THESIS

THE FECAL AND LIVER ABSCESS MICROBIOTA OF FEEDLOT STEERS MANAGED IN NATURAL AND CONVENTIONAL PROGRAMS

Submitted by

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ABSTRACT

THE FECAL AND LIVER ABSCESS MICROBIOTA OF FEEDLOT STEERS MANAGED IN NATURAL AND CONVENTIONAL PROGRAMS

The fecal and liver abscess microbiota of steers managed in a single commercial feedlot in natural and conventional programs were evaluated. Steers managed in the conventional program (n = 7 pens) were administered hormonal implants, tylosin, monensin, and ractopamine; steers managed in the natural program (n = 7 pens) were raised without growth-promoting technologies or antimicrobials. The objective of the first study was to characterize the longitudinal changes in the fecal microbiota and to evaluate differences between management programs after placement (T1), after transition to the finishing diet (T2), immediately before the beta-adrenergic agoinst feeding period (T3), and immediately before shipment for harvest (T4). The phyla Firmicutes and Bacteroidetes composed greater than 85% of the fecal microbiota over the entire feeding period. Across both programs, 23 families were differentially abundant from T1 to T2; 13 families were differentially abundant from T2 to T3; one family was differentially abundant from T3 to T4. From T1 to T2, an increase in the relative abundance of Prevotellaceae (W = 117) and a decrease in the relative abundance of Bifidobacteriaceae (W = 126) were observed. At T1, the fecal microbiota of naturally managed steers had greater alpha diversity (measured as Shannon Diversity Index) than that of conventionally managed steers (P = <0.001), but no difference was detected between programs for Shannon Diversity Index (P = 0.774) at T4. Across both management programs, greater Shannon Diversity (based on pen-level composited samples) at T4 was associated with reduced liver abscess prevalence (r_s = -0.438).

The objective of the second study was to characterize the microbiota of liver abscess purulent material and to evaluate differences between microbial communities from steers in natural and conventional management programs. The dominant phyla included *Fusobacteria* (64.42% of reads) and *Bacteroidetes* (34.87% of reads). The genera identified in greater than 1% relative abundance of all reads included *Fusobacterium* (64.18% of reads; 100% of samples), *Bacteroides* (33.59% of reads; 93.33% of samples), and *Porphyromonas* (1.25% of reads; 7.62% of samples). Rare genera (identified in less than 1% abundance of all reads) included bacteria of the Ruminococcaceae, Provotellaceae, Clostridiaceae 1, Spirochaetaceae, Erysipelotrichaceae, and Peptostreptococcaceae families. The rare taxa discovered had been previously identified in rumen contents and on the rumen epithelium. The rare families present in liver abscess purulent material were also identified in the fecal samples from the same population of steers. While a greater taxonomic resolution is needed to identify potential homology between families present within both feces and liver abscess purulent material, the results suggest that escape of bacteria from the hind gut could be a factor in the formation of polymicrobial liver abscesses.

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CHAPTER I: INTRODUCTION

Liver abscess occurrence in fed steers is associated with a reduction in live animal performance, hot carcass weight, and visceral value (Brink et al., 1990; Brown and Lawrence, 2010). Prevalence of liver abscesses among fed cattle has risen from the earliest reports of 5.3% (Smith, 1940) to 17.8%, reported in the results of the most recent National Beef Quality Audit (Eastwood et al., 2017). The proposed etiology of liver abscess formation has been described since Smith (1944) and Jensen et al. (1954b) established a link between liver abscess incidence, ruminal health, and feeding of high-grain and low-roughage diets. Increasing the inclusion rate of high-fiber feedstuffs has been shown to reduce liver abscess prevalence (Foster and Woods, 1970; Holland et al., 2018). Though conservative estimates indicate that liver abscesses effectively cost the U.S. beef industry over 60 million dollars per year (Brown and Lawrence, 2010; Herrick et al., 2018), high grain-diets remain the standard for finishing steers as they result in favorable impacts on efficiency of beef production and beef quality (Galyean and Rivera, 2003; Reinhardt and Hubbert, 2015; Holland et al., 2018). Tylosin phosphate supplementation remains the most effective method of control for liver abscesses when sound bunk management is practiced (Brown et al., 1975; Nagaraja and Lechtenberg, 2007a), but feeding antibiotics to livestock has become increasingly scrutinized (Aarestrup, 1999; Beukers et al., 2015).

Across most scientific disciplines, an increasing amount of research is focused on characterizing microbial communities using next-generation sequencing (Jones, 2013); this trend is largely due to a reduction in the cost of 16S rRNA gene sequencing (Minich et al., 2018). Bovine rumen and fecal microbiota have been investigated using next-generation sequencing (Shanks et al., 2011; Henderson et al., 2015; Azad et al., 2019; Holman and Gzyl, 2019), but few

studies have investigated the microbiota that compose the liver abscess purulent material by community-level sequencing (Weinroth et al., 2017). With the established rumenitis-liver abscess complex (Jensen et al., 1954b) and the bacterial etiology of liver abscess occurrence (Scanlan and Hathcock, 1983), next-generation sequencing could be valuable as researchers seek to describe the microbial communities of the gastrointestinal tract and liver abscess purulent material.

The following review was completed to compile and summarize research pertaining to liver abscesses in fed cattle, including prevalence, economic significance, pathogenesis, etiologic agents, preharvest diagnosis, prevention, and opportunities for future research. The overarching objective of the presented research was to utilize 16S rRNA sequencing to describe the microbiota of feedlot steer feces (as a conveniently sampled gastrointestinal component) and to describe the microbiota of liver abscess purulent material to better understand the relationship between the gastrointestinal tract and liver abscesses in feedlot steers.

CHAPTER II: REVIEW OF LITERATURE

2.1 HISTORICAL OVERVIEW

Liver abscesses result from establishment of pyogenic bacteria in the liver followed by necrosis of local tissue, inflammation, and immune responses by the host (Nagaraja et al., 1996). Pyogenic abscesses are known to affect cattle of all ages and classes (Nagaraja and Lechtenberg, 2007a). Though observed in beef and dairy cows, liver abscesses are most commonly identified in cattle managed in feedlots and fed high-concentrate diets (Rezac et al., 2014). Liver abscesses pose potential challenges related to production efficiency, animal welfare, and food safety.

Liver abscesses in fed cattle were initially described independent of other disease challenges in the 1930s. Many early studies consistently identified *Fusobacterium necrophorum* as a component of liver abscess purulent material (Feldman et al., 1936; Frederick, 1943; Smith, 1944). The economic impact of liver abnormalities in fed cattle was originally scientifically reported by Smith (1940) and Frederick (1943); abscesses were identified as the leading cause of beef and calf liver condemnation in federally inspected packing plants with direct loss estimated near two million dollars annually. In 1944, Smith reported that 5.3% of livers were condemned for liver abscesses and hypothesized that reduced live cattle performance due to this disease state could be more costly than the value of the condemned livers.

Smith (1944) was also the first to connect ruminal lesions with abscessed livers in fed cattle, though a similar connection was drawn in the 1800s in human medicine (Finlayson, 1873). Jensen et al. (1954b) confirmed a link between damage to the rumen lining and formation of liver abscesses and coined the phrase *rumenitis-liver abscess complex*. Furthermore, Smith observed that regions feeding grains had greater prevalence of liver abnormalities (Smith, 1940).

Consequently, researchers began to investigate the value of roughage for prevention of rumenitis; findings indicated that both the form of roughage and inclusion level were related to development of rumen parakeratosis, physiological properties of the epithelium acting as a barrier to portal circulation, and to liver abscess prevalence (Harvey et al., 1968; Haskins et al., 1969; Brent, 1976; Colling et al., 1979). However, reduced performance is also associated with greater roughage inclusion (Haskins et al., 1968).

In the late 1950s, subtherapeutic feeding of antimicrobial compounds to decrease liver abscess incidence in fed cattle began. Chlortetracycline was among the first antibiotics investigated for liver abscess prevention (Matsushima et al., 1954). Later reports indicated that only two of 80 steers fed chlortetracycline exhibited liver abscesses compared to 21 out of 80 steers fed no antibiotic (Harvey et al., 1968). Early reports of inclusion of bacitracin, another antimicrobial compound, indicated numerically similar liver abscess prevalence between treated and control groups (Haskins et al., 1967). The most presently relevant antimicrobial advancements in liver abscess prevention were published by Brown et al. (1973), who found that both tylosin phosphate and tylosin phosphate urea adduct were effective at reducing liver abscess prevalence by approximately 80%.

