. Additionally, in the same study, a PCoA failed to show significant beta diversity differences in the rumen microbial communities of the two groups of steers (33). Finally, Zhang (33) also concluded the relative abundance of two eukaryotic taxa (kingdom *Fungi* and genus *Entodinium)* were effected by steers divergent in feed efficiency status. These studies are great assets in identifying critical components of feed efficiency when applied in feed lot production.

Production traits, such as feed efficiency, fertility, and ribeye area, have been found to be variable in heredity, and are often referred to as expected progeny differences (EPDs) (34). Specifically, it has been found that feed efficiency is a moderately heritable trait (0.2-0.4) (35, 36). To add to that point, Sasson (37) was able to find that there is a group of rumen microbes that are relatively heritable. Additionally, there were some production traits that were able to be independently associated with rumen microbes as well, such as DMI, energy-harvesting efficiency, and milk protein. In sheep, Ellison (38) identified a group of microbes, including *Methanobrevibacter smithii* and *Mitsuokella jalaludini*, that differed in abundance in high and low feed efficiency groups that were also heritable. In dairy cattle, Shabat (39) identified microbes that differed in high and low feed efficiency groups, as well as differences in abundance and richness within those phenotypic groups.

Microbiome-focused feed efficiency studies have most commonly taken place in a dry lot setting where diets are controlled, and intake can be monitored individually and accurately (4). Dry lot

research directly reflects the management and feeding strategy utilized by cattle feeding operations. Cattle can be pushed to maximize performance in terms of growth, red muscle tissue development, and adipose fat tissue deposition. To achieve these outcomes, diets consist of greater quantities of a high fiber and protein diet as a cattle grower ration, largely consisting of a forage base, to promote frame growth and muscle development in younger cattle (40). As calves age and gain weight, cattle are transitioned to a high energy finishing diet to maximize yield and quality grades (41), and minimize energy lost processing high fiber diets. Research has demonstrated the benefits of this diet transition process from an economic, health, and performance standpoint (42-44). This management is unique to cattle feeding operations aimed typically at fed-cattle development.

Diets vary among producers, geographical locations, and varying production operations (45). Diet is also one of the greater factors impacting the rumen microbiome (46). This is influenced by a variety of factors. Rumen incubation period of different forages, as described by Elliot and others (47), impact the temporal stability of the rumen, as well as the timeline of rumen colonization events. Fernando (48) demonstrated when examining eight ruminally cannulated beef steers, a significant shift of rumen microbial population in favor of amylolytic and other starch digesting bacteria, during a diet transition stage from forage to high grain diet and suggests this is likely in response to the increase in fermentable substrates in a high concentrate diet. At the phylum level, an increase in Bacteroidetes populations and a decrease in Firmicutes and Proteobacteria populations are a common effect of transitioning cattle from forage-based diets to concentrate rich diets that fed cattle typically undergo when entering the feedlot (49, 50). In some cases, the rapid transition of cattle diets induces subacute ruminal acidosis (SARA) where the rumen pH decreases and affects the capability of the rumen to function normally by damaging the lining of the gastrointestinal tract, limiting the absorption of volatile fatty acids, formation of liver abscesses, and ultimately inhibiting optimal performance (51). When more readily fermentable carbohydrates, as found in concentrate diets, are increased, the rumen is likely to enter an acidotic state which hinders bacterial fibrolytic activity and is the ideal environment for amylolytic and lactic acid utilizing bacteria (52-55);. Ogunade (56) demonstrated several commensal bacterial species, such as *Fibrobacter succinogenes* and two *Ruminococcus* species, were reduced with the

onset of SARA in feed lot steers indicating a reduction of diversity with the onset of acidotic changes. Microbiome and rumen chemistry changes due to SARA can also impact ruminal tissue, further exacerbating the deleterious effects of a rapid dietary transition. For example, when cattle were transitioned over 10 months from forage diets (90.6% dry matter, 114 g crude protein/kg dry matter, 600 g neutral detergent fiber/kg dry matter, 173g nonfiber carbohydrate/kg dry matter, 69 g starch/kg dry matter; 1.91 mCal/kg dry matter) to grain diets (35% chopped hay and 65% mixed grain; 88.9% dry matter, 117 g crude protein/kg dry matter, 307 g neutral detergent fiber/kg dry matter, 504 g nonfiber carbohydrate/kg dry matter, 409 g starch/kg dry matter; 2.60 mCal/kg dry matter), the rumen epithelium was compromised due to the reduction of cell adhesions in stratified squamous epithelial layers and sloughing of the stratum corneum (57). These findings are useful for explaining dietary impacts on the rumen microbiome commonly associated with the fed cattle system. However, the cow-calf enterprise does not share all commonalities with cattle feeding operations in diet or other management practices, but this research is beneficial in demonstrating the differences in the two, as well as the management of the products, calves, of productive cow-calf operations.

Pregnancy Effects on Gut Microbes

As previously stated, the majority of beef cattle nutritional, feed efficiency, and rumen microbiome research focuses on steers (3, 4). Few studies have focused on the rumen microbiome of breeding female cattle that consume roughly 70% of feed supply across sectors of the beef industry (58). The few studies that utilize female cattle tend to occur within dairy breeds of cattle (59, 60). In beef production, cow-calf operations are more commonly based around pasture and forage-based nutritional programs (61, 62). This sector of the beef industry focuses on meeting the nutritional needs of the animal to enhance reproductive performance, and in turn, profitability (63).

Successful reproductive performance in the cow-calf sector results in a health pregnancy and live, marketable calf. Bovine prenatal development was first outlined by Winters et al. (64), and Swett et al. (65), and have more recently studied by Lyne (66), Ferrell et al. (67), and Prior and Laster (68), with a particular point of interest being the physiological changes throughout pregnancy, especially from day 220 of pregnancy to the end of term. This period is critical as the fetus

undergoes the largest growth transformation and final organ development (69). With the increase in fetal growth and development, the female's nutritional requirements are expected to also increase. On average, it has been reported that pregnant cows should receive 20% or more dietary energy and protein to aid in fetal growth, maintaining proper body condition going into calving, and preparing for lactation, an energy demanding stage of production (70). Ensuring maternal nutritional requirements are met, will offset potential negative effects, such as malformation and other nutritional deficiencies in times of nutrient restriction (71-73). Weller (71) demonstrated when examining 62 multiparous crossbred cows detrimental maternal effects from overnutrition on offspring fertility such as a reduction of the number of follicles in female fetuses and the disruption of testicular development which reduces fertility in bull calves. Wu (73) demonstrated restriction of feed in early pregnancy inhibits muscle and adipose tissue performance in resultant calves. Gionbelli (72) further acknowledged advantages of meeting maternal nutritional needs for skeletal muscle tissue, adipose fat, and connective tissue development during the fetal stage when examining zebu and crossbred cattle.

The gut microbiome is expected to acclimate in response to dietary changes imposed by the change in nutrient demands and dietary nutrient profile during pregnancy (74). Differences in rumen microbiome during the prepartum and post-partum interval in dairy cattle have been identified, specifically as an indicator of production traits such as milk production (75). Lima (75) demonstrated differences in the relative abundance of amylolytic and cellulolytic bacteria as well as a shift of fungi and protozoal abundances between the prepartum and postpartum period in dairy cattle, which were highly influential in milk production outputs. Beyond the limited work in dairy cattle, the effect of pregnancy on the gut microbiome of women and other vertebrate species has been reported (76-78). Koren (76) discovered that the gut microbiota of 256 pregnant women during the first trimester of pregnancy was similar to that of a healthy, non-lactating, unpregnant woman; however there was a shift of phylogenetic composition and microbial function similar to disease-associated dysbiosis over the course of pregnancy. Despite this change, the motive behind the mechanism failed to be identified. In the same study, it was found that by the third trimester, there was a noticeable increase in Proteobacteria and Actinobacteria. Women in this study often had symptoms of gut inflammation, which was further supported by Mukhopadhya (79) who noted

similar abundances tied to gut inflammation, when populations exceeded a defined threshold of commensal proteobacterial colonization. To date, the human gut microbiome composition and functional responsiveness to the physiological changes occurring throughout pregnancy has been described, however there remains a gap in connecting established and expected changes in the rumen microbiome in beef cattle during pregnancy.

Pregnancy Effects on Rumen Microbiome and Metabolic Status in Cattle

Metabolic health and feed conversion remains a consistent goal especially with regard to reproduction in the cow-calf sector. Studies from nearly the last fifty years have proven the relevance of hormones and metabolic products in cattle productivity and performance (80-90). In terms of reproduction, metabolic products are a driving factor stimulating ovulation, embryonic development, ovarian structures, steroid production, and estrus length in beef cattle (91).

A cow's demand for glucose and other nutrients is expected to increase to support fetal growth and lactation after calving (92). Unfortunately, there is often a decrease in appetite and feed intake later in pregnancy, potentially in relation to fetal size or estrogen secretion (93). Cattle become more reliant upon the rumen microbes to effectively metabolize feed stuffs into usable nutrients to support maternal and reproductive function (94). Beever (95) explained the production practice of increasing dietary protein and energy in late gestation to counteract the expected decrease in intake is beneficial up to calving. After calving, the negative effects of the altered diet including inhibited fertility and decreased production outputs are present.

Additional research highly supports the importance of meeting nutritional requirements during gestation. When carbohydrate intake is not sufficient to meet the demands of production, metabolic diseases in response to nutrient deficiencies are detrimental to the animals continued production (96). During times of nutrient restriction, cows will undergo metabolic changes where blood glucose and insulin secretion decreases, and as a result, adipose tissues are mobilized for increased ketogenesis and the production of ketone bodies occur, and in extreme cases referred to as ketosis (97, 98). When cows are not receiving nutrition sufficient to sustain a new pregnancy, it has been demonstrated internal signaling involving the somatotrophic axis (GH, IGF1, insulin, IGFBP2) and

leptin reduces fertility when the cow's metabolic state is not sufficient to maintain pregnancy (99). Regardless, metabolic outputs are significant for any stage of production, but especially for successful reproductive activity and healthy pregnancy.

The onset of pregnancy instigates a variety of hormonal fluctuations. The exact effect all of pregnancy related hormones on the beef cattle microbiome composition remain to be clarified. Mulak and others (100) suggested that pregnancy hormones, such as progesterone and estrogen, impact bacterial metabolism, growth, and virulence of pathogens in the gastrointestinal tract. Another study demonstrated that the host's sex hormone levels and diet impact the gut microbiome in 341 female and 348 male mice (101). Additionally, host's gut microbiota could assist in regulating and modulating steroid sex hormone levels (102).

Several studies to date have aimed to examine the association of hormone levels and host microbiomes in both the both rumen and reproductive tract. Henniger and others (18) utilized 50 crossbred steers on either a moderate or aggressive growth hormone implant strategy containing estradiol and trenbolone acetate. They failed to find many significant differences in the rumen bacterial composition of steers differing in hormonal supplementation (18). The authors reasoned this to be in response to the lack of sex hormone receptors in the rumen epithelium. However, when looking at the effects of protein supplementation on rumen and uterine microbiomes in 60 commercial Angus heifers in both pre and post pubertal stages, and researchers again failed to find significant variation between the two maturity groups (103). This further supports the lack of relationship between sex hormone fluctuations and microbiome response. Unlike the rumen, the uterus does have sex hormone receptors and therefore is more sensitive to hormonal fluctuations (104) however the microbiome variation in the 60 Angus heifers was minimal. Additionally, another study indicated eight of the most abundant OTUs present in the vaginal microbiome were also found in greater abundance in several segments of the ruminant gastrointestinal tract or fecal matter (105). Ruminococcaceae, a highly abundant bacterial family in the rumen, has also been found to be the most abundant in the female reproductive tract, namely the vagina (106). It is thought that the presence of microbes in both physiological tracts is part a result of the location of the vagina and its allowance for microbes to enter from the digestive tract and other parts of the

body (105). Laguardia-Nascimento and others (105), concluded that there was no evidence of hormonal differences, associated with pregnant or non-pregnant cattle, throughout puberty or pregnancy influencing the microbiome of the vaginal tract. However, it was noted that more research should be performed to clarify and validate their findings on other physiological changes and processes. Undoubtedly, pregnancy induces a variety of physiological changes, and the host response, with an emphasis on nutrition, metabolism, and reproduction, in cattle has been described. However, the lack of understanding of the rumen environment response to pregnancy garners attention from research moving forward in search of determining the differences in beef cattle.

Next Generation and Future Work

With the growing demand for sustainable practices in agriculture, feed efficient cattle hold more emphasis. The rumen microbiome composition of a cow is critical for host health (107), productivity (9), and feed efficiency (3, 4). Identifying the rumen microbiome and feed efficiency status of a cow through stages of production enhances a producer's opportunity to produce the next generation of feed efficient cattle. Research exploring the relationship of the rumen microbiome changes during pregnancy with a focus of feed efficiency status will provide novel insight to improve the economic and environmental sustainability of beef cattle production.

CHAPTER TWO

PREGNANCY INFLUENCES ON THE RUMEN ENVIRONMENT OF ANGUS HEIFERS DIFFERING IN FEED EFFICIENCY

Introduction

Improving the sustainability across the US beef industry is important due the expected increased demand for high-quality protein sources. The US beef industry must continue to produce environmentally sound, socially responsible, and economically viable beef (108). Roughly 70% of beef cattle production costs are associated with feed and forage production and procurement (25). To improve upon the profit margins and sustainability of beef production, the efficient use of a variety of inputs are necessary for effective production, including costs, arable land, and natural resources (108). With rising input costs and decreases in arable land to produce feed and forages, the importance of sustainable practices in agriculture to produce feed efficient cattle are paramount.

Feed efficiency in ruminant animals considers their unique ability to convert feed stuffs and what would be considered as a low-quality nutrient source for humans, such as forages, and its conversion into a high-quality end product, such as meat or milk. Ultimately, the rumen microbiome is responsible for the fermentation, conversion and processing of feedstuffs into usable compounds for the ruminant to absorb and utilize for energy (109, 110). Research regarding the rumen microbiome to date has had a heavy emphasis on demonstrating the relationship between feed efficiency and the rumen microbiome to describe further sources of variation in feed efficiency (3, 4, 111). Specific ruminal microorganisms and metabolites have been associated with the feed efficiency status in beef cattle. Myer and others (4) found *Succiniclasticum, Lactobacillus, Ruminococcus*, many *Prevotella*, and the family Veillonellaceae with different abundances in steers divergent in feed efficiency. Clemmons and others (111) demonstrated pantothenate and abundances of *Flavobacteriia* were significantly different in steers with differing feed efficiency status. To date, research has been thorough in exploring and explaining the relationship of the rumen environment in steers and fed cattle, but fewer have examined these relationships on the productive brood cow.

To be considered productive, a cow must consistently raise a calf. Reproductive failures, such as failure to conceive or gestational losses, account for $471 million annually (112). The emphasis of maintaining health during pregnancy has the utmost importance from a profitability standpoint.

The physiological changes that occur during pregnancy (64-68), and ultimately the suggested changes nutritional requirements and intake (92) could influence the gut microbiome leading to alterations in the metabolic outputs, but the relationship between pregnancy and the rumen environment remain unclear.

The focus on the rumen microbiome of fed cattle has likely been driven from an economic standpoint, as the majority of revenue in the US beef industry derives from the sale of beef as a consumable commodity (113). Very few studies investigate the cow-calf sector of the beef industry. The impact of pregnancy on rumen health, especially in relation to the feed efficiency status of the female has yet to be clarified. Understanding of the cow's feed efficiency status and rumen microbiome will enhance the selection process and ability to produce the next generation of highly productive beef cattle required to meet the increasing demand for a quality protein source such as red meat. Therefore, the hypothesis of this study was that feed efficiency status and pregnancy in Angus heifers will impact rumen environment features. To determine the rumen environment changes during pregnancy and their correlation with previously established feed efficiency status, the objectives of this study were to determine key changes in the rumen microbiome and environment during pregnancy in Angus heifers and determine the correlation of key ruminal environment changes to their previously established feed efficiency status.

Materials And Methods

This study was approved and carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville protocol number 2639-0818.

Animals, Management, and Sample Collection

For this study, 17 previously cannulated registered Angus heifers of approximately 2 years of age and 678 ± 54 kg were placed on a 70-day feed efficiency trial at the Plateau Research and Education Center, (Crossville, TN). The trial utilized a GrowSafe System© (GrowSafe Systems Ltd., Airdrie, Alberta, Canada) to determine individual feed intake and resultant feed efficiency status based on residual feed intake. As described by Clemmons et al, 2022 (114), the diet

composed of corn silage and a custom blend mixed based on guidelines from the National Research Council's recomendations for cattle growth. Body weights were recorded on days -1, 0, 35, 70, and 71 throughout the feed efficiency trial. Day 0 denoted the first day of the feed efficiency trial and was used to calculate average daily gain (ADG). Average daily feed intake (ADFI) based on dry matter intake and ADG were used to determine RFI and feed efficiency status (24, 114). At the completion of the study, heifers were transported to the East Tennessee Research and Education Center (Louisville, TN) and an industry standard 7-day co-synch + CIDR and timed artificial inseminations protocol (115) was followed to prepare for breeding. All heifers were artificially inseminated and turned out with two fertile Angus cleanup bulls. Bred heifers were maintained on mixed cool season pasture and with ad libitum access to a loose mineral supplement (#678, Co-op Supreme Cattle Mineral, Protrition Feeds, LaVergne, TN) and supplemented with mixed grass hay when forage was limited. Prior to breeding (Week -1) and on the week of timed AI (Week 0), rumen content was removed by a rumen grab method from the ventral sac of the rumen via cannula access. The rumen grab collection was lightly squeezed through four layers of cheese cloth to collect 15mL of rumen fluid to identify the rumen fermentative and metabolomic profile. With the remainder of the sample, 40mL of rumen content was placed into a separate conical tube for microbial DNA extraction and identification. Both samples were immediately flash frozen in liquid nitrogen and stored at -80°C for further analysis. Beginning with Week 4 after pregnancy confirmation, the same samples were collected from all bred heifers every other week.

Non-pregnant cows were removed from the study after pregnancy confirmation via blood test on day 30 after timed AI or upon bull removal. 13 heifers carried full term and 1 heifer experienced a midterm abortion and was removed from the trial at that point in time. Final samples for this project were obtained prior to calving, up to Week 38 in some animals. Samples were not collected from animals that failed to meet health and wellness criteria on sampling days for reasons that include off behaviors, illness, and labor.

DNA Extraction, Library Preparation, and Sequencing of Rumen Content

Rumen samples were processed at the University of Tennessee, Knoxville for DNA extraction and purification. Microbial DNA was extracted and purified by the rumen digesta extraction protocol detailed by Yu and Morrison (116). Briefly, approximately 0.2g of rumen content was placed into a 2ml beaded screw cap tube with 0.5mm ZR bead bashing matrix (Zymo Research, Irvine, CA, USA), 1ml of lysis buffer (500 mM NaCl, 50mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate [SDS]) and homogenized for 3 minutes at 21 Hz. Samples were incubated at 70°C for 15 minutes with inversions of sample tubes at 5 minutes intervals. Samples were then centrifuged at 16,000 x g for 5 minutes and supernatants were transferred into separate 2ml Eppendorf tubes. Lysis buffer (300 μL) was added into the beaded screw cap tube, and the steps were repeated. After centrifugation, individual samples were pooled, and lysis tubes were discarded. A total of 260 μL of 10-M ammonium acetate was added to each individual sample for nucleic acid precipitation. Samples were mixed and put on ice for 5 minutes. Tubes were then centrifuged at 4°C for 10 minutes at 16,000 x g. The supernatant was divided evenly into two fresh tubes with an equal volume of isopropanol. Samples were put on ice for 30 minutes before being centrifuged 4°C for 15 minutes at 16,000 x g resulting in a nucleic acid pellet formation. Excess supernatant was discarded, and the pellet was washed with 70% ethanol and dried for 5 minutes. The pellet was then dissolved in 100 μL of Tris-EDTA (TE) buffer and aliquots were pooled. After completion of nucleic acid precipitation, QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA) was utilized for purification. Proteinase K (15 μL) and 200 μL of Buffer AL were added to eliminate protein contamination. Samples were then incubated at 70°C for 10 minutes. Ethanol (200 μL of 100%) was added and mixed before being transferred into a QIAamp column (QIAGEN, Valencia, CA, USA). Columns were then centrifuged for 1 minute at 4°C at 16,000 x g. Flow through was discarded, and the process was repeated twice more, with the addition of 500 μL of Buffer AW1 the first repetition, and 500 μL of Buffer AW2 the second. After discarding final flow through, columns were centrifuged for 1 minute at 16,000 x g to dry the column. Columns were then placed into fresh 1.5mL tubes and 70 μL of Buffer AE was added to the column and incubated at room temperature for 2 minutes. Following the 2 minutes, 30 μL of Buffer AE was added and incubated at room temperature for 2 minutes. Finally, samples were centrifuged at

4°C for 1 minute at 16,000 x g and DNA was retained in 1.5mL tubes. Quality of extracted DNA was checked via gel electrophoresis. Samples were diluted to 10 ng/ul in 1.5mL cryovials. Samples were stored at -20°C until amplification and library prep.

Samples were thawed and diluted to 10 ng/ul in 1.5mL cryovials. Amplicon library preparation for the 16S rRNA region was performed at the University of Tennessee Genomics Core Laboratory, following their standard operating procedures of a two-step polymerase chain reaction (PCR). The V4 region of the 16S rRNA gene was amplified from extracted DNA, using primers 515Fb (GTGYCAGCMGCCGCGGTAA) (117) and 806Rb (GGACTACNVGGGTWTCTAAT) (118), modified with adapters for Illumina MiSeq sequencing. The first PCR used the V4 amplicon primers (2 mM), Kapa HiFi master mix (Roche, Indianapolis, IN, United States), and 2.5 uL of DNA. The reaction consisted of 3 min at 95°C, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR product was purified with 20 uL Agencourt Ampure XP beads (Beckman Coulter) and eluted in 50 uL of 10 mM Tris, then each sample was indexed with a unique combination of forward and reverse Nextera XT v2 indexes (Illumina) using Kapa HiFi master mix, and 8 cycles of the above PCR. The indexed PCR product was again purified with 56 uL of Agencourt Ampure XP beads and eluted in 25 uL of Tris. Final libraries were visualized and assessed for quality control and quantified with a combination of nanodrop and Agilent Bioanalzyer. The libraries were sequenced at a final loading concentration of 4 pM using 250 paired-end reads on an Illumina MiSeq instrument with version 2 reagents and 20% PhiX spike-in.

Metabolite Extraction and Analysis of Rumen Content

After thawing from storage, 2mL of rumen fluid were placed into two 2mL microcentrifuge tubes and both were centrifuged at 6,000 x g for 15 minutes at 4°C. Following centrifugation, the supernatant was aspirated from both tubes and pooled before being filtered through a 0.22 μM syringe filter (MidSci, St. Louis, MO, USA) and placed into a 2mL cryovial. All samples were stored at -20°C until metabolite analysis could be conducted.

Samples were processed by the Biological and Small Molecule Mass Spectrometry Core (BSMMSC), University of Tennessee, Knoxville, TN (RRID: SCR_021368). Water soluble metabolites were extracted from 100 µL of rumen content using 4:4:2 acetonitrile/methanol/water with 0.1 M formic acid as previously described by Domingo-Almenara and others (119). Following extraction, the supernatant was dried under N2 then resuspended in 300 µL water. All solvents used were HPLC grade and purchased from Fisher Scientific (Hampton, NH, USA).

To identify metabolites, ultra-high performance liquid chromatography high resolution mass spectrometry (UHPLC-HRMS) (Thermo Scientific, San Jose, CA, USA) was utilized using an untargeted metabolomics method decribed by Lu and others (120). Metabolites were separated based on polarity by reverse-phase (RP), ion-paring chromatography. Chromatographic separation utilized a Synergi Hydro RP column (100 mm x 2.1 mm, 2.6 µm, 100 Å) and an UltiMate 3000 pump (Thermo Fisher Scientific, Waltham, MA). Finally, mass analysis was performed by using Exactive Plus Orbitrap MS (Thermo Fisher Scientific, Waltham, MA) in conjunction with the UHPLC system.

16S rRNA Gene Sequence Processing and Analysis

An R pipeline as described by (121) was used to process the Illumina 2x250 fastqc sequencing files. The R package 'fastqcr' v0.1.2 (122) was used to assess quality. Samples with less than 10,000 reads were removed from the data (n=7).

Open-source R packages 'phyloseq' v1.40.0 (123) and 'DADA2' (124) were used to filter, merge, and perform taxonomic assignment for the Fast Illumina files. The parameters for *filterandTrim* were set with a truncLen of 240 for forward and reverse reads.

Forward and reverse reads without either a quality score of 25 or greater, more than 2 expected errors in a forward read, or more than 2 expected errors in a reverse read were removed (125). The *learnErrors* function in the 'DADA2' package v.1.24.0 (124) was utilized to identify error rates. Amplicon sequence variants (ASVs) were computated by the *dada* function.

‘DADA2’ was then used to correct for amplicon errors, and forward and reverse reads were merged using the *mergePairs* function with a 12 base pair overlap requirement. Following, all sequences were put into a sequence table, and sequence lengths were checked. Chimeras and Eukaryota were removed from data set by the removeBimeraDenova method.

Taxonomy to the genus level was assigned using the SILVA v138.1 database (126) using *assignTaxonomy*. All known and assigned data was inputted into a phyloseq object for further analysis.

Alpha diversity was measured using the *estimate_richness* function from the ‘phyloseq’ package in R to calculate the observed ASVs, Chao1 metric, and Shannon Diversity Index. Beta diversity was measured by using a Bray-Curtis Dissimilarity Matrix to identify distances for a principal coordinate analysis (PCoA) in the ‘vegan’ package in R v2.6.2 (127).

Statistical Analyses

Downstream analysis for bacterial sequences, alpha- and beta- diversity were performed in R v2.6.2. A histogram was created to visually assess the normality, quality, and distribution of scores.

Alpha Diversity

Visual assessment of alpha diversity measurements of Observed ASVs, Chao1, and the Shannon Diversity Index were performed before using a Shapiro-Wilks test (W) to test for normality at a $W>0.85$ and $P<0.05$. To identify differences in alpha diversity measurements, a linear mixed effects model was used. The resultant alpha diversity index, being observed, Chao1, or Shannon, were tested as a response of time point, being week of measurement, and blocked by individual animal to account for repeated measurements. Following, an ANOVA was used to test for differences across means in alpha diversity measurements. Pairwise comparisons between weeks were calculated using linear hypothesis testing and Tukey's test. Alpha was set $P<0.05$ for all analyses.

Beta Diversity

Samples were then transformed from total counts and a Bray-Curtis Dissimilarity Matrix was used to determine distance between samples for beta diversity measurements. Dispersion estimates were determined, and a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was performed using the *adonis* function in the 'vegan' package in R (127) with significance of $P<0.05$ for all analyses. To test pairwise comparisons in the Bray-Curtis Dissimilarity Matrix between time points, the *pairwise.adonis* function was used (128).

Differential Abundance

The 'ANCOMBC' package v2.0.2 (129) was used to globally test for differential abundance of bacterial communities from each sample across different time points. The phyloseq object was utilized in the *ancombc2* function. The fixed effect was time point in pregnancy and the random effect was individual animal to adjust for repeated measures. Taxa with less than 10% abundance were removed from the data set. For this data set, there were no assumed structural zeroes. The adjusted p-value (q-value) was set to $q<0.05$ for all analyses. Following, pairwise comparisons were conducted and Benjamini-Hochberg (130) was used to adjust for multiple testing. $P<0.05$ for all analyses.

Visualizations for ASV and metabolite abundances by week based on continuous residual feed efficiency were generated in JMP v15.2.0 (SAS Institute Inc., Cary, NC, 1989–2023).

Metabolites

Metabolomic Analysis and Visualization Engine (MAVEN) was used to visualize raw UHPLC-HRMS data and identify rumen metabolites. Metabolites were identified based on the exact mass and retention time in accordance with an in-house database (127). Variable importance of projection (VIP) scores were determined in Metaboanalyst v5.0 via partial least squares discriminant analysis.

Correlation

Analysis to determine the relationship between ASV abundance, metabolite prevalence, and residual feed efficiency status was performed in Metaboanalyst v5.0. The pattern search function using the Pearson R method was used to identify correlation patterns $P<0.05$ and FDR<0.05.

Results

Animal Data

At the completion of a 70-day feed efficiency trial, 13 cows received residual feed intake values based on the metrics calculated from the GrowSafe 6000 system (GrowSafe Systems Ltd., Airdrie, Alberta, Canada). These values fell on a continuous scale from -3.3 to 3.5. A standard deviation of ±0.5was used to determine RFI classification, where three animals ranked in the High-RFI category, eight animals ranked in the Mid-RFI category, and two animals ranked in the Low-RFI category (Table 1). However, for this analysis, the animals were not binned into respective groups, but analyzed on a continuous scale (114).

Sequencing Results

An average of 80,353 read pairs per sample were produced from the libraries sequenced on the Illumina MiSeq. The minimum number of reads was 23,809 and the maximum was 164,552. A total of 32 unique phyla and 372 unique genera were identified from 207 samples. Overall, 88,410 taxa were identified.

Alpha Diversity of Bacterial Communities

Alpha diversity analyses examining the observed ASVs (richness), chao1 (anticipated richness), and Shannon diversity index (richness and evenness) among all weeks are displayed (Figures 1-3, respectively). The linear hypothesis of the pairwise comparisons of observed ASVs, Chao1, and Shannon Diversity Indices indicated numerous significant differences among weeks ($P<0.05$; Tables 2-4, respectively). The number of pairwise differences among weeks in all alpha diversity metrics increased throughout pregnancy weeks.

Table 1. Feed Efficiency Classification using Residual Feed Intake (RFI) of 13 Angus Heifers

COW ID	RESIDUAL FEED INTAKE[2]	CLASSIFICATION[1]
3876	-3.3	LOW
3796	-1.5	LOW
3886	-1.1	MID
1027	-1	MID
3546	-0.9	MID
3816	-0.7	MID
3326	0.1	MID
1537	0.2	MID
1107	1.1	MID
3316	1.1	MID
3456	1.7	HIGH
3446	3.1	HIGH
3336	3.5	HIGH

[1]RFI Classification determined by 0.5 Standard deviation for previous trial
[2]RFI viewed on continuous scale for this trial