When tylosin and chlortetracycline were fed in a single trial by Brown et al. (1975), prevalence of liver abscesses was 56.2% for the control diet, 44.2% for the diet including chlortetracycline, and 18.6% for the diet including tylosin. Later research indicated no interaction between tylosin and the ionophore monensin fed to cattle to prevent coccidiosis and promote feed efficiency (Heinemann et al., 1978; Pendlum et al., 1978, Schelling, 1984). Potter et al. (1985) completed a meta-analysis of 14 previous studies with a tylosin treatment including 821 cattle and reported the average liver abscess prevalence for control and tylosin-fed cattle as

28.7% and 8.7%, respectively. Virginiamycin was later researched and was deemed to have similar efficacy to chlortetracycline (Smith et al., 1989; Gill et al., 1990; van Koevering et al., 1991; Rogers et al., 1995).

Though well-reviewed by Nagaraja and Chengappa (1998), renewed interest in liver abscess prevention was driven by an increase in liver abscess prevalence in Holstein fed cattle in the early 2000s. However, techniques to reduce current antimicrobial use rather than test new antimicrobials have been investigated due to increasing scrutiny of feeding antimicrobials to livestock (Haskell et al., 2018; Chen et al., 2019). Feeding tylosin intermittently throughout the feeding period (Hans Christian Müller et al., 2018) and withdrawing tylosin at the end of the finishing period (Walter et al., 2018) have demonstrated a reduction in liver abscess prevalence that was similar to the reduction seen with continuous feeding of tylosin. Furthermore, research has indicated that withdrawing tylosin at the end of the feeding period reduced the quantity of antimicrobial-resistance genes in fecal bacteria at time of slaughter (Beukers et al., 2015). Increased roughage concentrations (of adequate particle size) have been shown to control liver abscess prevalence, but the severity of liver abscesses remains unaffected (Holland et al., 2018). Additionally, economics do not favor replacing concentrate feedstuffs with high-fiber feedstuffs (Reinhardt and Hubbert, 2015; Holland et al., 2018).

Antimicrobial alternatives have been researched in an attempt to mitigate the negative effects of long-term antimicrobial exposure on microbial ecosystems. Essential oils (Meyer et al., 2009; Elwakeel et al., 2013), direct fed microbials (Scott et al., 2017; Huebner et al., 2019), supplemental antioxidants (Krumsiek and Owens, 1998; Hans C Müller et al., 2018), and supplemental minerals (Van Bibber-Krueger et al., 2015; Van Bibber-Krueger et al., 2016; Van Bibber-Kruege et al., 2017a; Lundy et al., 2017) have displayed some potential for liver abscess

reduction, but lack of widespread adoption by commercial cattle feeders is indicative of limited desirability for use across the industry as a whole.

Instead of novel feed additives, the majority of cattle feeders continue to feed tylosin and to meticulously manage ration formulation and delivery to prevent liver abscess occurrence (Reinhardt and Hubbert, 2015). However, liver condemnations remain costly for the beef industry in the United States, with conservative estimates near 60 million dollars annually (Herrick et al., 2018). The Canadian cattle industry, which operates under a similar structure to that of the United States, reports the opportunity cost of liver condemnations at greater than 20 Canadian dollars, or 15 US dollars, per head (Canadian Cattlemen's Association and Beef Cattle Research Council, 2018). As such, liver abscess occurrence remains a challenge for the cattle industry.

2.2 PREVALENCE

2.2.1 National Occurrence

Prevalence of liver abscesses in feedlot cattle ranges from 1% to over 95% (Nagaraga and Lechtenberg, 2007a), demonstrating the complex nature of liver abscess formation and management. Typically, liver abscess prevalence in feedlot settings for individual pens of cattle averages between 10 and 32% (Nagaraja and Chengappa, 1998; Amachawadi and Nagaraja, 2016). Findings of recent National Beef Quality Audits (NBQA) for fed cattle are consistent with the literature review performed by Nagaraja et al. (1998). In 2011, the NBQA reported total liver condemnations of 20.9% of the 17,926 head observed; 13.7% of livers observed were condemned due to abscesses (McKeith et al., 2012). Interestingly, the 2016 NBQA reported total liver condemnations of 30.8% of 24,366 head observed (Eastwood et al., 2017). It is noteworthy that the 10-percentage-point increase in liver condemnations over five years was accompanied by

an increase in the proportion of observations from Holstein cattle (5.5% in 2011 to 20.4%). In the 2016 audit, 17.8% of livers were condemned for liver abscesses (Eastwood et al., 2017).

A similar liver abscess prevalence rate of 13.7% was reported by Brown and Lawrence (2010) from databases that account for over 75,000 observations. Elanco Animal Health's Liver Check data indicated an average liver abscess prevalence rate between 16 and 18% for non-Holstein fed cattle based on a ten-year average of at least 1.5 million head surveyed per year (Elanco, 2014; 2016). Generally, recent publications indicate an overall liver abscess prevalence between 10 and 20% for fed cattle in the United States.

2.2.2 Severity

In order to characterize differences in abscess severity, a standardized scoring system was developed by Elanco Animal Health (Elanco, 2016). Elanco Animal Health (2014) defines livers free from abscesses as normal (score of 0); livers with inactive scars, no more than four abscesses of 2 cm or less in size, or one to two large abscesses no greater than 4 cm in size are classified as mild or moderate (score of A); livers with at least one large or multiple small, active abscesses are classified as severe (A+). The greatest effects on live cattle performance are associated with liver abscesses classified as severe (Nagaraja and Lechtenberg, 2007a). Similarly, the greatest impacts on fed beef carcasses are attributed to severe abscesses, especially when the liver abscess or surrounding liver tissue is adhered to the diaphragm or other internal organs (Brown and Lawrence, 2010). For the purpose of research, mildly or moderately abscessed livers are further divided into categories of A- (scarred livers) and A (abscessed livers) (Brown et al., 1975). Typically, prevalence of severe liver abscesses in fed cattle is near 5% with close to half being adhered to viscera and/or internal tissues (Davis et al., 2007; Brown and Lawrence, 2010; Rezac et al., 2014).

2.2.3 Variability of Occurrence

Prevalence and severity of liver abscesses is highly variable across cattle populations with differing characteristics. Generally, cattle fed high-concentrate diets for longer periods of time have a greater prevalence and severity of liver abscesses (Gill et al., 1979; Roberts, 1982). Common recommendations to reduce liver abscess occurrence are to increase roughage level and to practice sound bunk management in attempt to promote consistent time and amount of feed delivered to the pen (Reinhardt and Hubbert, 2015). Though increasing roughage content of the diet is typically effective, reducing liver abscess prevalence can be achieved more economically by adding antimicrobial feed additives to feedlot diets (Brown et al., 1973).

In addition to nutritional management, cattle type and location influence liver abscess prevalence. Typically, liver abscess prevalence among steers is one to two percentage points greater than heifers (Elanco, 2016). Liver abscess prevalence in Holstein fed cattle averages near 30%, close to double the prevalence of fed beef cattle (Herrick et al., 2018). While liver abscess prevalence in non-Holstein cattle has remained relatively steady over the past ten years, the liver abscess prevalence of Holstein fed cattle appears to have fluctuated markedly (Elanco, 2016). Elanco Liver Check data released in 2014 indicated that cattle slaughtered in the Central Plains of the United States had a total liver abscess prevalence of 22%, with 14% of the total exhibiting severe abscesses (Reinhardt and Hubbert, 2015). In contrast, cattle slaughtered in the Midwest, Southern Plains, and Desert Southwest regions had a total liver abscess prevalence of 13% total; 4% of the total cattle population had severe abscesses (Reinhardt and Hubbert, 2015). A higher liver abscess prevalence rate in the central plains may be historically supported in that initial liver abscess investigation originated in Colorado (Newsom, 1938; Smith, 1944). In 1944, Smith reported liver abscess prevalence in Denver, Colorado, to be 13.3% while the national averge

liver abscess prevalence of all cattle slaughtered was reported to be 5.3%. Smith (1944) noted that the greatest liver abscess prevalence across the United States was observed in Colorado.

2.3 SIGNIFICANCE

On a national level, the economic effect of liver abscesses in fed cattle is difficult to quantify. The published discrepancies in effects of liver abscesses on live cattle performance, the variable marketing systems for fed cattle, and the difference in the value of beef liver for domestic or international consumption all contribute to the complexity of producing an accurate estimate.

2.3.1 Preharvest

First suggested by Smith (1940), liver abscess presence is thought to reduce efficiency of feedlot cattle. Though the exact mechanisms of reduced growth are not well studied, it is likely that the repartitioning of nutrients to support inflammation and healing of the rumen and liver (Gifford et al., 2012) and altered feed intake following incidence of acidosis contribute to a reduction in available nutrients above maintenance requirements to support growth (González et al., 2009). Brown et al. (1973) documented a 7.7 kg reduction in weight gain over the feeding period for cattle with abscessed livers compared to cattle with normal livers. Later, Brown et al. (1975) reported that the most drastic effects of performance reduction occurred among cattle with severe liver abscesses; these cattle had a 12.7% reduction in average daily gain compared to cattle with normal livers.