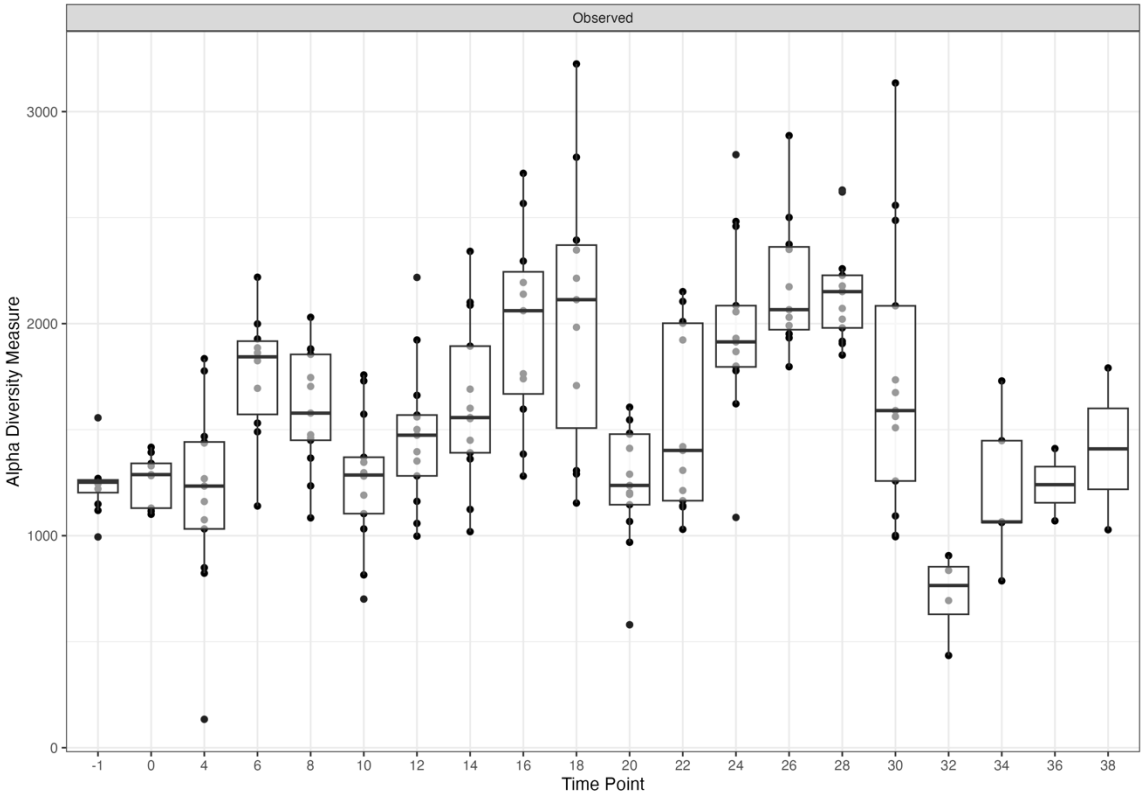

Figure 1. Observed Amplicon Sequence Variants (ASV) Alpha Diversity by Week

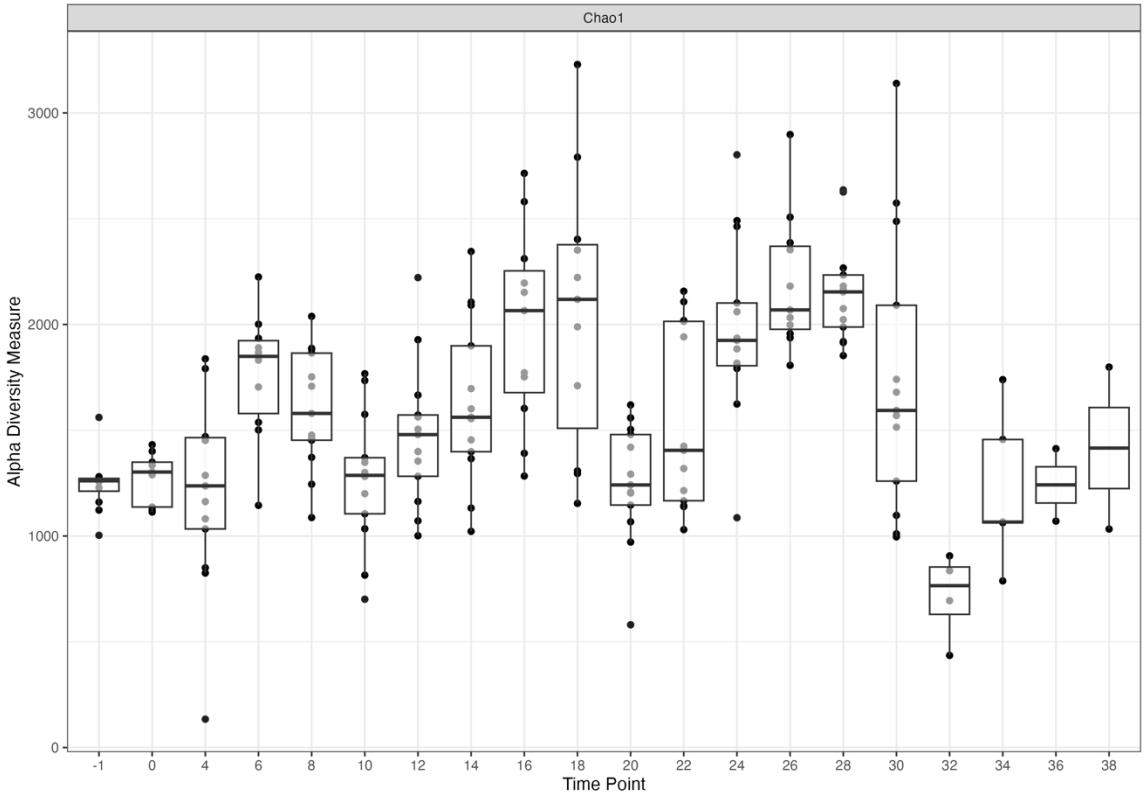

Figure 2. Chao1 Alpha Diversity by Week

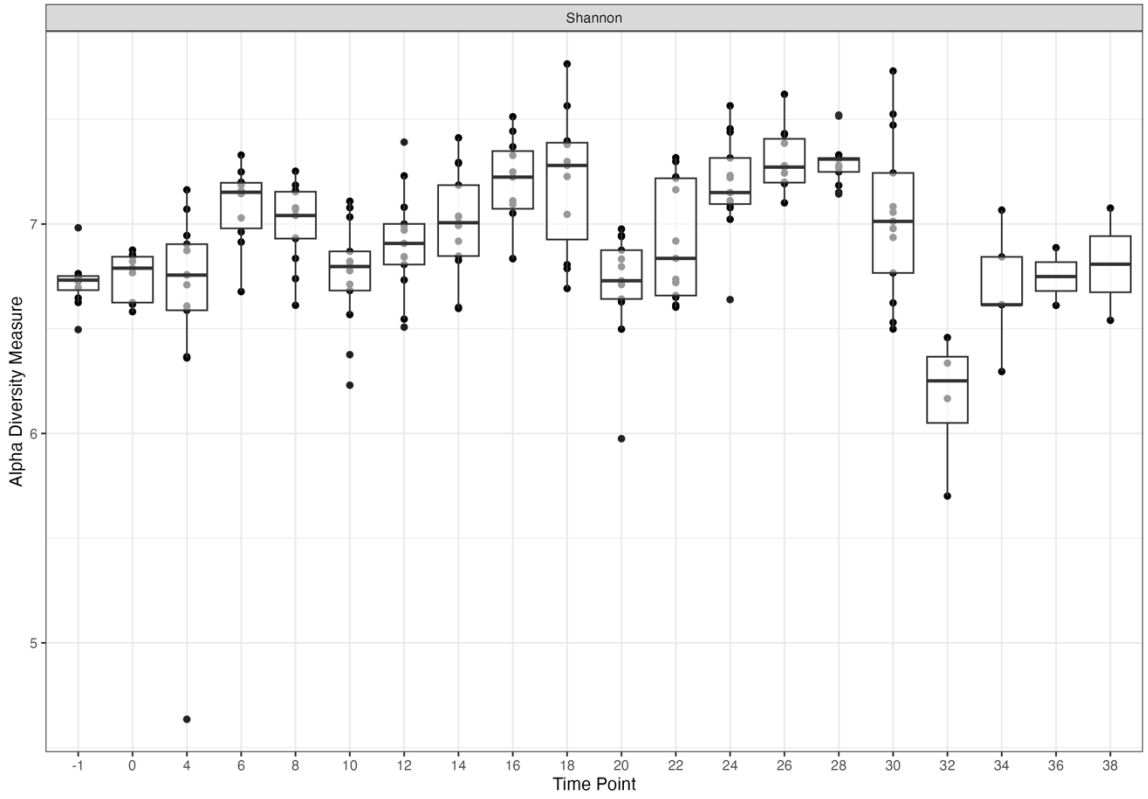

Figure 3. Shannon Diversity Alpha Diversity by Week

Table 2. Significant Pairwise Comparisons of Observed Amplicon Sequence Variants (ASV) by Week[1, 2]

WEEK	WEEK
-1	16, 18, 24, 26, 28
0	16, 18, 24, 26, 28
4	6, 16, 18, 24, 26, 28, 30
6	4, 32
8	26, 28, 32
10	16, 18, 24, 26, 28
12	18, 26, 28, 32
14	26, 28, 32
16	-1, 0, 4, 10, 30, 32, 34
18	-1, 0, 4, 10, 12, 20, 32, 34
20	24, 26, 28
22	26, 28, 32
24	-1, 0, 4, 10, 20, 32, 34
26	-1, 0, 4, 8, 10, 12, 14, 20, 22, 32, 34
28	-1, 0, 4, 8, 10, 12, 14, 20, 22, 32, 34
30	4, 16, 32
32	6, 8, 12, 16, 18, 22, 24, 26, 28, 30
34	16, 18, 24, 26, 28
36	-
38	-

[1] Linear hypothesis testing

[2] For all pairwise comparisons significance set to $P<0.05$

Table 3. Significant Pairwise Comparisons of Chao1 by Week[1, 2]

WEEK	WEEK
-1	16, 18, 24, 26, 28
0	16, 18, 24, 26, 28
4	6, 16, 18, 24, 28, 30
6	4, 32
8	26, 28, 32
10	16, 18, 24, 26, 28
12	18, 24, 26, 28, 32
14	28, 32
16	-1, 0, 4, 10, 20, 32
18	-1, 0, 4, 10, 12, 20, 32, 34
20	16, 18, 24, 26, 28
22	26, 28, 32
24	-1, 0, 4, 10, 12, 20, 32, 34
26	-1, 0, 8, 10, 12, 20, 22, 32, 34
28	-1, 0, 4, 8, 12, 14, 20, 22, 32, 34
30	4, 32
32	6, 8, 12, 14, 16, 18, 22, 24, 26, 28, 30
34	18, 24, 26, 28
36	-
38	-

[1] Linear hypothesis testing

[2] For all pairwise comparisons significance set to $P<0.05$

Table 4. Significant Pairwise Comparisons of Shannon Diversity by Week[1, 2]

WEEK	WEEK
-1	16, 18, 24, 26, 28, 32
0	16, 18, 24, 26, 28, 32
4	6, 8, 14, 16, 18, 24, 26, 28, 30
6	32
8	32
10	16, 18, 24, 26, 28, 32
12	26, 28, 32
14	4, 32
16	-1, 0, 4, 10, 20, 32
18	-1, 0, 4, 32, 34
20	24, 26, 28, 32
22	26, 28, 32
24	-1, 0, 4, 10, 20, 32, 34
26	-1, 0, 4, 10, 12, 20, 22, 32, 34
28	-1, 0, 4, 10, 12, 20, 22, 32, 34
30	4, 32
32	-1, 0, 6, 8, 10, 12, 16, 18, 20, 22, 24, 26, 28, 30
34	18, 26, 26
36	-
38	-

[1] Linear hypothesis testing

[2] For all pairwise comparisons significance set to $P<0.05$

Beta Diversity of Bacterial Communities

When analyzing beta diversity using Bray-Curtis-based principal coordinates analysis, there was a significant difference among week after PERMANOVA analysis with 999 permutations ($P < 0.05$; Figure 4). The circles represent a 95% confidence interval around the means of week. Pairwise comparisons of Bray-Curtis Dissimilarity Matrices indicated numerous significant differences among weeks (Table 5).

Abundance of Bacterial Communities

Based on the global test in the ANCOM-BC analysis, 10 ASVs were confirmed to be differentially abundant across time points (Table 6; $q<0.05$).). At the genus level either, seven ASVs were taxonomically classified to *Prevotella* and three to *Succiniclasticum.* Differential abundance pairwise comparisons indicated 25 significant comparisons (Table 7).

Metabolite Analysis Results

After determining abundances of metabolites, a one-way ANOVA indicated 90 metabolites with significant difference across time points (FDR<0.05; Figure 5). For the sake of this study, the 10 metabolites with the highest variable importance in projection (VIP) scores were utilized in final analysis (Figure 6).

Correlation

The 10 significant ASVs identified in the ANCOMBC global test and top 10 metabolites identified by VIP scores in Metbaolanalyst v5.0 were used for correlation analyses. JMP v15.2.0 (SAS Institute Inc., Cary, NC, 1989–2023) was used to create images for visual assessment of trends of ASV and metabolite abundances by RFI status (Figures 7-26). The pattern search function in Metaboanalyst v5.0 identified correlation values across multiple weeks. Significant correlations occurred in Weeks 4, 12, 16, 18, 20, 22, 24, 26, and 30. ($P<0.05$; Table 8; Figures 27-35). When adjusting for FDR, significant correlations existed for Week 26 (p-adjusted<0.05; Table 8; Figures 34).

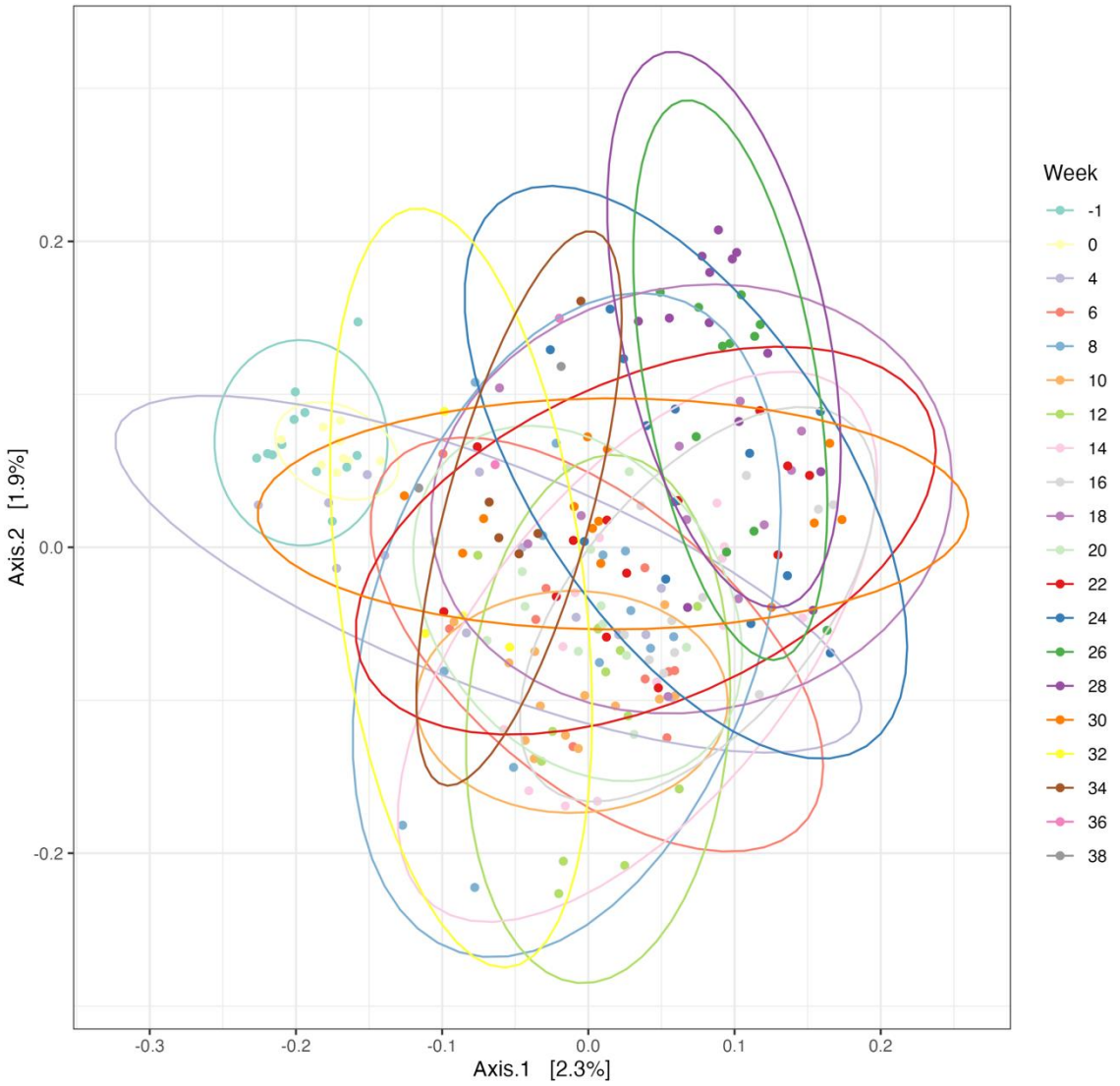

Figure 4. Bray-Curtis Dissimilarity Matrix PCoA with 95% confidence intervals. Individual animals are represented by dots. Weeks are represented by colors.

Table 5. Significant Beta Diversity Pairwise Comparisons based on Bray-Curtis Dissimilarity Matrix[1,2,3]

WEEK	**WEEK**
-1	0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38
0	-1, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38
4	-1, 4, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32
6	-1, 0, 10, 12, 16, 18, 20, 22, 24, 26, 28, 30, 34, 36
8	-1, 0, 4, 10, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36
10	-1, 0, 4, 6, 8, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38
12	-1, 0, 4, 6, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38
14	-1, 0, 4, 10, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38
16	-1, 0, 4, 6, 8, 10, 12, 18, 20, 24, 26, 28, 30, 32, 36, 38
18	-1. 0, 4, 6, 8, 10, 12, 14, 16, 20, 22, 24, 26, 28, 30, 32, 34, 36
20	-1, 0, 4, 6, 8, 10, 12, 14, 16, 18, 22, 24, 26, 28, 30, 32, 36
22	-1, 0, 4, 6, 8, 10, 12, 14, 18, 20, 26, 28, 30, 32, 36
24	-1, 0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 28, 32, 36
26	-1, 0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 30, 32, 34, 36
28	-1, 0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 30, 32, 34, 36
30	-1, 0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 26, 28, 32, 36
32	-1, 0, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32
34	-1, 0, 6, 8, 10, 12, 14, 16, 18, 26, 28
36	-1, 0, 6, 8, 10, 12, 14, 18, 20, 22, 24, 26, 28, 30, 34
38	-1, 0, 10, 12, 14, 16

[1] Bray-Curtis Dissimilarity Matrix was used to determine distance between samples for beta diversity measurements
[2] Permutational multivariate analysis of variance (PERMANOVA) with 999 permutations
[3] For all pairwise comparisons significance set to $P<0.05$

Table 6. Taxonomic Identification of Globally Significant Amplicon Sequence Variants (ASV)

ASV	Phylum	Class	Order	Famliy	Genus	W
ASV9	Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	54.6
ASV15	Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	58.3
ASV35	Firmicutes	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	51.7
ASV46	Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	55.8
ASV59	Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	59.0
ASV83	Firmicutes	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	44.6
ASV106	Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	50.4
ASV196	Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	47.4
ASV245	Firmicutes	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	47.6
ASV1166	Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	51.3

[1] Analysis conducted in 'ANCOMBC' package v2.0.2
[2] Significance set to $q<0.05$
[3] W-value is the coefficient obtained from the ANCOM-BC log-linear model divided by their standard error.

Table 7. Significant Differential Abundance Pairwise Comparison of Weeks [1,2,3]

ASV	Week
ASV9	8, 10, 12, 14, 16, 20, 22, 26
ASV35	28
ASV46	10, 12
ASV59	8, 10, 12, 14, 16, 20, 22, 26, 30

[1]ANCOM-BC Test
[2]All weeks compared to Week -1
[3]For all pairwise comparisons significance set to $P<0.05$

Table 8. Significant Differential Abundance Pairwise Comparison of Weeks [1,2,3]

ASV	Week
ASV15	12

[1]ANCOM-BC Test
[2]All weeks compared to Week 0
[3]For all pairwise comparisons significance set to $P<0.05$

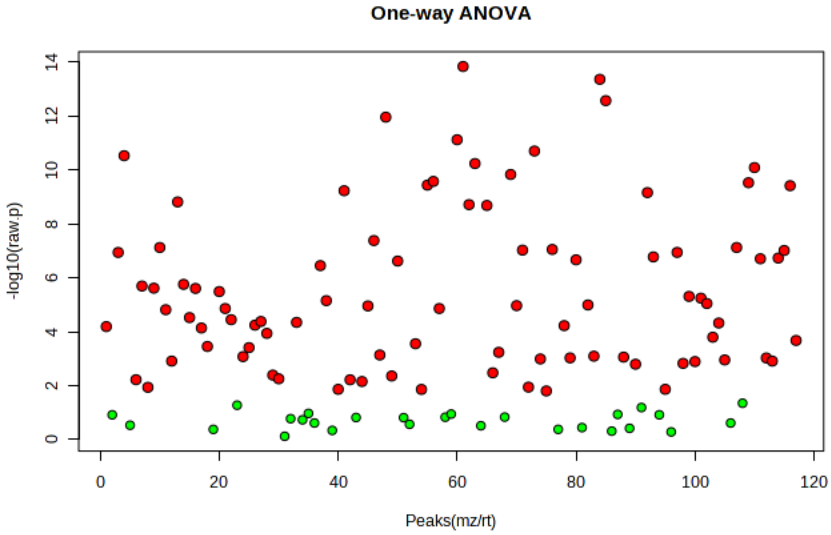

Figure 5. One-Way Analysis of Variance (ANOVA) for Rumen Metabolites. Red dots (n=90) indicate significant metabolites; green dots indicate non-significant (n=27). Significance set to $P<0.05$.