Rust et al. (1980) reviewed 11 studies that included 2,055 feedlot cattle. Across all cattle with liver abscesses, daily gain was 0.9% lower than cattle with normal livers. When liver abscess score was considered, cattle with severely abscessed livers (which accounted for 6.5% of all cattle) were the only animals that experienced reduced overall daily gain (5.2%). However,

cattle that developed liver abscesses experienced numerically more rapid gain than all other cattle during the first 56 days of the feeding period and markedly decreased average daily gain (9.9% lower than those with normal livers) during the final 90 days of the feeding period. Similar observations by Fox et al. (2009) demonstrate reduced growth of cattle with liver abscesses late in the feeding period.

A review of 12 studies by Brink et al. (1990) that included 566 individually fed cattle receiving no antimicrobials, is frequently cited for conclusions on liver abscesses and live cattle performance. For analysis, nine studies were pooled into two groups based on homogeneity of variance; the average liver abscess prevalence was 32.1% for group one and 77.7% for group two (Brink et al., 1990). In group one, no differences were found between cattle with abscessed and normal livers; however, it is noteworthy that only nine head of cattle with severely abscessed livers were included in group one (173 total head). The results from group two (that included 62 of 247 head with severely abscessed livers) are more often discussed; cattle with severely abscessed livers gained 8.2% less and weighed less at slaughter than cattle with normal livers. Additionally, cattle with mild or severe abscesses exhibited a 6.7% decrease in dry matter intake compared to cattle with normal livers, particularly late in the feeding period. These results demonstrating reduced feed intake late in the feeding period support findings of slower gain late in the feeding period (Rust et al., 1980; Fox et al., 2009). Brink et al. (1990) suggested that this reduction in weight gain may be due to a reduction in net energy consumption rather than decreased efficiency of nutrient utilization.

It is evident that cattle with severely abscessed livers exhibit the greatest reductions in average daily gain in tandem with reduced feed intake. On an individual basis, cattle with reduced daily gain could be fed longer to reach a target endpoint, permitting a comparable final

weight to cattle with normal livers. However, large-pen commercial feedlots cannot adjust marketing strategies to accommodate for light weight cattle when they compose a minority of a pen. Therefore, rather than increased days on feed, reduced final weight is likely the largest cost of cattle with severe liver abscesses.

2.3.2 Postharvest

The effects of liver abnormalities on carcass value were first thoroughly described by Montgomery (1985) and more recently by Brown and Lawrence (2010). Based on observations of greater than 75,000 carcasses from two databases, Brown and Lawrence (2010) provided the most relevant estimates of financial loss incurred due to liver abscess presence by summarizing findings related to the impact of abscesses on carcass characteristics. Parallel to reduced live growth rate, the hot carcass weight of cattle with severely abscessed livers was shown to be approximately five kg lower than cattle without liver abscesses (Brown and Lawrence 2010). Carcasses exhibiting A+ abscesses with adherence to the viscera, diaphragm, or flank (A+ AD), were 13.7 kg lighter, on average, compared to those with normal livers (Brown and Lawrence, 2010). The reduction in hot carcass weight is often due in part to increased carcass trimming to eliminate contamination (Nagaraja and Chengappa, 1998). A reduction of 0.25 to 0.75% in dressing percentage may also contribute to reduced hot carcass weight (Brown and Lawrence, 2010; Elanco, 2016). Together, reduced live growth and reduced dressing percentage associated with liver abscesses result in decreased hot carcass weight.

In addition to carcass weight, yield grade and quality grade may be marginally affected by liver abscess occurrence. Although Brown and Lawrence (2010) found that ribeye area was not affected by liver abscess presence when adjusted for carcass weight, reductions in subcutaneous fat depth and percentage of KPH (kidney, pelvic and heart) fat resulted in a slightly

more favorable mean USDA yield grade (no more than 0.1 unit difference in yield grade) for cattle with liver abscesses compared to those without. Average marbling score was generally slightly lower for carcasses with liver abscesses (by no more than 10 marbling score units) compared to carcasses with normal livers (average of SM[16], Brown and Lawrence, 2010). These effects of liver abscesses on carcass quality factors appear to be reflective of reduced energy intake during the finishing period. Despite significant statistical differences in yield and quality grade factors when comparing cattle with and without liver abscesses (Brown and Lawrence, 2010), the difference has limited economic significance in comparison to differences in hot carcass weight.

When considered cumulatively, the effects of carcass weight, quality grade, and yield grade reflect a large discount for carcasses from cattle with liver abscesses. Though dependent on fluctuating market prices, carcasses with liver scores of A- or A (mild to moderate), A+ (severe), or A+AD (severe with adhesion) are worth approximately $5, $10, and $35 less than carcasses with liver scores of 0, respectively (Brown and Lawrence, 2010). When discounts associated with liver abscess classes are applied as a weighted average to an annual average slaughter of 26 million fed steers and heifers (Brown and Lawrence, 2010), total carcass and performance losses due to liver abscesses likely exceeds $50 million dollars.

The condemnation of beef livers leads to significant economic losses. Estimating 18.1% of livers are inedible, Brown and Lawrence (2010) project that total liver value lost due to condemnation exceeds $15 million, with meat processing companies losing $3.25 for each condemned liver. However, this value may be drastically higher depending upon export conditions; the value of livers for export, particularly to Egypt, can exceed $20 per liver (USDA FAS, 2018a; 2018b). The collective effects of liver abscesses in fed cattle are conservatively

estimated to cost the US beef industry upwards of $60 million dollars (Herrick et al., 2018). While this figure represents the financial impact associated with liver abscesses, the effects of this disease state are not limited to economics. Challenges related to antibiotic stewardship are evident but more difficult to quantify.

2.4 PATHOGENESIS

The pathogenesis of liver abscesses was first hypothesized when Smith (1944) published evidence of a possible link between lesions of the rumen and the incidence of liver abscesses. Later, Jensen et al. (1954b) observed a greater prevalence of liver abscesses in cattle with ruminal lesions compared to cattle with no lesions (43% versus 23%). Braun et al. (1995) commonly found gastrointestinal tract lesions in cows upon necropsy after liver abscesses were identified in the live animal by ultrasound, thereby adding to the body of evidence that supports this relationship. More recently, Rezac et al. (2014) found a similar association between ruminal health and liver abscess incidence in cull cows; cows noted to have mild or severe ruminitis had a greater abscess prevalence than cows with healthy rumen epithelium (32% versus 19%).

However, not all studies have demonstrated a strong relationship between ruminal lesions and liver abscess incidence. Weiser et al. (1966) found no correlation between rumen lesions and liver abscess incidence in early-weaned steer calves. To explain the inconsistent findings concerning rumen health and liver abscess occurrence, Brent (1976) reasoned that acidotic events early in the feeding period could allow bacteria to enter the portal vasculature and inoculate the liver while allowing time for the rumen to heal.

Regardless, it is well supported that poor health of the ruminal epithelium is a predisposing factor for liver abscess formation (Jensen et al., 1954b; Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007b). Damage to the rumen lining by acidosis commonly

precedes the formation of a liver abscess (Nagaraja and Chengappa, 1998). Acid accumulation (from fermentation of high-concentrate feedstuffs to organic acids) reduces pH of the rumen contents, increases osmotic pressure, and increases molality in the rumen, typically resulting in some degree of rumenitis (Steele et al., 2011; Meissner et al., 2017). Sub-acute acidosis is identified when the ruminal pH drops below 5.6, and acute acidosis is identified when ruminal pH drops below 5.0 (Owens et al., 1998). Acidosis can be induced by rapid changes to high-concentrate diet formulations or sudden changes in feed consumption (Elam, 1976); therefore, the negative effects of acidosis also likely begin during periods of diet transition. During the first week of feeding a high-grain diet, the depth of the rumen epithelium decreases, specifically in the stratum basale, spinosum, and granulosum layers (Steele et al., 2011). Thinning of the rumen wall is accompanied by rupture of cells on the stratum lucidum and consequent sloughing of the stratum corneum from the papillae (Thomson, 1967).

Upon degradation of cellular junctions, bacterial entry into the rumen epithelium is permitted (Thomson, 1967; Steele et al., 2011). Though microbes are commonly commensally associated with the stratum corneum of the ruminal epithelium (Church, 1988), parakeratosis of the ruminal mucosa compromises the physical barrier of the tight junctions within the stratum granulosum (Haskins et al., 1967; Salcedo et al., 2012). Damage to the ruminal epithelium can also occur due to punctures or particles becoming embedded in the rumen wall (Fell et al., 1967). Regardless of the initial cause of ruminal damage, lesions and ulcers may result and are susceptible to colonization by *Fusobacterium necrophorum* (Kanoe et al., 1978). Li et al. (2019) found increases in *Fusobacterium* bacteria associated with the rumen epithelium in dairy calves when fed a high starch diet intended to initiate acidosis for research purposes. Acidosis can also

reduce innate immune response to bacterial infection, enabling higher survival rates of invading bacteria within the rumen wall (Alarcon et al., 2011).

Once foci of infection are established in the portal-drained viscera, it is believed that bacteria enter circulation in the portal blood (Nagaraja and Chengappa, 1998). Bacteria are transported to the liver as an embolus once passed from the rumen or dislodged from the rumen wall (Nagaraja and Lechtenberg, 2007a). A study conducted by Narayanan et al. (1997) provided further evidence of this phenomenon; researchers found that ribotyping of *F. necrophorum* isolates from liver abscesses and ruminal walls yielded indistinguishable isolates.

Additionally, inoculation studies support the link between bacterial entry into portal circulation and formation of liver abscesses. Jensen et al. (1954a) first induced liver abscesses in cattle by portal inoculation. Shinjo et al. (1981) inoculated mice with a bovine liver abscess strain of *F. necrophorum* into the portal vein and observed liver abscesses in all mice sacrificed five days following inoculation. Interestingly, the researchers also identified the isolate in the spleen, kidney, and lungs, but no abscesses were observed in other organs (Shinjo et al., 1981).

In 1983, Scanlan and Berg experimentally induced liver abscesses in cattle by inoculation of *F. necrophorum* into the portal venous system. Microabscesses and microthrombi were observed in the liver, followed by the incidence of abscesses and coagulative necrosis. Nakajima et al. (1986) documented similar findings of granular tissues surrounding abscesses and intravascular coagulation. Though the progression from coagulative necrosis to abscess formation typically takes three to ten days, formation of a necrotic core occurs as abscesses mature (Nagaraja and Lechtenberg, 2007a). If ample time is allowed for healing, abscesses eventually become sterile, are replaced by fibrous scar tissue, and resorb (Nagaraja and Lechtenberg, 2007a). Initiation of scar formation has been observed from 45 to 180 days after

experimental inoculation (Jensen et al., 1954a). Though the connection between liver abscesses and the lymphatic system is uncharacterized, liver abscesses have also been associated with hyperplasia of portal lymph nodes (Eleni et al., 1994). Saginala et al. (1997) induced liver abscesses by portal inoculation of *F. necrophorum* in steers for the purpose of studying vaccines; all steers in the control group had abscessed livers upon necropsy. It is noteworthy that the mentioned studies successfully produced liver abscesses by portal inoculation; Robinson et al. (1951) did not produce liver abscesses by intragastric or intrajugular inoculation. Takeuchi et al. (1984) similarly determined that portal injection of *F. necrophorum* produced liver abscesses while jugular injection produced no abscesses.

Although the link between presence of *F. necrophorum* in portal blood and formation of a liver abscesses is well supported (Shinjo et al., 1981; Nakajima et al., 1986; Saginala et al., 1997), the previously described rumenitis-liver abscess complex is only associated with foregut acidosis and its potential to promote liver abscess formation. Since portal-drained viscera includes the hindgut, it is possible that hindgut acidosis could have similar pathological effects as ruminal acidosis and provide a route for causative agents of liver abscesses to enter splanchnic tissue.

2.5 PROPOSED ETIOLOGIC AGENTS

Bacteria enter the liver and embed in hepatic parenchyma before becoming encapsulated to form an abscess (Scanlan and Hathcock, 1983). Resulting abscesses are typically polymicrobial infections dominated by anaerobes with more than half of the bacteria being classified as gram-negative (Calkins and Dewy, 1968; Nagaraja and Chengappa, 1998; Amachawadi and Nagaraja, 2016). Due to the role of bacteria in abscess formation, the bacterial flora of the liver has been well investigated; findings implicate *F. necrophorum* (formerly

Spherophorus necrophorus) as the primary etiological agent with *Trueperella pyogenes* (formerly *Actinomyces pyogenes* and *Corynebacterium pyogenes*) being a commonly found constituent (Newsom, 1938; Jensen et al., 1947; Scanlan and Hathcock, 1983; Tan et al., 1996; Lechtenberg et al., 1998; Amachawadi and Nagaraja, 2015; Weinroth et al., 2017).

Using culture-based techniques, *F. necrophorum* is typically isolated from 80 to 100% of liver abscesses (Nagaraja and Chengappa, 1998). In the single published study that used molecular techniques to characterize the constituents of liver abscesses in fed cattle, 16S rRNA sequences recognized as *F. necrophorum* were identified in all liver abscesses (Weinroth et al., 2017). Liver abscesses have also been reported to contain *Salmonella enterica* (Amachawadi and Nagaraja, 2015), *Pasteurella spp.* (Simon and Stovell, 1971)*, Clostridium spp.* (Simon and Stovell, 1971)*, Streptococcus spp.* (Calkins and Dewey, 1968; Simon and Stovell, 1971; Lechtenberg et al., 1988)*, Staphylococcus spp.* (Lechtenberg et al., 1988)*,* and *Bacteroides spp.* (Newsom, 1938; Simon and Stovell, 1971; Berg and Scanlan, 1982; Weinroth et al., 2017). Together these findings indicate that liver abscesses are typically polymicrobial, but aside from *F. necrophorum*, the genera present within liver abscesses demonstrate variable patterns of detection.

2.5.1 *Fusobacterium necrophorum*

Fusobacterium necrophorum, a gram-negative, nonmotile, nonsporulating anaerobe, is known to affect cattle as the primary pathogen of foot rot, foot abscesses, necrotic laryngitis (calf diphtheria), and liver abscesses (Emery et al., 1985; Tan et al., 1996). A single Canadian annual agricultural report indicated that liver abscesses in cattle were common in areas where foot rot was present (Gunn, 1956). However, no studies have demonstrated links between liver abscess occurrence and other diseases in which *F. necrophorum* is implicated as an etiologic agent.

Fusobacterium necrophorum is part of the normal flora of the bovine gastrointestinal tract, with populations specifically noted in the rumen (Robinson et al., 1951; Langworth, 1977; Berg and Scanlan, 1982; Tan et al., 1994b). Generally, the concentration of *F. necrophorum* in the rumen is 10^5 to 10^6 colony forming units (CFU) per gram of rumen contents (Nagaraja and Chengappa, 1998). Tan et al. (1994c) found that the concentration of *F. necrophorum* increased ten-fold when cattle were transitioned from an all roughage diet to a high-concentrate diet. During transitions to high-energy diets, short-chain fatty acid (SCFA) concentration in the rumen increases and pH decreases due in part to accumulation of lactate (Elam, 1976; Owens et al., 1998). The elevated concentration of lactate provides a competitive advantage for *F. necrophorum* to multiply given that lactate is the preferred energy substrate (Lechtenberg et al., 1988). The production of SCFAs, especially butyrate, is beneficial for epithelial cell maintenance at moderate levels (Lechtenberg et al., 1988; DeLong, 2012); however, rapid increases in the concentration of *F. necrophorum* at times of potential acidotic damage to rumen epithelium could increase the probability of *F. necrophorum* reaching the portal blood.

In addition to residing in rumen fluid, *F. necrophorum* has been found adhered to the rumen wall (Kanoe et al., 1978). Using 16S ribosomal RNA sequencing, Liu et al. (2016) found greater relative abundance of the phylum *Fusobacteria* associated with the ruminal epithelium than in rumen contents. When quantified by Bedwell et al. (1998), *F. necrophorum* was found in the highest concentration (6×10^5 CFU per square centimeter) on epithelium samples from the ventral sac and in lowest concentration (2×10^3 CFU per square centimeter) on epithelium samples from the dorsal sac. Although the epimural bacteria (bacteria associated with the rumen wall) only comprise one percent of the ruminal microbes, this bacterial population is critical as it digests sloughed epithelial cells and maintains the anaerobic environment of the rumen (Cheng

and McAllister, 1997). *Fusobacterium necrophorum* produces proteases likely capable of digesting epithelial debris and is involved with lysine metabolism (Nakagaki et al., 1991; Elwakeel et al., 2013). Similarly, *F. necrophorum* has been shown to be aerotolerant and could potentially survive amidst the oxygen gradient of the epimural surface (Hofstad, 1984).