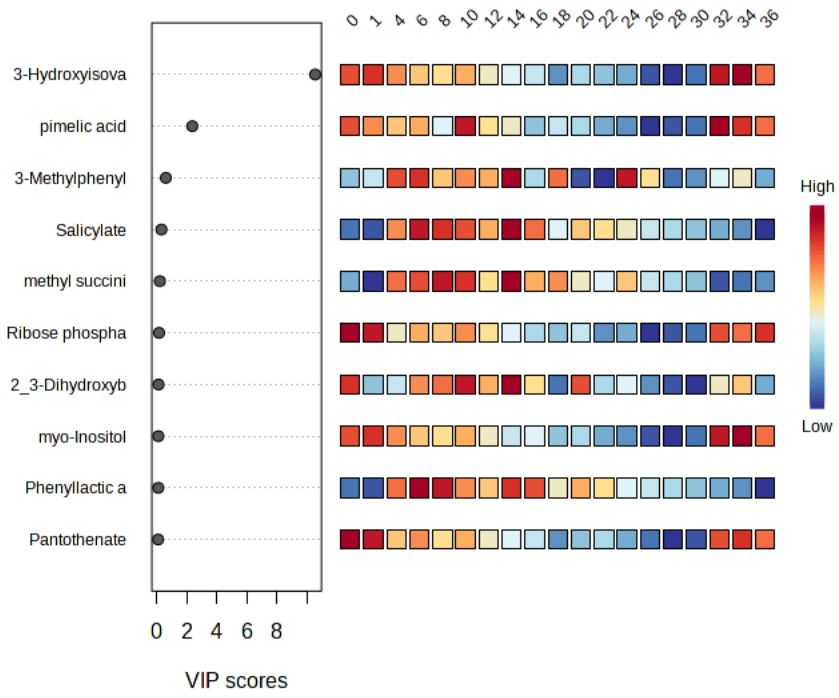

Figure 6. Variable Importance of Projection (VIP) Scores of the Top Ten Metabolites identified from Partial Least Squares Discriminant Analysis (PLS-DA).

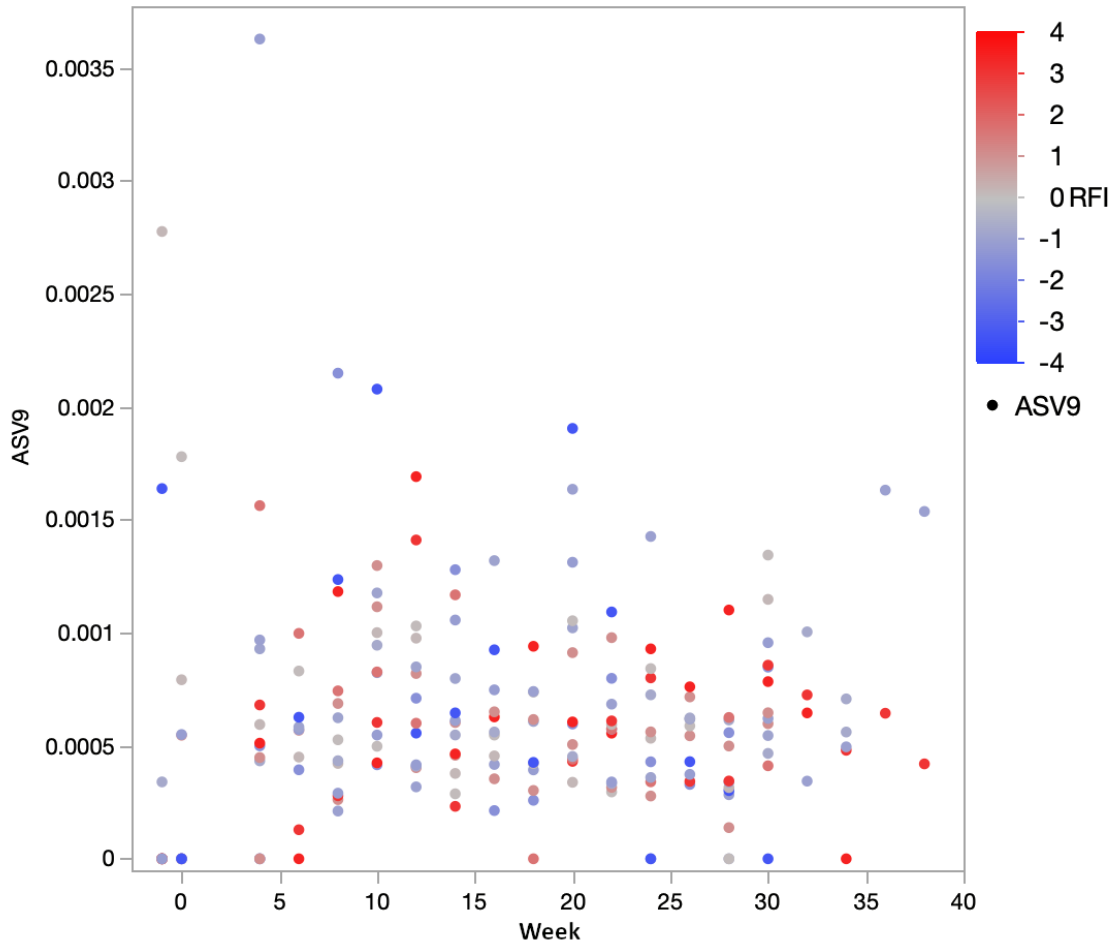

Figure 7. Residual Feed Intake (RFI) Status of ASV9 *Prevotella* Abundance by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

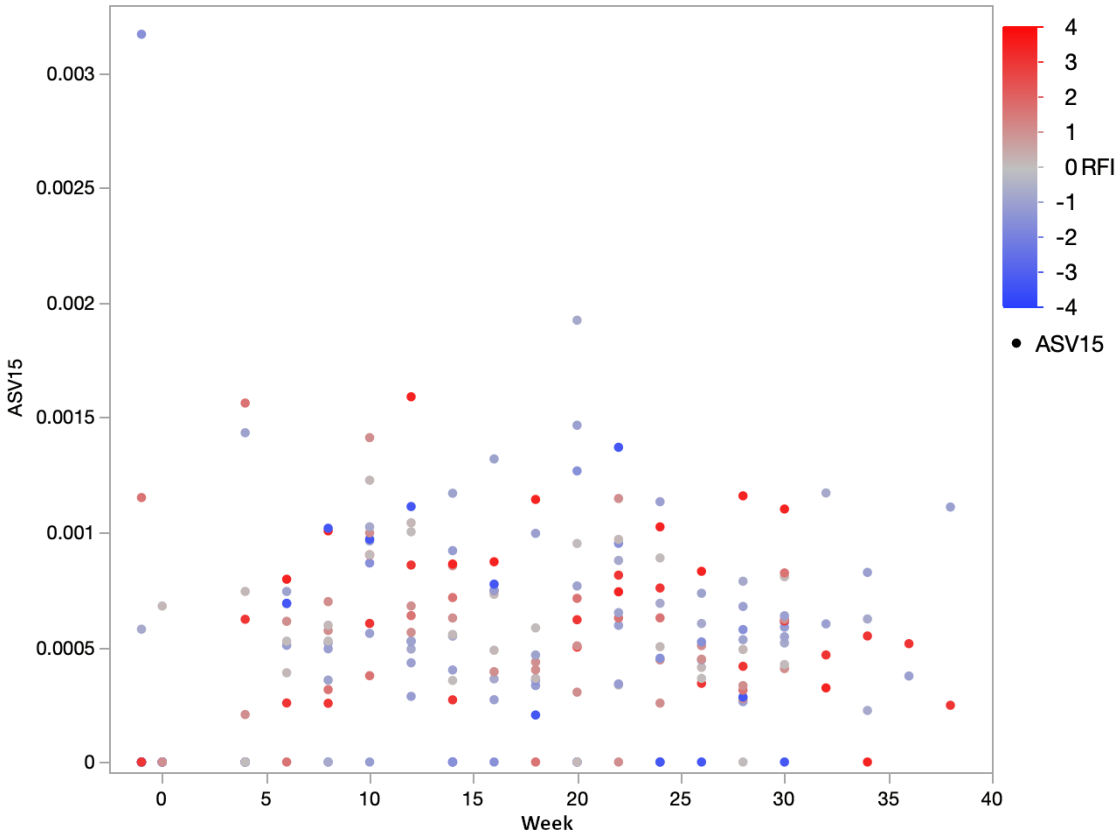

Figure 8. Residual Feed Intake (RFI) Status of ASV15 *Prevotella* Abundance by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

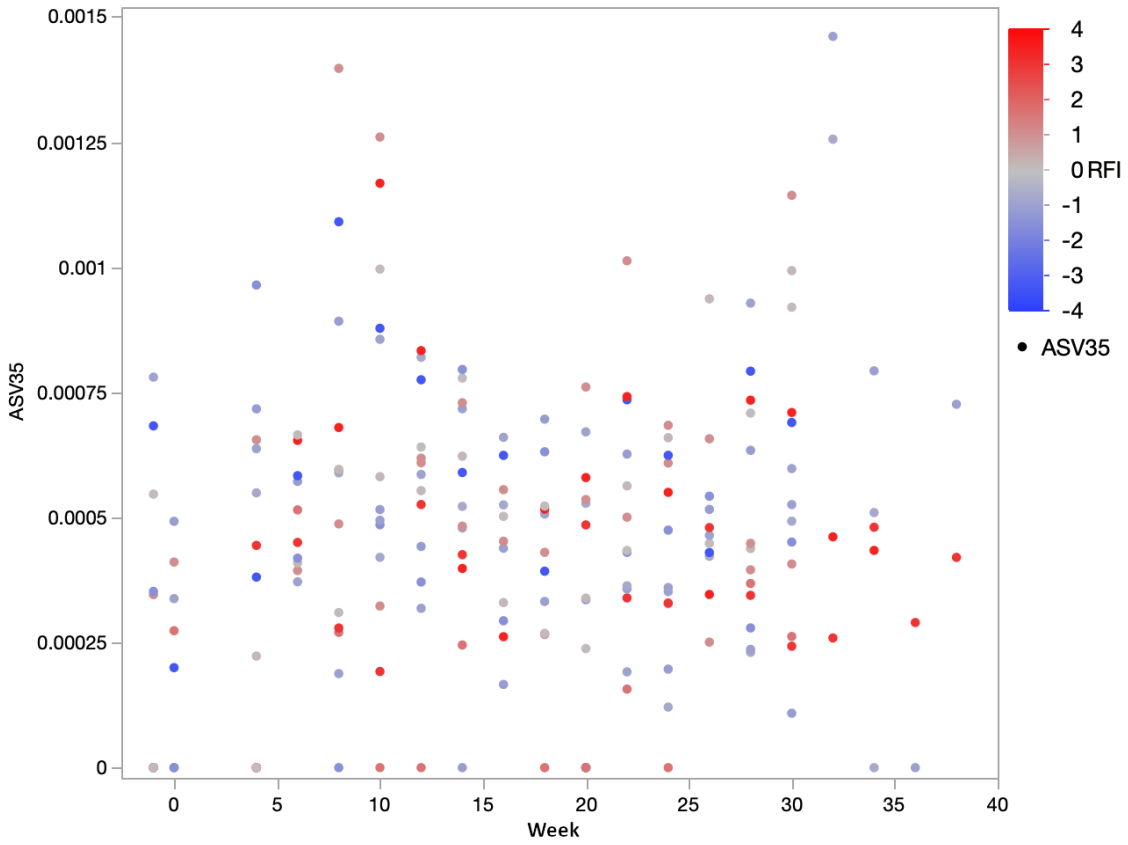

Figure 9. Residual Feed Intake (RFI) Status of ASV35 *Succiniclasticum* Abundance by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

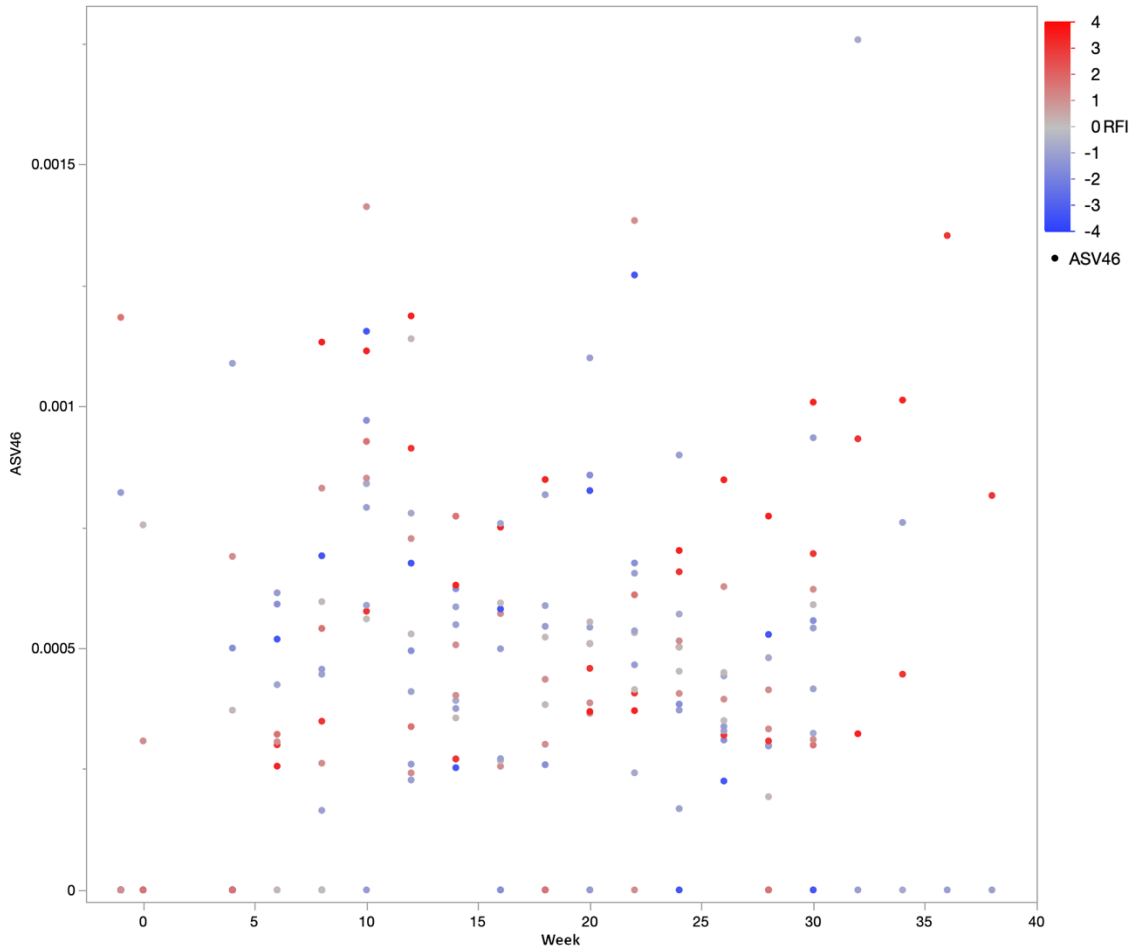

Figure 10. Residual Feed Intake (RFI) Status of ASV46 *Prevotella* Abundance by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