The presence of *F. necrophorum* in portal blood has not been confirmed through published research; however, it is likely present as bacterial emboli have been shown to produce liver abscesses in portal inoculation studies (Shinjo et al., 1981; Takeuchi et al., 1984; Nakajima et al., 1986; Saginala et al., 1997). Upon entering the liver sinusoid, virulence factors of *F. necrophorum* assist the bacteria in overcoming a phagocytic and aerobic environment to allow for initiation of abscess formation (Nagaraja and Chengappa, 1998). Protection from innate immune responses is attributed to leukotoxin and endotoxic lipopolysaccharide production (Nagaraja and Chengappa, 1998). Leukotoxin is considered the major virulence factor than enables *F. necrophorum* to survive in the liver (Nagaraja et al., 2005). Noted by Roberts (1967), leukotoxin exerts cytotoxic effects of apoptosis or lysis on polymorphonuclear leukocytes and hepatocytes (Tan et al., 1994d; Narayanan et al., 2002; Tadepalli et al., 2009). The pathogenicity of *F. necrophorum* is correlated with leukotoxin production; a higher rate of liver abscesses was seen following inoculation with *F. necrophorum* isolates with greater leukotoxin production (Coyle-Dennis and Lauerman, 1979; Emery et al., 1985). Endotoxic lipopolysaccharide is similarly destructive and can induce necrosis and promote intravascular coagulation (Berg and Loan, 1975; Garcia et al., 1975; Warner et al., 1975).

The ability of *F. necrophorum* to overcome the high oxygen content of the portal blood and liver parenchyma has been attributed to hemolysin, hemagglutinin, platelet aggregation factor, and proteases (Nagaraja and Chengappa, 1998). Hemolysin activity is positively

correlated to *F. necrophorum* growth and is highest at a pH near neutral when activated by bovine serum (Amoako et al., 1994). It is plausible that hemolysin reduces the oxygen concentration surrounding *F. necrophorum* emboli in the liver by impairing the ability of erythrocytes to carry oxygen by lysing the cells. Hemolysin has been previously identified within the purulent material of liver abscesses (Kanoe, 1990). Hemagglutinin allows *F. necrophorum* cells to adhere to surfaces of both the rumen wall and the liver (Kanoe and Iwaki, 1987). Hemagglutinin is also considered a platelet aggregation factor, serving to slow blood flow and further perpetuate the formation of a necrotic microenvironment (Kanoe and Yamanaka, 1989). As oxygen concentration is reduced and hepatocytes become exposed to ischemic conditions, extracellular proteases of *F. necrophorum* begin to break down hepatic proteins and cause local tissue damage (Nakagaki et al., 1991; Amoako et al., 1993; Tan et al., 1994b).

The degree of pathogenicity of *F. necrophorum* is driven by subspecies phylogeny (Tan et al., 1992). *Fusobacterium necrophorum* is classified into three relevant biotypes: A, B, AB (Langworth, 1977; Nagaraja and Chengappa, 1998). Biotypes A and B are most commonly identified in liver abscesses and have been classified as subspecies *necrophorum* and *funduliforme*, respectively (Shinjo et al., 1991). Subspecies *necrophorum* demonstrates greater production of leukotoxin (Berg and Scanlan, 1982; Tan et al., 1992; Tan et al., 1996); consequently, subspecies *necrophorum* has been isolated from 71 to 95% of liver abscesses while subspecies *funduliforme* has been isolated from 5 to 29% of liver abscesses (Lechtenberg et al., 1988). Biotype AB (an intermediate to biotypes A and B) is most commonly identified in foot lesions from both sheep and cattle (Emery et al., 1985) but is rarely found in liver abscesses (Berg and Scanlan, 1982). Based on 16S rRNA sequencing, biotype AB is phylogenetically more closely related to subspecies *funduliforme* (Nicholson, et al., 1994).

2.5.2 *Trueperella pyogenes*

Trueperella pyogenes (formerly *Actinomyces pyogenes* and *Corynebacterium pyogenes*) has historically been identified as the second most commonly isolated bacteria from liver abscesses (Lechtenberg et al., 1988; Tan et al., 1996). *Trueperella pyogenes*, a gram-positive facultative anaerobe, has been associated with various bovine infections including mastitis, endometritis, and lung lesions, as well as spontaneous abortions (Bretzlaff, 1987; Semambo et al., 1991; Madsen et al., 1992; Leifsson et al., 1995). In relation to liver abscesses, *T. pyogenes* is more frequently identified in the slightly aerobic environment of the rumen wall than in the anaerobic environment of rumen contents (Narayanan et al., 1998). Similar to *F. necrophorum*, *T. pyogenes* isolates from liver abscesses and the rumen wall are indistinguishable by ribotyping (Narayanan et al., 1998).

Roberts (1967) suggested that a synergistic relationship exists between *T. pyogenes* and *F. necrophorum*. *Trueperella pyogenes* utilizes oxygen that diffuses through the rumen wall and produces lactate as a byproduct of metabolism (Tadepalli et al., 2009; Reinhardt and Hubbert, 2015), creating a more favorable anaerobic environment that is rich in energy substrates for *F. necrophorum* (Nagaraja and Chengappa, 1998). The presence of *F. necrophorum* could lend protection to *T. pyogenes* against host immune defenses by a diverse set of virulence factors.

Though *T. pyogenes* has been isolated as a pure culture from liver abscesses (Nagaraja and Lechtenberg, 2007a), it is unlikely that *T. pyogenes* is capable of initiating the formation of an abscess. When steers were inoculated with a pure culture of *T. pyogenes*, no liver abscesses were produced; however, when a mixed culture of *T. pyogenes* and *F. necrophorum* or *T. pyogenes* with leukotoxin was administered, abscesses were formed (Lechtenberg et al., 1993 in Nagaraja and Lechtenberg, 2007a). This demonstrates the reliance of *T. pyogenes* on the

virulence factors of *F. necrophorum* to withstand host innate immune responses. *Trueperella pyogenes* has been isolated more often in liver abscesses of cattle fed tylosin compared to cattle not fed tylosin (Nagaraja et al., 1999a).

2.5.3 *Salmonella enterica*

Recently, *Salmonella enterica* has emerged as a constituent of polymicrobial liver abscesses (Amachawadi and Nagaraja, 2015). The first report of *Salmonella* in liver abscesses of fed cattle was by Amachawadi and Nagaraja (2015). These findings were confirmed by identifying *Salmonella* in liver abscesses of both Holstein and crossbred beef steers with prevalence near 25% (Amachawadi et al., 2017). However, *Salmonella* prevalence was lower in tylosin-fed cattle compared to non-tylosin-fed cattle (17% compared to 33%; Amachawadi et al., 2017). Herrick et al. (2018) also found *Salmonella* in liver abscesses of fed cattle with prevalence near 25%. Though no regional differences in *Salmonella* prevalence in liver abscesses of fed cattle have been reported (Herrick et al., 2018), regional differences in isolated serotypes have been identified (Amachawadi et al., 2017). It is likely that cattle within contemporary groups will display more similar phylogeny among isolated serotypes (Amachawadi and Nagaraja, 2015).

The role of *Salmonella* in liver abscess etiology is unknown. *Salmonella* is tolerant of varying oxygen levels but can be more virulent under anaerobic conditions (Yamamoto and Droffner, 1985; Schiemann and Shope, 1991). The role of *Salmonella* as an intracellular pathogen leaves question regarding its mechanism of entrance into liver abscesses (Lee and Falkow, 1990). Inflammation of the ruminal epithelium, and possibly the mucous membrane of the hindgut, associated with ruminal and hindgut acidosis could allow for entrance of *Salmonella* into splanchnic tissues (Amachawadi and Nagaraja, 2015).

However, *Salmonella* has also been identified in other tissues of fed cattle, specifically in peripheral lymph nodes surrounded by adipose tissue (Arthur et al., 2008). The pathogen is of concern in the realm of food safety as lymph nodes containing *Salmonella* may be included in trimmings designated for ground beef (Brichta-Harhay et al., 2012). Though similar pulsed field gel electrophoresis banding patterns have been identified from *Salmonella* isolates from lymph nodes and liver abscesses, the relationship between the microbiome of the hepatic and lymphatic systems is largely unknown (Amachawadi and Nagaraja, 2015)

2.6 PREHARVEST DIAGNOSIS

2.6.1 Observation

A major challenge of reducing liver abscess rates in fed cattle is understanding the timing of liver abscess formation. Hickey (1963) reported that fed cattle with liver abscesses had no "outward sign of disorder" and were otherwise healthy. More recently, a case report of a bull with slow and progressive loss of condition and anorexia was found to have liver abscesses upon post-mortem examination (Tromp et al., 2005). Similarly, retrospective study of Holstein cows diagnosed with liver abscesses found anorexia as the most frequent cause of presentation for examination (Dore et al., 2007). Though reduced appetite may be identified as a sign of liver abscesses in intensively managed systems, prolonged individual observation is not practical in the large-pen settings that are common to the cattle feeding industry in the United States.

2.6.2 Ultrasonography

Particularly in veterinary medicine, ultrasonography has been used to attempt to visualize liver abscesses in the live animal. In research settings, experimentally induced abscesses have been visualized as early as three days following inoculation (Lechtenberg and Nagaraja, 1991); abscesses appeared as a hyperechoic center of cellular debris surrounded by hypoechoic fluid.

Braun et al. (1995) examined the livers of cows by ultrasound of the right side and found large variation in rates of detection dependent on probe location (from the sixth to twelfth intercostal spaces), abscess size, and abscess characteristics (capsule identification, sites of echoic and anechoic content). Similarly, Franz (2008) noted variability in the appearance of liver abscesses in ultrasound images. Variation in abscess appearance is not surprising given the complexity of the host response to infection and its role in abscess formation (Nagaraja and Lechtenberg, 2007a). An additional limitation of ultrasonography to detect liver abscesses is the inability to evaluate regions of the liver obstructed by the lungs and other internal organs (Braun, 2009). As a diagnostic tool, ultrasonography can identify liver abscesses on the right side of the liver in regions not obstructed by the lungs or kidneys and offers valuable confirmation of inoculation in liver abscess experimentation. However, ultrasonography for liver abscess diagnosis of cattle in commercial feeding systems has questionable applicability and economic feasibility.

2.6.3 Blood Markers

To better understand and identify the systemic effects of liver abscess, blood metabolite and enzyme concentrations have been investigated in relation to liver abscess incidence. Japanese studies have identified increases in the glycan-related compounds (sialic acid and mucoprotein) and in proteases (kallikrein and prolidase) in cattle with liver abscesses (Motoi et al., 1985; Itabisashi et al., 1987; Motoi et al., 1987; Motoi et al., 1989). Motoi et al. (1992) also identified an increase of alpha-1-acid glycoprotein (AGP) in cattle with naturally occurring or induced liver abscesses. As an acute phase protein (APP), AGP is affected by inflammatory cytokines (Fournier et al., 2000) that have demonstrated a negative effect on animal performance (Gifford et al., 2012). Serum amyloid A, another APP, was identified by Tajik et al. (2013) to be moderately correlated with liver abscess size. Serum adenosine deaminase (ADA) activity was

observed by Ellah et al. (2004) to be increased with liver abscesses and ADA was noted in higher concentrations with increasing diffuse tissue damage. Presence of ADA indicates lymphocyte activity and is common in tissues with a high rate of cellular turnover (Van der Weyden and Kelley, 1976).

More recently, research focus has returned to general markers of liver function. In an effort to identify measurements that could indicate the presence of liver abscesses late in the finishing period, Macdonald et al. (2017) monitored 29 beef bulls fed a high-concentrate finishing diet; plasma samples were obtained at slaughter and at eight time points over the feeding period. At harvest, nine bulls exhibited abscessed livers. Analysis of plasma metabolites over the 56-day sampling period indicated an average increase in cortisol and aspartate aminotransferase and a decrease in albumin and cholesterol. At the time of slaughter, bulls with abscessed livers had lower levels of albumin and cholesterol. These data indicate metabolic stress existed in bulls with liver abscesses, both prior to and at harvest. Though potentially valuable for understanding how to identify liver abscesses before harvest, the study limitations of a small sample size, sex differences in comparison to most fed cattle, and application of blood sampling in commercial feeding scenarios must be realized.

2.7 PREVENTION AND CONTROL

Observation, ultrasonography, and blood metabolite analysis have been used to attempt to identify existing liver abscesses, but reduction of liver abscesses in cattle is centered around prevention and control rather than treatment. In a 2003 review of nutritional disorders affecting feedlot cattle, Galyean and Rivera summarized that detection of liver abscesses was impractical in large-pen settings and listed prevention as the only feasible approach. Control of liver abscesses in feedlot cattle has historically depended on antimicrobial use and sound nutritional

management. Amid pressures to reduce antimicrobial use, vaccines and an array of feed supplements have been researched as potential methods for liver abscess prevention.

2.7.1 Antimicrobial Feed Additives

The ruminant animal relies on a symbiotic relationship with microbes for production of useable metabolites and proteins (Hungate, 1966). The microbial ecology of the rumen is driven by adaptation to niche environments and is therefore unique with regard to substrate availability, oxygen levels, particle size, and passage rate (Yokoyama and Johnson in Church, 1988). Recent research has focused on manipulation of the rumen microbiome (Clemmons et al., 2019) specifically in response to dietary changes (Henderson et al., 2015). However, this concept of microbiome alteration has been practiced with fed antimicrobials since the 1950s, particularly in relation to rumen microbial development and liver abscess prevention (Mann et al., 1954; Matsushima et al., 1954; Flint and Jensen, 1958). By altering rumen microbial communities, researchers have hypothesized that levels of potentially pathogenic *F. necrophorum* can be reduced (Nagaraja and Chengappa, 1998). Though other feed additives attempt to alter the microbiome of post-weaning cattle, antimicrobials administered in feed are the most direct and well-researched method.

The United States Food and Drug Administration has approved bacitracin methylene disalicylate, chlortetracycline, oxytetracycline, neomycin sulfate with oxytetracycline, tylosin (as tylosin or tylosin phosphate), and virginiamycin for use in type B and C medicated feeds to reduce liver abscess incidence in feedlot cattle (US FDA, 2019). The susceptibility of *F. necrophorum* to various microbials has been thoroughly tested (Simon, 1977; Berg and Scanlan, 1982; Tan et al., 1994b; Mateos et al., 1997; Lechtenberg et al., 1998; Nagaraja et al., 1999). *Fusobacterium necrophorum* is vulnerable to macrolides (tylosin phosphate), tetracyclines

(chlortetracycline and oxytetracycline), streptogramins (virginiamycin), and penicillin but is resistant to aminoglycosides and most ionophores (Lechtenberg et al., 1998). However, the approved antibiotics differ in their inhibitory effects of *F. necrophorum* specific to its role in liver abscess formation (Nagaraja and Chengappa, 1998). Generally, tylosin phosphate is the most effective and bacitracin is the least effective with virginiamycin and chlortetracycline being intermediate in their effect on liver abscess incidence (Haskins et al., 1967; Brown et al., 1973; Brown et al., 1975; Smith et al., 1989; Rogers et al., 1995).

2.7.1.1 Tylosin

The most commonly fed antimicrobial for liver abscess prevention is tylosin phosphate (Reinhardt and Hubbert, 2015). In large feedlots (at least 1,000 head capacity), tylosin is fed to greater than 70% of cattle (USDA, 2011). Based on USDA (2011) survey data, cattle fed in feedlots with capacity of at least 8,000 head and cattle fed in the central region (Colorado, Nebraska, Kansas, Oklahoma, and Texas) were much more likely to receive tylosin. Due to survey-determined importance of macrolide use and lack of effective therapeutic alternatives, the World Organization for Animal Health classified macrolides including tylosin as a Veterinary Critically Important Antimicrobial Agent (OIE, 2018). In short, tylosin is currently widely fed to feedlot cattle in the United States but is scrutinized due to the importance of macrolide antibiotics for treating disease in livestock and human populations (Beukers et al., 2015).

The first set of feeding trials aimed to test the effect of tylosin in feedlot cattle with respect to liver abscesses was conducted by Brown et al. in 1973. Tylosin was found to reduce prevalence of liver abscesses to below 5% when prevalence of liver abscesses among control cattle exceeded 20% (Brown et al., 1973). Since the initial study, many others have tested the effects of tylosin with typical reductions in liver abscess prevalence between 40 and 70% (Brown

et al., 1975; Heinemann et al., 1978; Brink et al., 1990; Bartle and Preston, 1991; Tan et al., 1994a; Nagaraja and Chengappa, 1998). Vogel and Laudert (1994) concluded from a meta-analysis of 40 studies including 6,971 cattle that fed tylosin at 90 mg per head per day throughout the feeding period reduced liver abscess prevalence by 73%, improved average daily gain by 2.1%, and increased feed conversion by 2.6%. A more recent meta-analysis evaluating the impact of modern technologies on beef production found that tylosin inclusion reduced risk of liver abscess incidence from 30% to 8% (Wileman et al., 2009); however, only six studies were included from the systematic review due to criteria seeking multiple technologies. Research indicates that while tylosin supplementation reduces liver abscess prevalence, liver abscess incidence is not eliminated (Brown et al., 1973; Brink et al., 1990; Wileman et al., 2009). Typically, feedlot steers fed tylosin exhibit liver abscess incidence between 12 to 15% (Elanco, 2016). Though this is a reduction from the typical feedlot prevalence range of 12 to 32% (Elanco, 2016), the feeding of tylosin does not eliminate liver abscess occurrence.