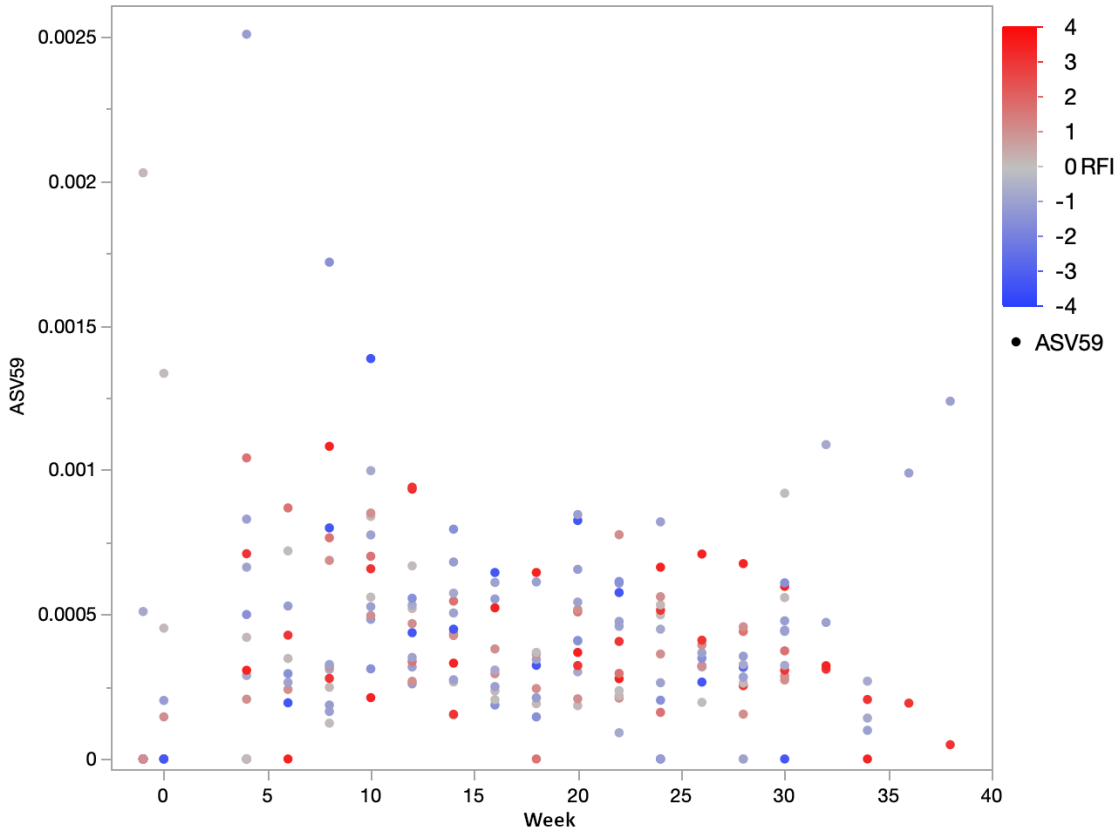

Figure 11. Residual Feed Intake (RFI) Status of ASV59 *Prevotella* Abundance by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

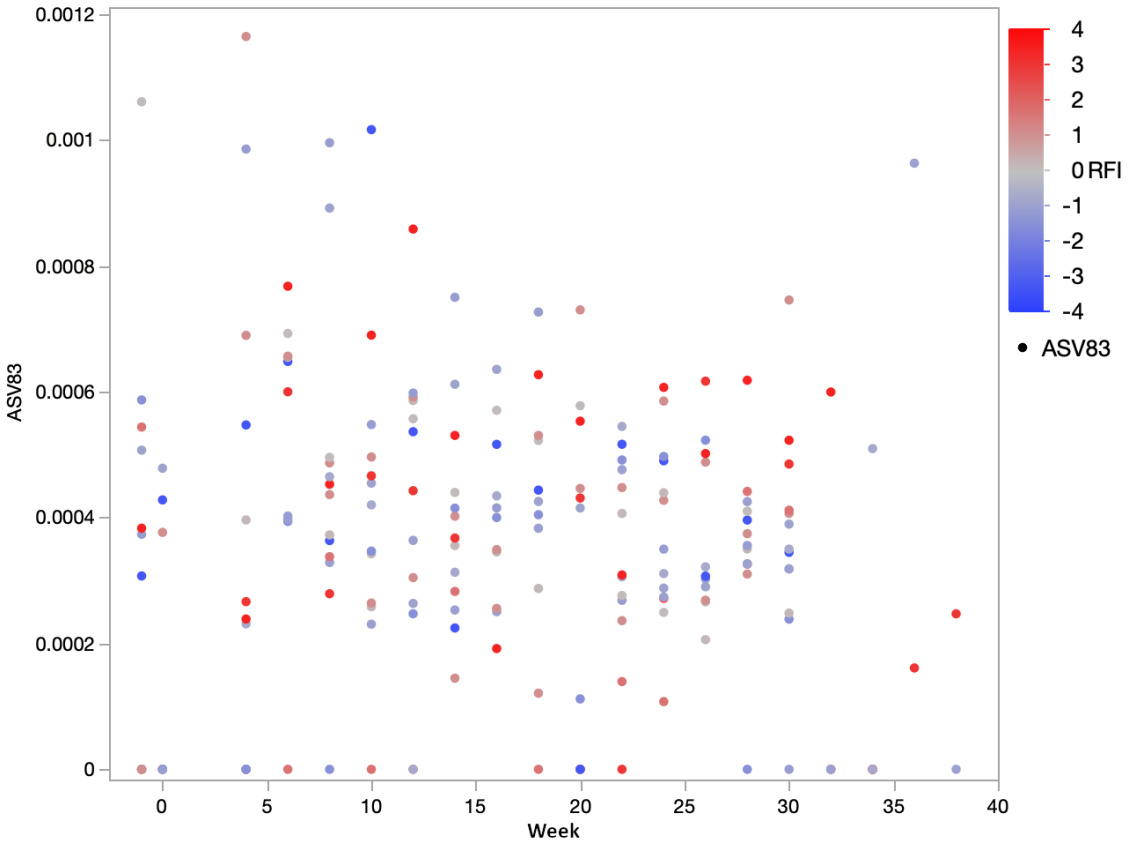

Figure 12. Residual Feed Intake (RFI) Status of ASV83 *Succiniclasticum* Abundance by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

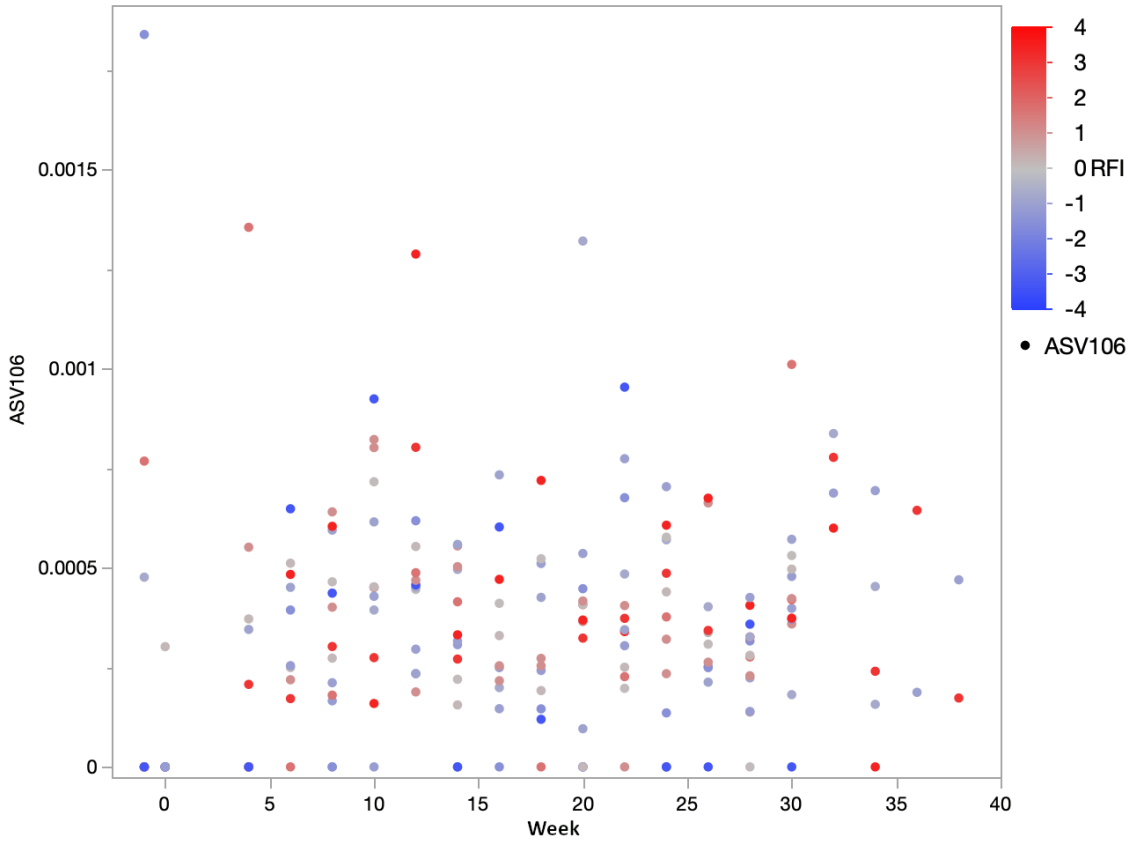

Figure 13 Residual Feed Intake (RFI) Status of ASV106 *Prevotella* Abundance by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

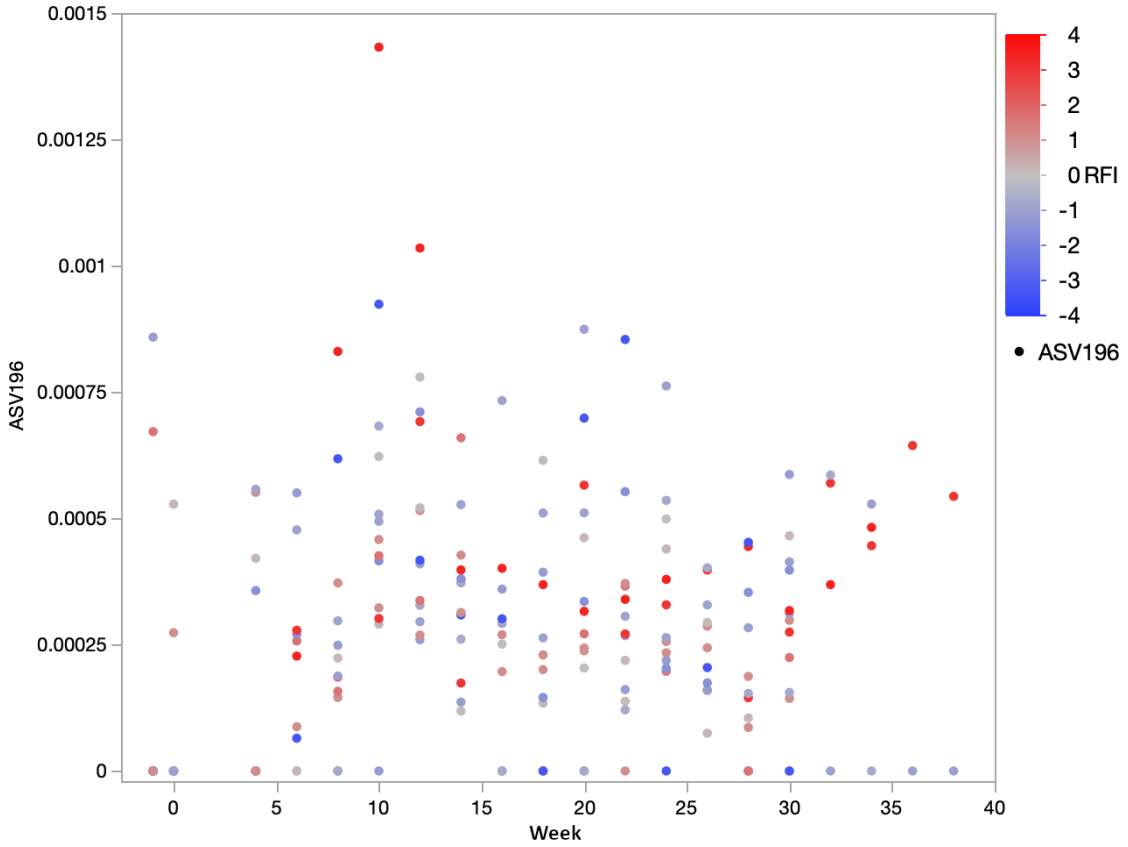

Figure 14. Residual Feed Intake (RFI) Status of ASV196 *Prevotella* Abundance by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

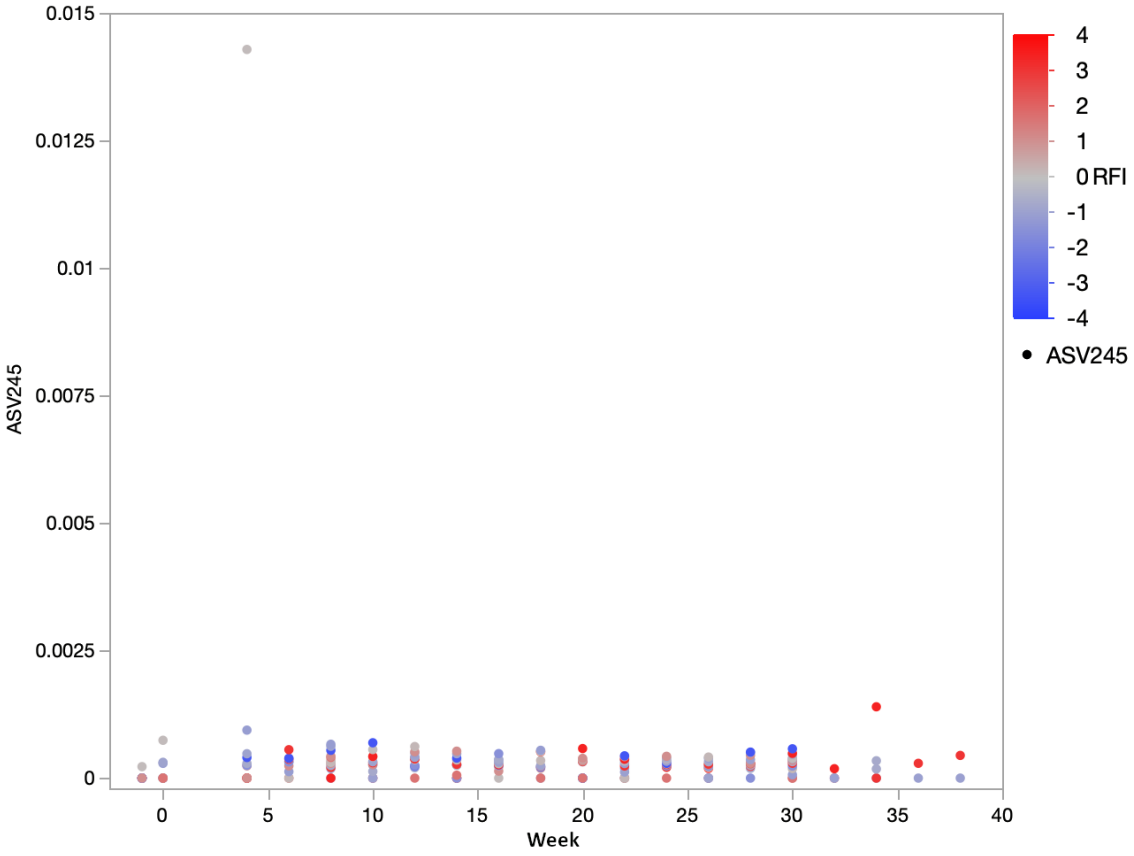

Figure 15. Residual Feed Intake (RFI) Status of ASV245 *Succiniclasticum* Abundance by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

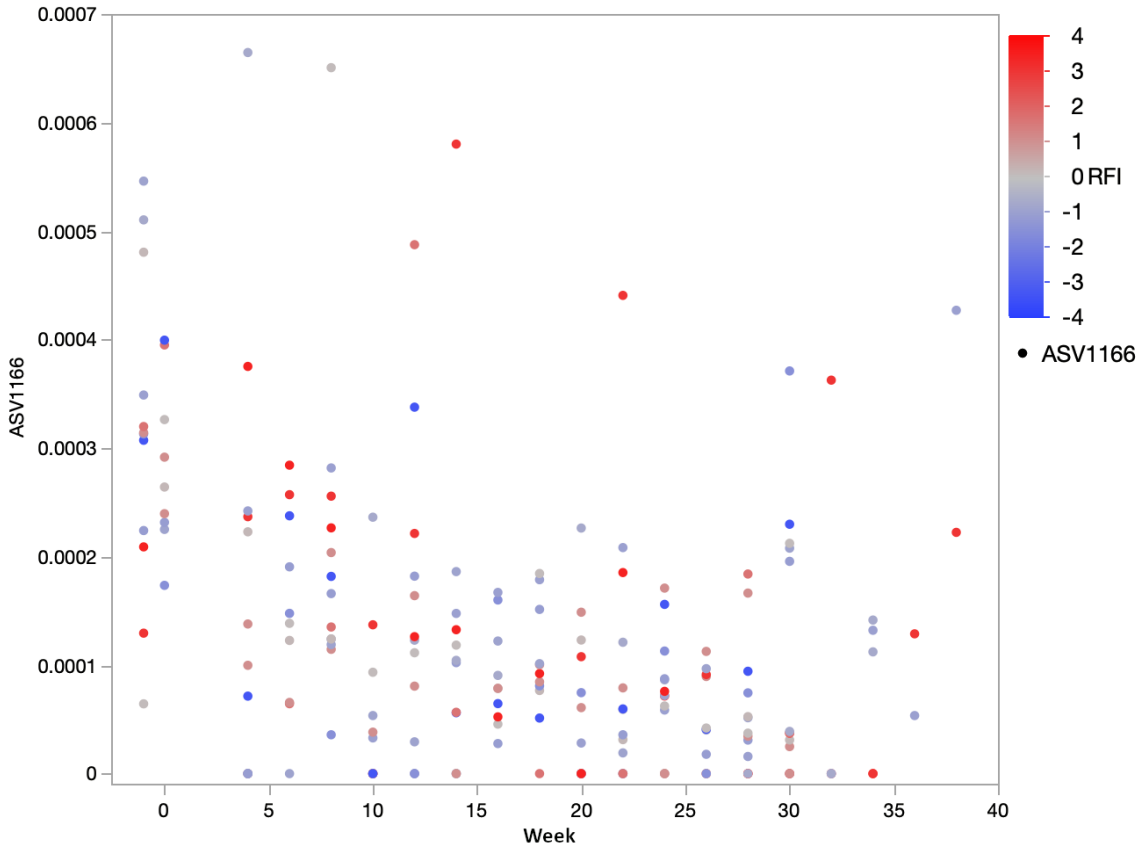

Figure 16. Residual Feed Intake (RFI) Status of ASV1166 *Prevotella* Abundance by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