Variability in liver abscess prevalence among cattle fed tylosin can be partially attributed to tylosin's mechanism of action. As a macrolide antibiotic, tylosin's hypothesized mechanism of action is to inhibit protein synthesis by reversibly binding to the bacterial ribosome (Boothe, 2016). It is suspected that the binding site for the 16-membered lactone ring of tylosin is located on the 23S rRNA molecule of the 50S ribosomal subunit (Mazzei et al., 1993; OIE, 2018). When a macrolide is bound to the ribosome, arrest of translation and dissociation of peptidyl-tRNA inhibits protein synthesis when specific short sequence motifs are detected (Kannan et al., 2014). As such, tylosin would be expected to function as a bacteriostat and limit bacterial proliferation (Endou et al., 1993).

In the rumen, tylosin has bacteriostatic properties on *Fusobacterium necrophorum* (Nagaraja et al., 1999b). During transition to high-concentrate diets, tylosin can reduce *F. necrophorum* concentrations by 80 to 90% (Nagaraja et al., 1999b). This indicates that tylosin inhibits the growth of *F. necrophorum* in the rumen under a physiological condition (presumptive high lactate concentration) that should promote logarithmic growth of the bacteria (Tadepalli et al., 2009). This indicates that tylosin could uniquely permeate the gram-negative cell envelope of *F. necrophorum*; macrolide antibiotics with known intracellular binding sites are typically thought to be mainly effective against gram-positive bacteria due to cell wall permeability (Hof, 1994).

While the effect of tylosin is thought to be primarily exerted in the rumen (Nagaraja and Chengappa, 1998), it is possible for macrolides to affect other locations in the body. Macrolide antibiotics are absorbed by the gastrointestinal tract (Gingerich et al., 1977; Scholar, 2007) and are widely distributed in tissues once absorbed (Boothe, 2016). Since tylosin is cleared by the liver, even minimal concentrations absorbed by the alimentary tract could concentrate in the liver (Boothe, 2016). Macrolide antibiotics have also been observed to concentrate in macrophages and polymorphonuclear leukocytes (Stein and Havlichek, 1992), directing bacteriostatic compounds to the site of infection. Though *F. necrophorum* and its leukotoxin can lyse cells of innate immune defense, the lysis could release tylosin near foci of infection. Tylosin likely inhibits *F. necrophorum* logarithmic growth in the rumen to prevent liver abscess formation and may slow *F. necrophorum* growth in the liver to reduce liver abscess severity.

Though historically effective and widely practiced (Reinhardt and Hubbert, 2015), feeding antimicrobials to livestock for liver abscess prevention is discouraged by growing concern of antimicrobial resistance. This is evidenced by governmental initiatives including the

National Antimicrobial Resistance Monitoring System in 1996 (CDC, 2019) and the Veterinary Feed Directive (VFD) in 2015 (US CFR, 2019). Following enactment of the VFD and removal of growth promotion (as opposed to therapeutic) claims on labels, use of antimicrobials important to human medicine in the food-animal industry dropped by over 30% (from 8,356,340 kg to 5,559,212 kg) in 2017 compared to the previous year (FDA, 2018). Across all food-animal species, macrolides accounted for 4% of total antibiotic use in 2017 with 274,479 kg of macrolides used specifically in the cattle industry (FDA, 2018).

Despite global rises in antimicrobial resistance, *F. necrophorum* has historically not demonstrated changes in susceptibility to tylosin. Minimum inhibitory concentrations for tylosin tested against *F. necrophorum* strains from cattle fed tylosin and strains from cattle not fed tylosin were similar, providing no evidence for selection of resistant *F. necrophorum* (Lechtenberg et al., 1988; Nagaraja et al., 1999a). However, *F. necrophorum* isn't the only microbe exposed to tylosin when the antibiotic is included in feedlot diets: all microbes in the rumen and feedlot ecosystem receive selective pressure from macrolide administration. *Trueperella pyogenes* has demonstrated macrolide resistance mechanisms (Jost et al., 2003; Jost et al., 2004). Similarly, resistant *Enterococcus spp.* (when used as an indicator bacterium) has been identified in feedlot cattle fed tylosin (Beukers et al., 2015). However, a 28-day withdrawal period prior to harvest was found to reduce levels of resistant *Enterococcus spp.* to levels observed in cattle not fed tylosin (Beukers et al., 2015). Using shotgun sequencing to characterize the resistome of steers fed tylosin and monensin, researchers found no difference in concentration of resistance genes in the rumen, cecum, or colon between steers fed antibiotics and steers not fed antibiotics; though macrolide resistance genes were found more frequently in

the rumen of steers fed antibiotics, no macrolide resistance genes were identified in the hind gut (Thomas et al., 2017).

Regardless, recent research findings suggest that the desired effects of tylosin may remain comparable even with a reduction in the duration of tylosin feeding. Müller et al. (2018) identified a similar reduction of liver abscess prevalence and severity in steers fed tylosin continuously or intermittently (every other week) with no effects on performance. Walter et al. (2018) studied the effects of feeding tylosin during different portions of the feeding period and found that the decrease in liver abscess prevalence and severity was similar when tylosin was fed for the first 126 days of the feeding period or continuously throughout the entire feeding period. Similarly, no increase in liver abscess prevalence was identified by Sides et al. (2009) when tylosin was removed from the diet the last 35 days of the feeding period. Additionally, Walter et al. (2018) concluded that their findings provided some evidence that the greatest risk for liver abscess formation exists early in the feeding period. However, previous observations indicated that liver abscess development most likely occurs during the last 60 days of the feeding period (Nagaraja and Lechtenberg, 2007a). Nonetheless, the beginning of the feeding period is an important period to consider in attempting to reduce liver abscess prevalence as tylosin is known to inhibit *Fusobacterium necrophorum* growth during step-ups to high-concentrate rations (Nagaraja et al., 1999b).

2.7.1.2 Other Antimicrobials

Though tylosin is the most effective and most commonly fed antimicrobial for liver abscess prevention, several other antibiotics have demonstrated efficacy in liver abscess prevention to some extent. The second most commonly fed antimicrobial labeled for liver abscess prevention is chlortetracycline (CTC), which is fed to approximately 18% of feedlot

cattle, specifically to those in smaller feedlots not in the central United States (USDA, 2011). When CTC and tylosin were fed as individual treatments in the same study, feeding CTC at 70 mg per head per day reduced liver abscess prevalence by 21% and decreased liver abscess severity by 35% (Brown et al., 1975). These reported reductions were less than half of the reduction in prevalence and severity observed for tylosin treatment groups (Brown et al., 1975). Oxytetracycline and virginiamycin are both fed to less than one percent of feedlot cattle (USDA, 2011). Reductions in prevalence and severity of liver abscesses when virginiamycin is fed to feedlot cattle are similar to when CTC is fed (approximately 30%); Smith et al., 1989; Rogers et al., 1995). Inclusion of the ionophore monensin in feedlot diets promotes consistent feed intakes and reduces incidence of acidosis by inhibiting rapid growth of Gram-positive lactate-producing bacteria (Heinemann et al., 1978; Pendlum et al., 1978, Schelling, 1984). However, monensin inclusion alone has not been effective for reducing liver abscess occurrence (Meyer et al., 2013). Though several antimicrobials have some level of efficacy in reducing liver abscess occurrence, tylosin remains the most widely used feed additive for liver abscess prevention.

2.7.2 Nutritional Management

Acidosis and the rumenitis-liver abscess complex have apparent ties to nutritional management (Reinhardt and Hubbert, 2015). Though nutritional decisions are largely based on economics of feeding efficiency, commodity availability, ingredient storage, and ingredient handling, sufficient research is available to consider liver abscess mitigation as part of the decision process.

2.7.2.1 Roughage

The inclusion level and form of roughage are included in most reviews of acidosis as key factors to prevent acidosis in feedlot cattle (Brent, 1976; Elam, 1976; Owens et al., 1998;

Galyean and Rivera, 2003; Nagaraja and Lechtenberg, 2007b; Galyean et al., 2010). As roughages are added to mixed rations in place of feed grains, fiber is added to the diet and the concentration of readily available carbohydrates is diluted (Smith et al., 1972; Gentry et al., 2016). Roughage feedstuffs are known to ferment more slowly in the rumen (Smith et al., 1972) and thus contribute to a more gradual decline in pH following consumption, in comparison to high-energy feeds. Additionally, higher roughage concentrations are associated with a higher ruminal pH and less time with ruminal pH depressed below 5.6, the threshold of sub-acute acidosis (Nagaraja and Lechtenberg, 2007b; Weiss et al., 2017). In addition to reducing acid accumulation in the rumen, including roughage of adequate particle size also increases rumination time (Mertens, 1997; Gentry et al., 2016). Rumination is associated with flow of saliva, a key buffer of the rumen environment (Erdman, 1988). By stabilizing the ruminal pH, roughage inclusion plays a critical role in maintaining consistent feed intake levels and mitigating acidosis (Fulton et al., 1979; Brown et al., 2000).