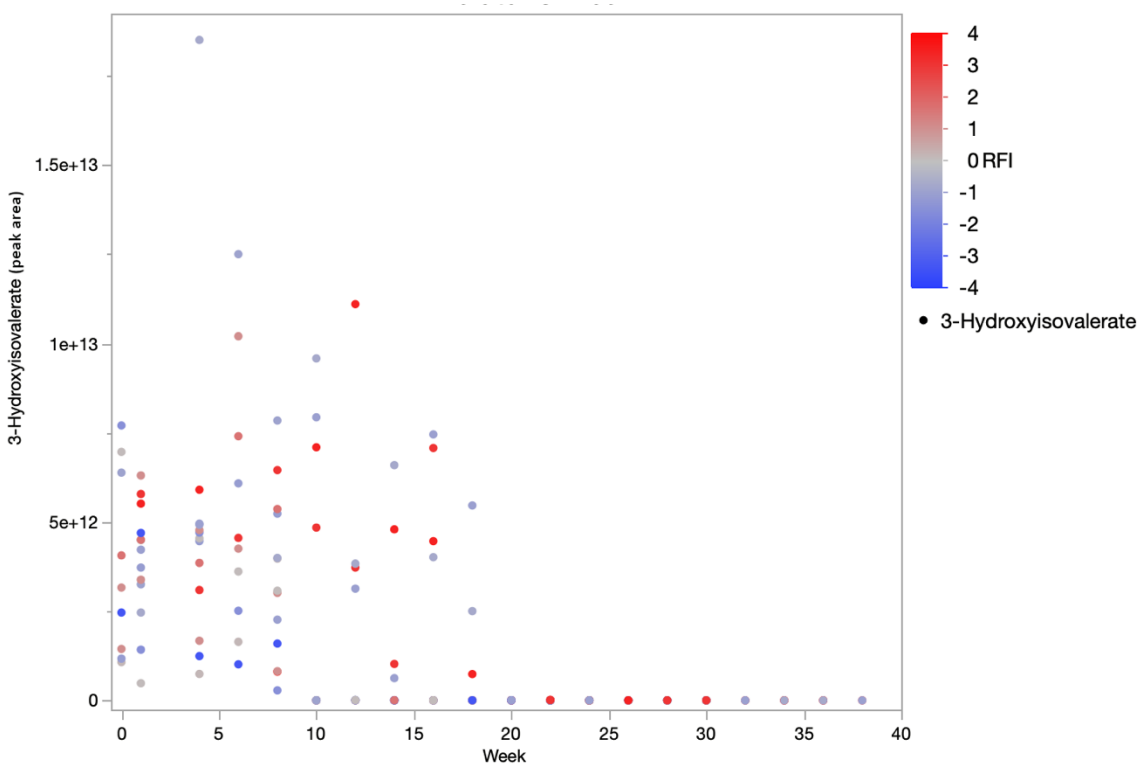

Figure 17. Residual Feed Intake (RFI) Status of 3-Hydroxyisovalerate by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

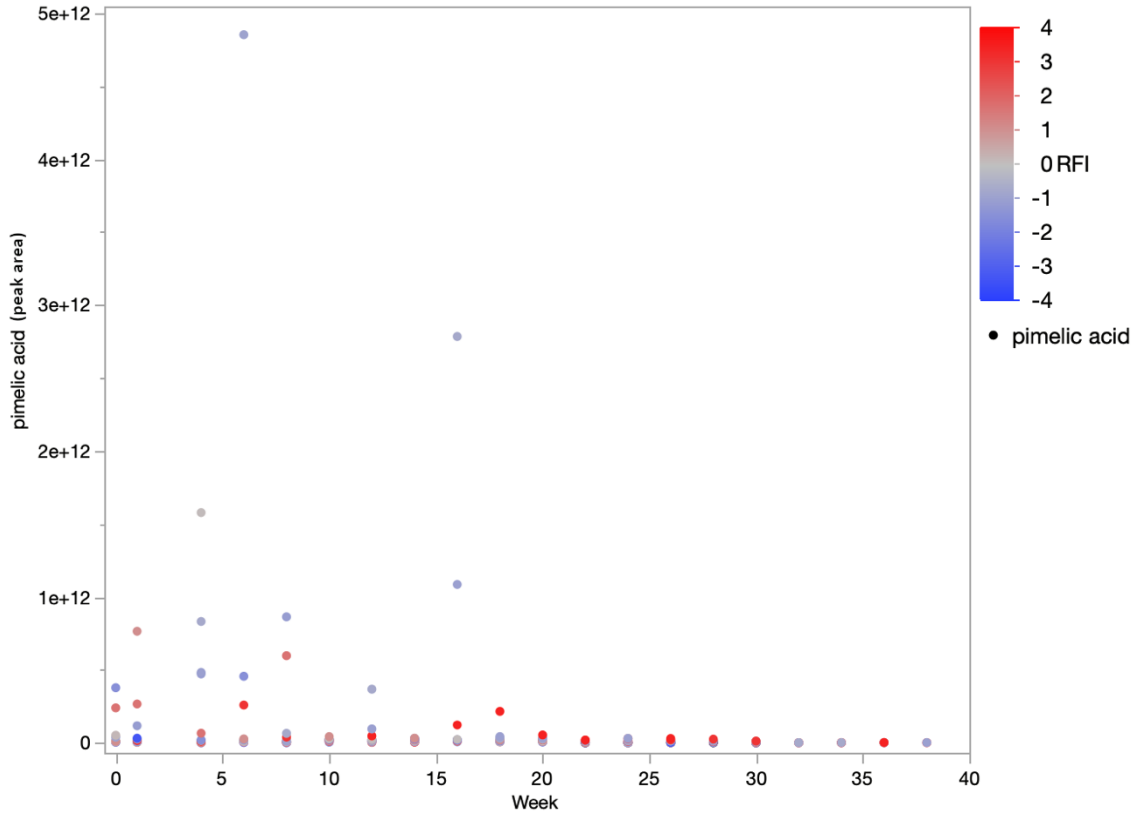

Figure 18. Residual Feed Intake (RFI) Status of Pimelic Acid by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

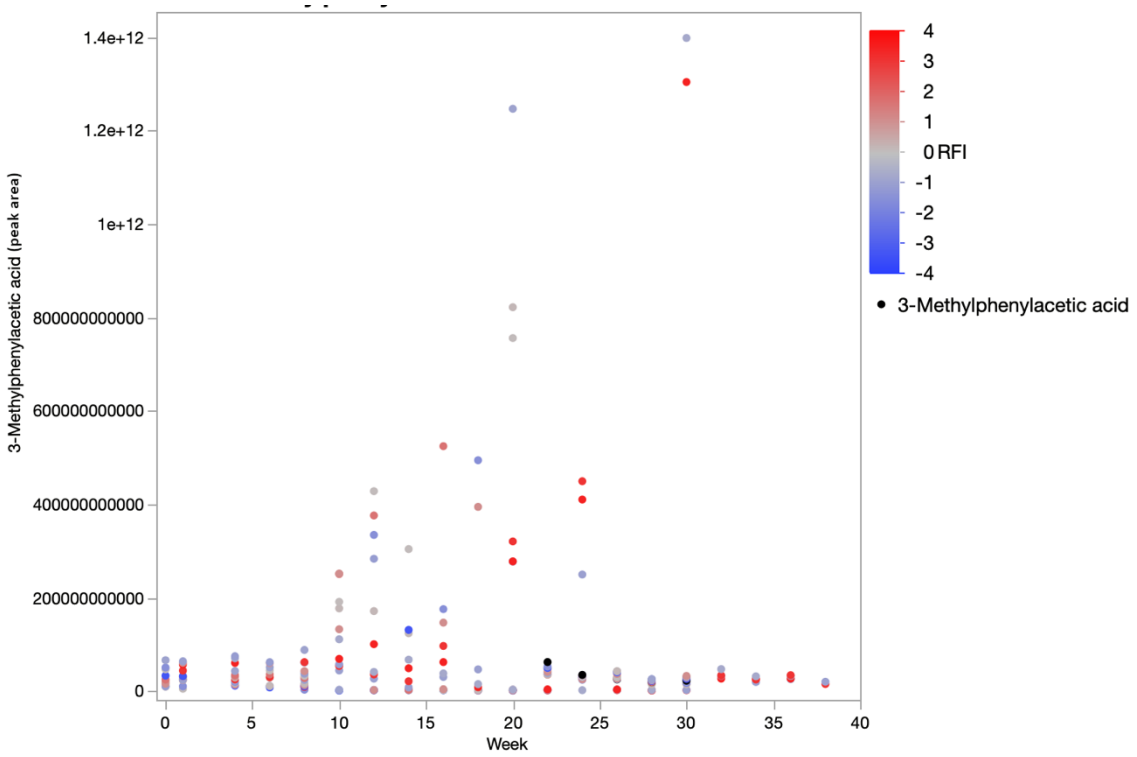

Figure 19. Residual Feed Intake (RFI) Status of 3-Methylphenylacetic Acid by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

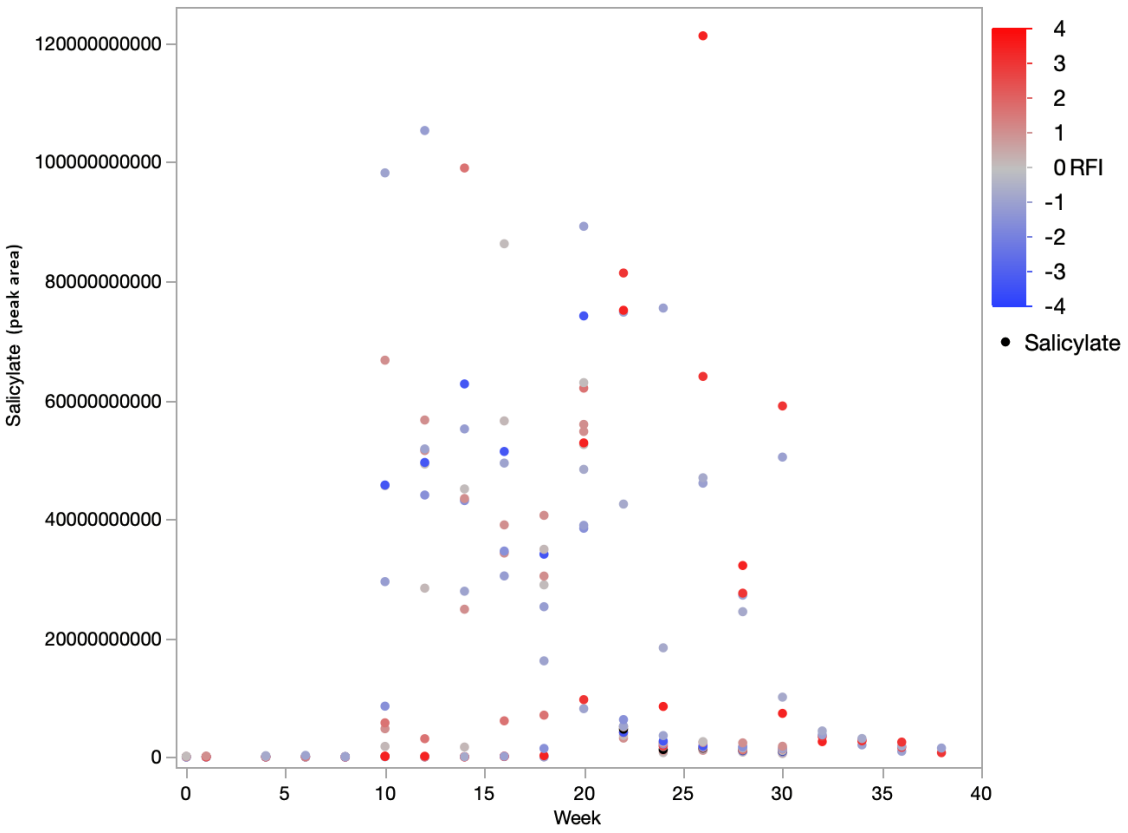

Figure 20. Residual Feed Intake (RFI) Status of Salicylate by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

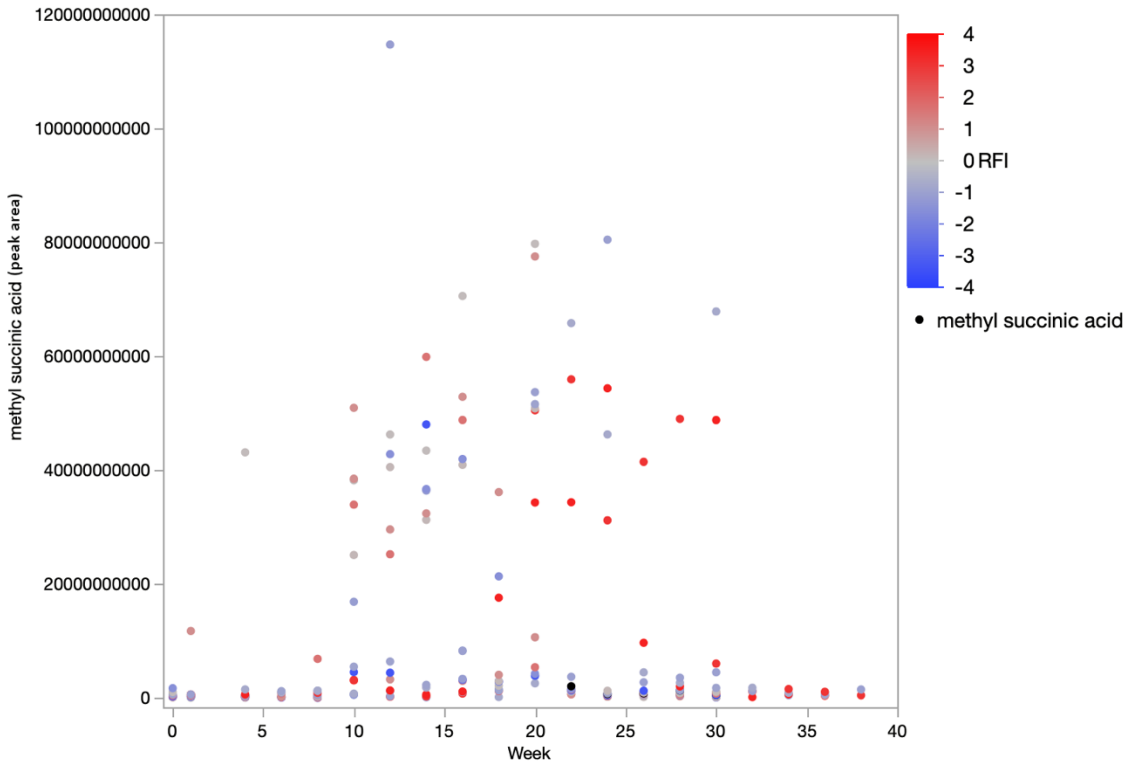

Figure 21. Residual Feed Intake (RFI) Status of Methyl Succinate by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

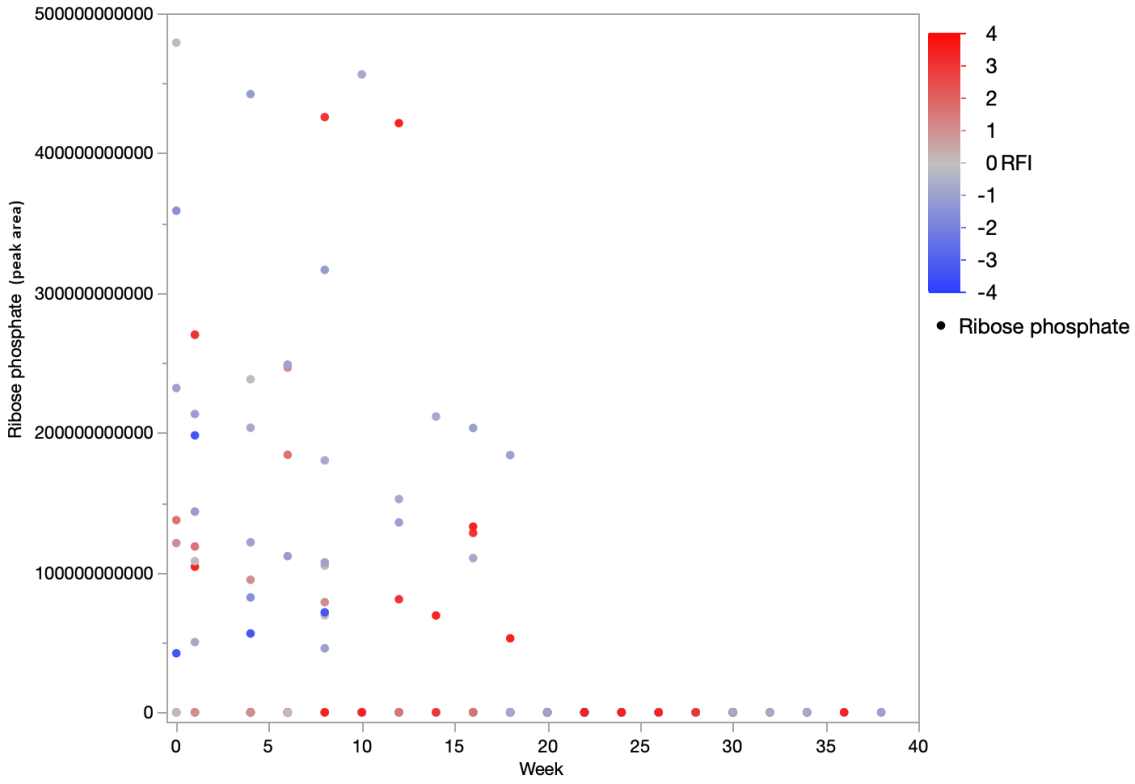

Figure 22. Residual Feed Intake (RFI) Status of Ribose Phosphate by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

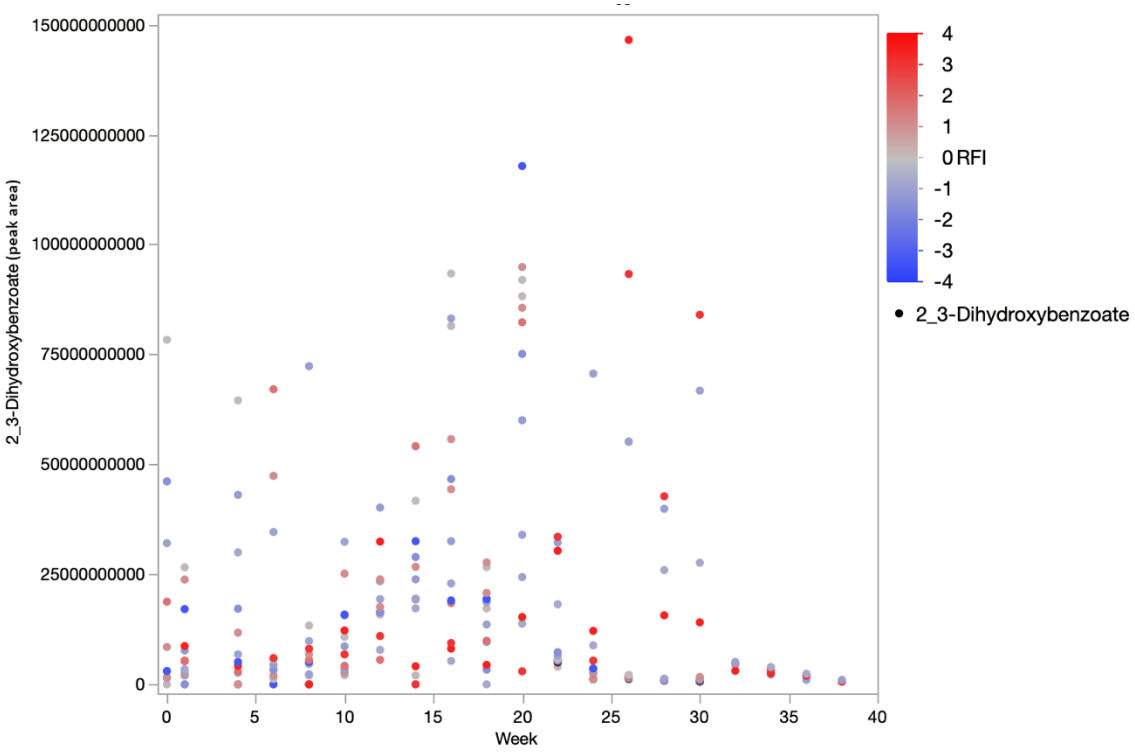

Figure 23. Residual Feed Intake (RFI) Status of 2_3-Dihydroxybenzoate by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

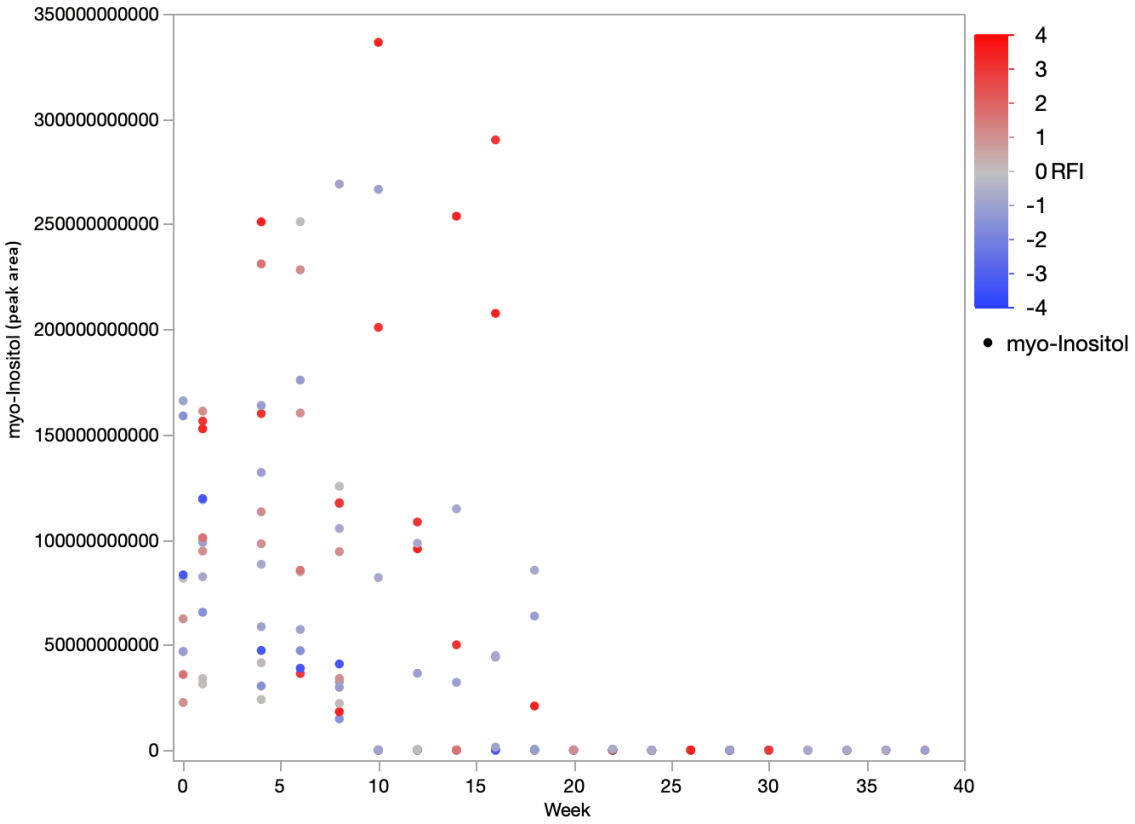

Figure 24. Residual Feed Intake (RFI) Status of myo-Inositol by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

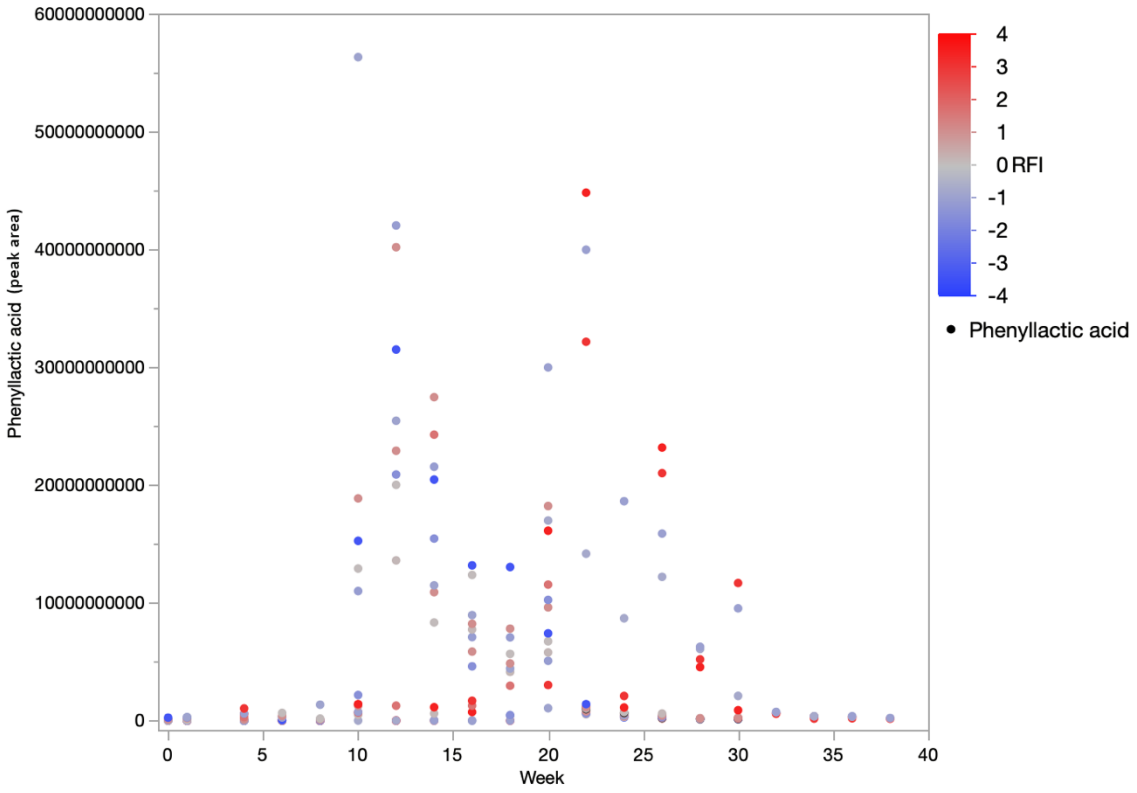

Figure 25. Residual Feed Intake (RFI) Status of Phenyllactic Acid by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

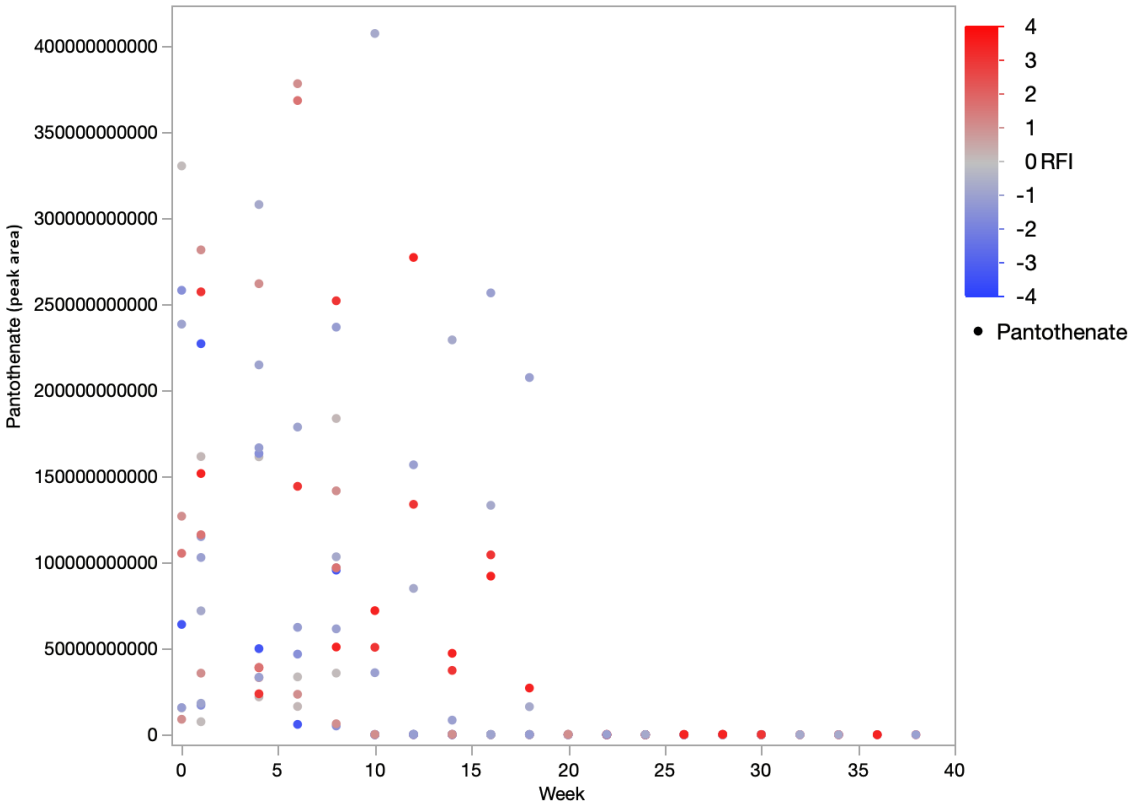

Figure 26. Residual Feed Intake (RFI) Status of Pantothenate by Week. Each dot represents a single animal. Color indicates RFI value. If values overlap, it is depicted by a single dot.

Table 9. Significant Correlations between Feed Efficiency and Bacteria/Metabolites among Weeks[1]

Week		Correlation Coefficient	p-value	FDR
4	myo-Inositol	0.665	0.013	0.138
4	Phenyllactic acid	0.631	0.021	0.145
12	ASV9	0.681	0.010	0.109
12	ASV106	0.606	0.028	0.193
12	ASV59	0.582	0.037	0.193
16	myo-Inositol	0.655	0.029	0.303
18	pimelic Acid	0.605	0.048	0.509
20	ASV245	0.666	0.013	0.101
20	ASV9	-0.658	0.014	0.101
20	ASV83	0.555	0.049	0.209
20	ASV59	-0.553	0.050	0.209
22	ASV83	-0.673	0.012	0.123
22	3-Hydroxyisovalerate	0.624	0.023	0.126
22	ASV106	-0.620	0.024	0.126
24	3-Methylphenylacetic acid	0.630	0.021	0.220
24	ASV46	0.567	0.043	0.246
26	ASV106	0.734	0.010	0.047
26	2_3-Dihydroxybenzoate	0.719	0.013	0.047
26	Phenyllactic acid	0.713	0.014	0.047
26	pimelic acid	0.706	0.015	0.047
26	myo-Inositol	0.705	0.015	0.047
26	Salicylate	0.696	0.017	0.047
26	3-Hydroxyisovalerate	0.693	0.018	0.047
26	ASV59	0.674	0.023	0.050
26	ASV46	0.671	0.024	0.050
26	Pantothenate	0.657	0.028	0.053
30	ASV15	0.692	0.009	0.092
30	ASV1166	-0.632	0.021	0.144

[1] Pearson r Correlation

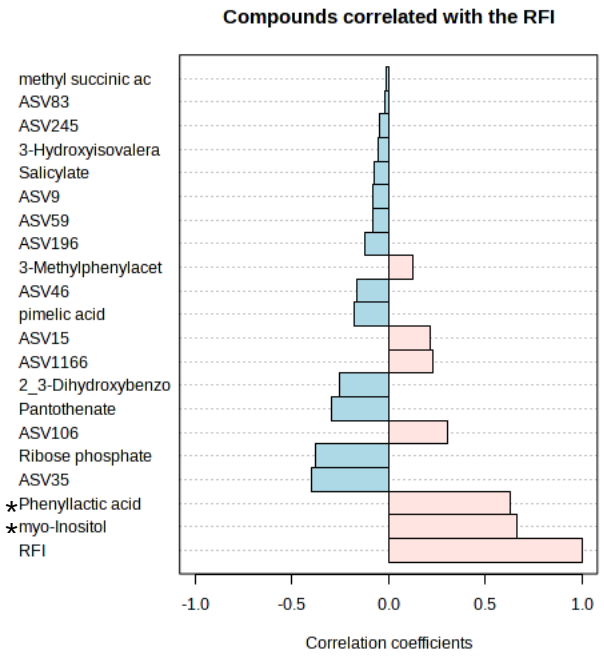

Figure 27. Week 4 Correlations of Amplicon Sequence Variants (ASV) and Metabolites with Residual Feed Intake (RFI). Asterisk (*) indicates significant correlation $P<0.05$

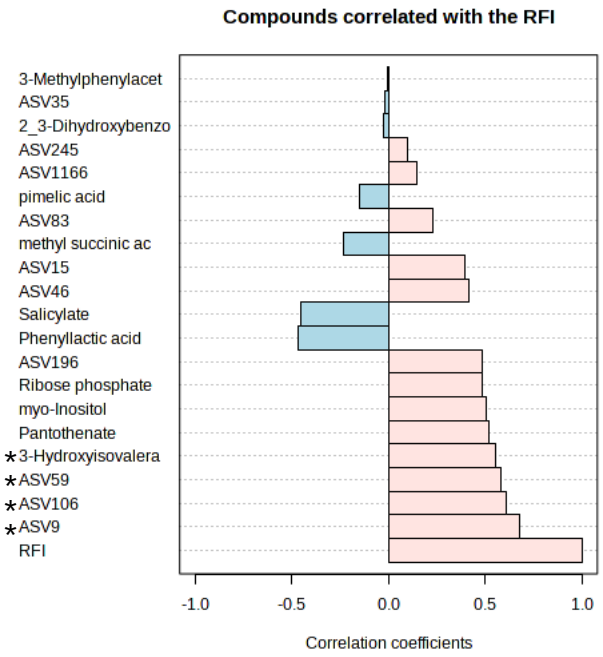

Figure 28. Week 12 Correlations of Amplicon Sequence Variants (ASV) and Metabolites with Residual Feed Intake (RFI). Asterisk (*) indicates significant correlation $P<0.05$

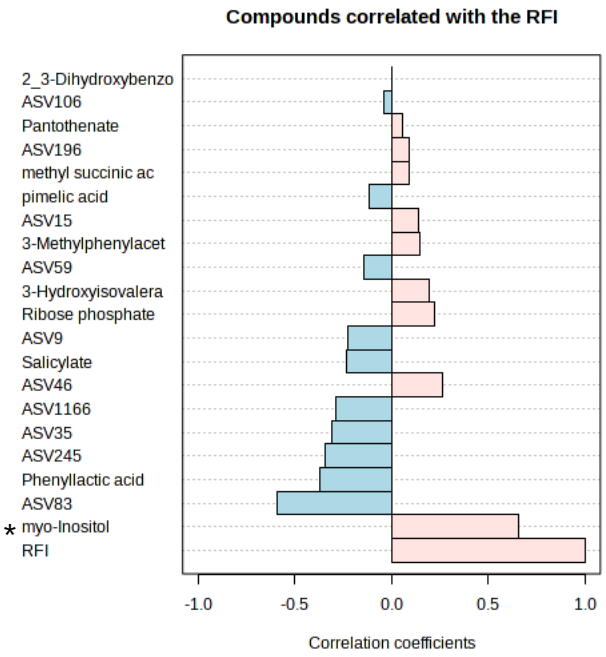

Figure 29. Week 16 Correlations of Amplicon Sequence Variants (ASV) and Metabolites with Residual Feed Intake (RFI). Asterisk (*) indicates significant correlation $P<0.05$

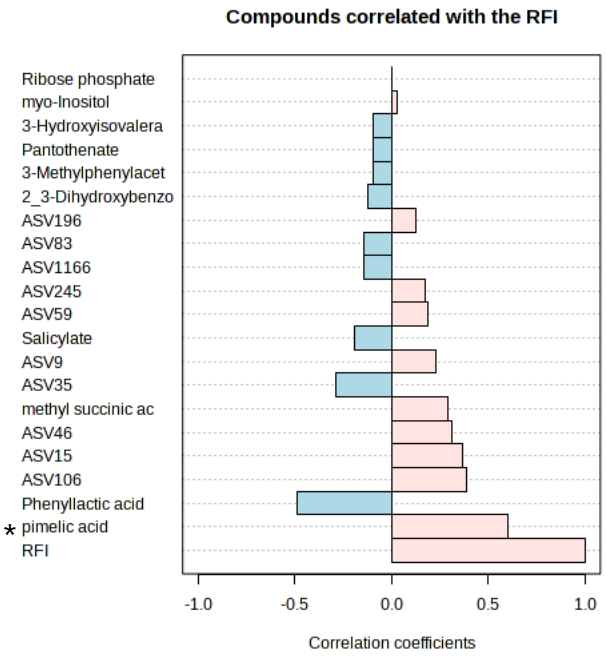

Figure 30. Week 18 Correlations of Amplicon Sequence Variants (ASV) and Metabolites with Residual Feed Intake (RFI). Asterisk (*) indicates significant correlation $P<0.05$

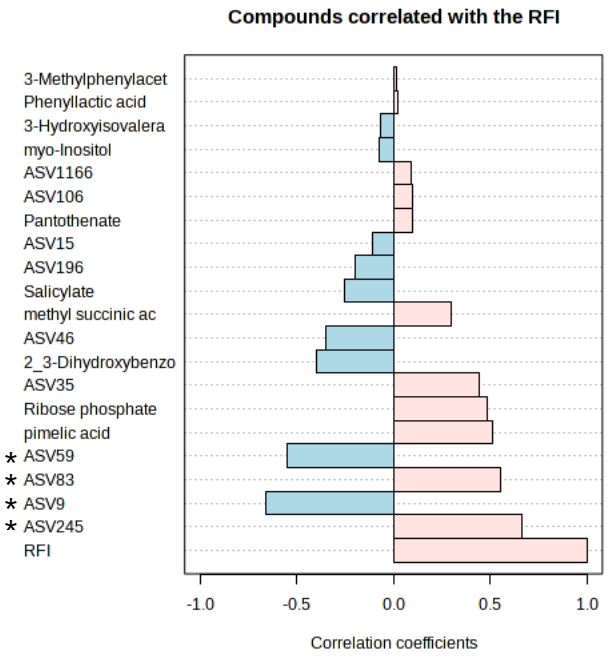

Figure 31. Week 20 Correlations of Amplicon Sequence Variants (ASV) and Metabolites with Residual Feed Intake (RFI). Asterisk (*) indicates significant correlation $P<0.05$

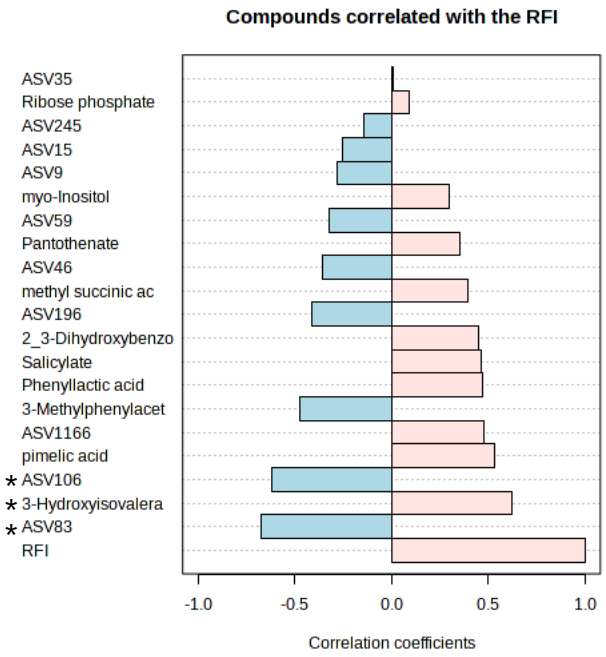

Figure 32. Week 22 Correlations of Amplicon Sequence Variants (ASV) and Metabolites with Residual Feed Intake (RFI). Asterisk (*) indicates significant correlation $P<0.05$

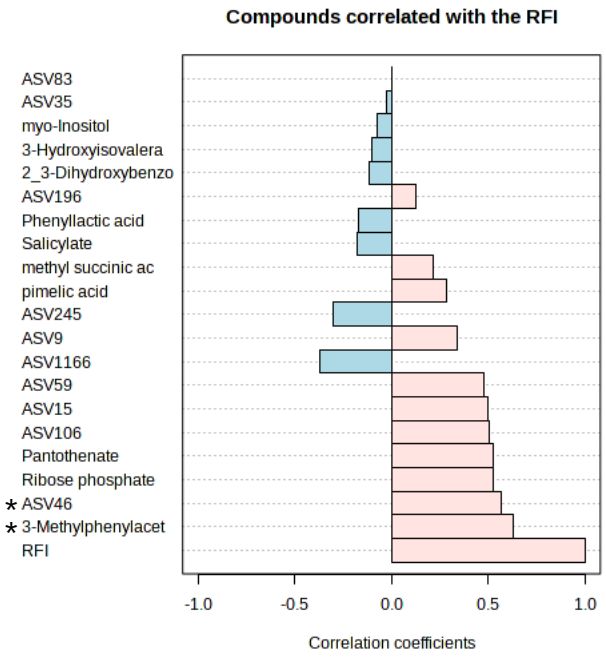

Figure 33. Week 24 Correlations of Amplicon Sequence Variants (ASV) and Metabolites with Residual Feed Intake (RFI). Asterisk (*) indicates significant correlation $P<0.05$

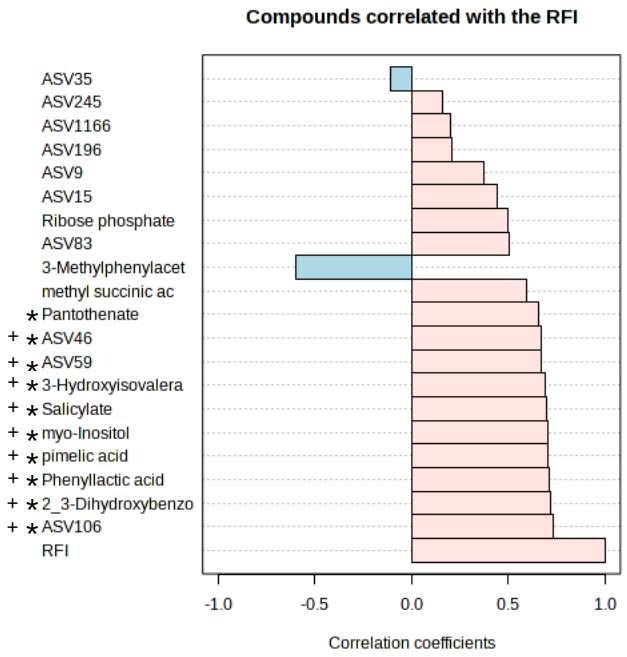

Figure 34. Week 26 Correlations of Amplicon Sequence Variants (ASV) and Metabolites with Residual Feed Intake (RFI). Asterisk (*) indicates significant correlation $P<0.05$. (+) indicates significant correlation FDR<0.05

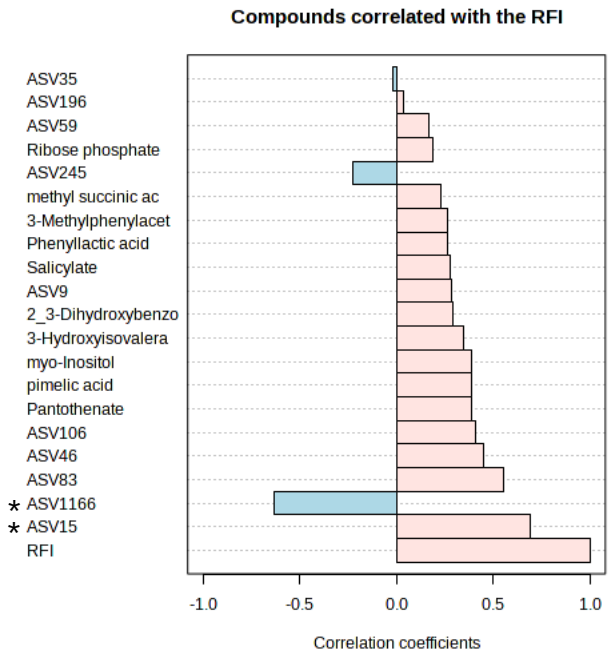

Figure 35. Week 30 Correlations of Amplicon Sequence Variants (ASV) and Metabolites with Residual Feed Intake (RFI). Asterisk (*) indicates significant correlation $P<0.05$

Discussion

Defining the rumen environment response to pregnancy is important for improving feed efficiency in beef production. This study identified rumen environment changes during pregnancy and determined correlations with established feed efficiency status in Angus heifers. The heifers in this study had numerous ASVs and metabolites that were different over time throughout pregnancy. However, of the 10 ASV abundances that were significantly different across all weeks and the 10 metabolites with the highest VIP scores, few were consistently significantly correlated with RFI across weeks in pregnancy. These data indicate that pregnancy physiologically impacts the rumen microbiome, and its fermentative activity based on feed efficiency status in heifers

Numerous significant alpha diversity pairwise comparisons for all three matrices (observed ASV, Chao1, and Shannon Diversity Index) occurred throughout pregnancy, and a notable increase in the number of significant comparisons through the progression of gestation occurred. From a physiological standpoint, the changes that occur throughout gestation tend to be more drastic beginning at roughly week 26 of gestation. At this point in pregnancy, rapid fetal growth is occuring for final organ, skin, and hair development, as well as the maternal preparation for parturition (64). This natural progression throughout pregnancy is congruent with the rumen environment changes occurring in this study with the increasing number of significant comparisons of bacterial richness and evenness throughout gestation. The mean averages for all three alpha diversity metrics shows a decrease in species richness and evenness later in gestation which is supported by previous work from Shabat (39) indicating that an efficient cow's rumen microbiome tends to be less diverse and more specialized to efficiently meet the animals nutritional requirements.

The dominance of *Prevotella* as a significant ASV in this study aligns with previous work indicating the role and function in the rumen. *Prevotella* has been reported as the dominant genus of the rumen and a large abundance of *Prevotella* is linked with a healthy rumen microbiome across different species including domestic cattle (131), sheep (132), and buffaloes (133, 134). *Prevotella* are unique in their ability to further process complex polysaccharides and produce

propionate, the most important substrate for gluconeogenesis (135). Additionally, *Succiniclasticum* also has a primary role converting succinate into propionate (136). Propionate is a critical energy source for ruminant animals (137), and is considered the primary substrate for gluconeogenesis in the liver (138, 139). The production of glucose in the liver is critical as ruminants do not consume adequate amounts in their diet and rely on microbial production to fuel a variety of physiological processes, including pregnancy. Increases in *Prevotella* and *Succiniclasticum* are commonly seen in animals on a more energy-intensive diet, rather than those grazing forages, similar to the heifers in this study (136, 140). Myer and others (4) indicated an associateion of increased *Prevotella* in greater feed efficient steers. The same study indicated *Succiniclasticum* was greater in the lesser feed efficient steers. However, Auffret (17) demonstrated higher abundances of *Succiniclasticum* in the greater feed efficient steers. In context of this study, the variation of feed efficiency status of the heifers in this trial in addition to the increased energy requirements during pregnancy should be considered before making associations between the dominance of the two genera present.

The heifers in this study weighed approximately 678 kg at the beginning of the trial and were likely of greater body condition score (BCS) than ideal, although exact BCS were not recorded. The excess BCS of the heifers on this trial likely impacted conception rates additionally, as with first exposure, less than 25% of all females conceived via artificial insemination by three different AI sires, and 76% total were confirmed pregnant with the utilization of a 60-day breeding season. Body condition score is a direct reflection of adipose fat deposition. Adipose fat tissue produces leptin (99). Increases in leptin levels in obese women, inhibits estradiol production and secretion and ovarian response to IGF-1, therefore providing no signal for the initiation of LH surges (141). The chain reaction of events from increased adipose fat prevents ovulation and therefore pregnancy (141). This connection is further supported by other research indicating a body condition score greater than 4 at first service negatively impacts first service conception rates in dairy cattle (142, 143). In beef cattle however, research has indicated a BCS>8 negatively effects conception, in addition to cyclicity, mobility, and dystocia (144, 145). Leptin also inhibits insulin production and promotes insulin resistance. Insulin in turn stimulates leptin production and secretion (146). Insulin also works to regulate blood glucose levels. Glucose is the key nutrient in fetal development (147).

And during pregnancy, increased levels of leptin (148), promote insulin resistance to allow more glucose to be free flowing and transported to the fetus.

The increased body weight of the heifers in this trial also likely influenced the metabolic outputs. The metabolite 3-Hydroxyisovalerate had the highest VIP score of the top 10 metabolites. VIP scores are indicative of their importance in the model (149). 3-Hydroxyisovalerate is a product of leucine degradation in an attempt to produce ATP for the Krebs cycle (150). In human studies, increased levels of 3-Hydroxyisovalerate from pregnant women was likely due to altered biotin, amino acid, and gut metabolism status, and potentially related to increased BMI status (151). Based on identified abundances in this trial, 3-Hydroxyisovalerate is the most abundant in the beginning of pregnancy. In early pregnancy, biotin serves as an important precursor for normal fetal growth (152) as well as an important factor for glucose regulation (153). In several species, a biotin deficiency in pregnancy can be teratogenic on the fetus (154). Mock (155) concluded that biotin metabolism is impacted by the alteration of steroid hormone concentrations that occurs with the onset of pregnancy. The production of 3-Hydroxyisovalerate and other metabolites regarding pregnancy in heifers and cows may be of interest for future research with regard to its impact on gut function, gut metabolism, biotin, and BCS.

This exchange of glucose and other nutrients becomes the most critical beginning as early as day 180 during gestation, to support rapid fetal growth. In this study, the shift in rumen environment complexity around the beginning of the third trimester could indicate the rumen's acclimation to the subsequent change of nutrient requirements. Fetal growth is dependent on nutrient availability, primarily glucose, and its ability to be transported from the maternal source through the placenta to the fetus (156). In humans, glucose uptake is dependent on maternoplacental and fetal placental blood flow (157). Inhibition of glucose exchange through the maternal-fetus placenta has been demonstrated with both maternal overnutrition and undernutrition in ewes (158). Restriction of nutrients in sheep prevents the production of substrates needed for nitric oxide dependent vasodilation to enhance blood flow to the conceptus (158). On the other end of the spectrum, overfed ewes exhibited a reduction in uterine and umbilical blood flow as well as glucose and oxygen uptake (159). Meeting maternal nutrient requirements for pregnancy can permit optimal

metabolism within the rumen environment and efficient transport of nutrients to support maintenance of pregnancy.

The rumen environment changes and metabolic outputs from the heifers in this study correlated with feed efficiency status. The majority of the abundances of ASVs and metabolites were in a positive relationship with an increase in RFI. The metabolites and microorganisms correlated with the heifer's previously established feed inefficiency could be an indicator of metabolic inefficiency. Although these positively correlated metabolites and microbes may have other functions or relationships with feed inefficiency (4), their increased prevalence may indicate production increases, decreases in absorption, production exceeding absorption capacity of the host (3), or reduced metabolism of the fermentative products and microbial growth (160) and lead to low feed efficiency. These inefficiencies may provide insight to the metabolic response to pregnancy in beef cattle.

In conclusion, several rumen environment changes in Angus heifers were identified throughout pregnancy and were potentially associated with nutrient requirements and physiological changes. These metabolic and microbial changes were correlated with the previously established feed efficiency status of the heifers. However, more research is necessary to further explain the alterations in the rumen environment during pregnancy in response to feed efficiency in Angus heifer to further improve the cow-calf enterprise.

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VITA

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