Generally, higher levels of roughage in the diet reduce liver abscess prevalence and severity (Nagaraja and Chengappa, 1998). Studies using a variety of roughage sources (including ground alfalfa hay, chopped alfalfa hay, cottonseed hulls, ground peanut hulls, and corn silage) have reported reductions in liver abscess prevalence with a greater inclusion level of roughage in the finishing diet (Harvey et al., 1968; Foster and Woods, 1970; Utley and McCormick, 1975; Gill et al., 1979; Bartle et al., 1994; Zinn and Plascencia, 1996; Loerch and Fluharty, 1998). Bartle et al. (1994) and Loerch and Fluharty (1998) also identified that decreasing the concentration of roughage in the diet throughout the feeding period resulted in greater liver abscess prevalence, while increasing concentration of roughage late in the finishing period resulted in reduced liver abscess prevalence. Still, elevated roughage levels during the growing

period still appear to be effective for controlling liver abscesses. Reinhardt et al. (1998) and Checkley et al. (2005) both found liver abscess occurrence to be reduced when cattle were grown on diets higher in roughage (either silage or grass hay and silage).

In addition to roughage inclusion, the degree to which roughage is processed seems to affect liver abscess prevalence. Utley et al. (1973; 1974) identified that grinding or grinding and pelleting peanut hulls reduced the effective value of the roughage and was associated with higher liver abscess prevalence. Similar conclusions were drawn by Calderon-Cortes and Zinn (1996) from a two-by-two factorial study that investigated particle size and inclusion of ground hay in feedlot diets. Calderon-Cortes and Zinn (1996) found hay ground through a 7.6-cm screen reduced liver abscess prevalence in comparison to hay ground through a 2.5-cm screen; interestingly, no effect of roughage level (from 8 to 16% ground hay inclusion) on liver abscess prevalence was demonstrated. These results indicated that 8% inclusion of hay could provide sufficient physical stimulation to maximize salivary buffering by rumination and optimize epithelial health in finishing cattle (Calderon-Cortes and Zinn 1996). This aligns with the typical roughage inclusion of roughly 9% in finishing diets reported by Galyean and Gleghorn (2001) in a survey of consulting feedlot cattle nutritionists.

Formally discussed by Mertens (1997), the concept of appropriate particle size in combination with adequate roughage levels is referred to as physically effective neutral detergent fiber (peNDF). Evidence for the positive effects of physical stimulation of the rumen is provided by comparisons of wheat straw and cottonseed hulls fed as roughage sources to beef steers; steers fed wheat straw demonstrated greater rumination time (Moore et al., 1990). Additionally, the addition of indigestible objects (plastic pot scrubbers) into the rumen for physical stimulation

has been associated with fewer liver condemnations in feedlot cattle fed an all concentrate diet (Loerch, 1991).

Though neutral detergent fiber (NDF) is typically a sufficient measure to determine adequate roughage levels (Galyean and Defoor, 2003), increasing NDF in the diet by supplementing a starch-depleted energy source for a high-starch feedstuff does not reduce liver abscess incidence. For example, when distillers grains replace a rolled feed grain in finishing diets of feedlot cattle, no reduction in liver abscess prevalence is observed (Yang et al., 2012; Meyer et al., 2013; He et al., 2014). In short, over-processing of fibrous feedstuffs can minimize the beneficial effects of roughage in feedlot diets.

Increased roughage level in the diet has been tested as a potential replacement for tylosin. Holland et al. (2018) assigned 3,340 steers to dietary treatments of 1) 7.1% corn stalks with tylosin, 2) 7.1% corn stalks with no tylosin, 3) 13.1% corn stalks with no tylosin, or 4) 19.1% corn stalks with no tylosin. In diets three and four, corn stalks replaced steam flaked corn on a DM basis. On average, Tylosin inclusion reduced liver abscess prevalence (from 19% to 13%). A linear decline in liver abscess occurrence was observed with increasing roughage level; liver abscess prevalence for the diet four treatment group was similar to that of the tylosin treatment group (Holland et al., 2018). Foster and Woods presented results in 1970 that suggested a decrease in liver abscess prevalence among cattle fed diets with increased roughage levels. Though roughage has been effective in reducing liver abscess occurrence, Holland et al. (2018) found increased roughage level did not reduce abscess severity. Additionally, increased roughage inclusion resulted in increased dry matter intakes and decreased hot carcass weight among cattle with similar days on feed (Holland et al., 2018). Generally, replacing feed grains with roughage

is costly on an energy basis and often results in reduced efficiency and therefore an increased cost of gain (Galyean et al., 2010; Reinhardt and Hubbert, 2015).

2.7.2.2 Grain Processing

Processing grains to be used for feedstuff affects starch availability and can consequently alter ruminal rate of fermentation and pH decline, two factors associated with acidosis (Reinhardt and Hubbert, 2015). Greater starch availability and a higher rate of fermentation is achieved with ground high-moisture corn and steam flaked corn (SFC) than with dry rolled corn with favorable impacts on nutrient absorption (Galyean et al., 1976; Cooper et al., 2002). Reducing bulk bushel weight of SFC (a greater degree of processing) increases starch gelatinization and ruminal starch availability (Zinn, 1990; Sindt et al., 2006). However, greater starch availability can lead to more rapid fermentation and pH decline, increasing the risk of acidosis. Though grain processing affects ruminal physiology, many studies have demonstrated that grain processing has no effect on liver abscess occurrence (Mader et al., 1991; Huck et al., 1998; Loerch and Fluharty, 1998). These results suggest that changes in fermentability of grain, as a result of processing, has a limited effect on liver abscess formation, especially in comparison to roughage level and form included in the diet (Reinhardt and Hubbert, 2015).

2.7.3 Bunk Management

Consistent management of feed delivery quantity and timing to control intake is critical for prevention of acidosis and liver abscesses (Nagaraja and Lechtenberg, 2007b; Elanco, 2016). The challenge for feedlot managers is that dry matter intake is highly variable in feedlot cattle (Stricklin and Kautz-Scanavy, 1984; Hicks et al., 1989; Cooper et al., 1998; Soto-Navarro et al., 2000). Delayed time of feeding induces greater variability in intake pattern (Nagaraja and Titgemeyer, 2007; González et al., 2009). Additionally, limiting linear bunk space per animal

increases competition for feed, and results in greater animal-to-animal intake variation and fewer and more rapid meals eaten per day (González et al., 2008a; González et al., 2008b). Large and rapidly consumed meals expose cattle to more drastic changes in ruminal pH and can be a predisposing factor for acidosis. Pritchard and Bruns (2003) reported that consistency of intake, and resulting stability of the rumen environment, is promoted by a consistent daily feed delivery time and adequate bunk space (Pritchard and Bruns, 2003). Additionally, mud and snow depth, temperature extremes, illness, and lameness affect bunk attendance and feed intake of feedlot cattle (Nagaraja and Lechtenberg, 2007b).

Similarly, feeding program can impact intake consistency (Pritchard and Bruns, 2003). Limit feeding (feeding around 80% of expected dry matter intake) and programmed feeding (feeding 90 to 95% of expected dry matter intake) reduces variation in daily dry matter intake by maintaining a consistent appetite in feedlot cattle (Pritchard and Bruns, 2003). However, expected dry matter intake can be difficult to estimate and underfeeding results in loss of potential performance and can increase days on feed or reduce carcass weight. As such, consistency of intake is typically achieved at the loss of maximal performance per day. Clean-bunk (or slick-bunk) feeding is performed by constantly adjusting feed delivery in order to provide cattle a level of feed equal to the desired intake, with no day-to-day carryover of feed in the bunk. Theoretically, this system avoids performance restriction while reducing feed waste; slick-bunk feeding is a common management practice in the United States (Pritchard and Bruns, 2003). Reactive in nature, slick-bunk feeding likely allows for greater day-to-day intake variation by individual animals and relies heavily on accuracy of feed delivery estimations. However, when feed delivery occurs twice daily, the system can result in smaller and more frequent meals (González et al., 2012). *Ad libitum* feeding programs offer unrestricted feed access to all animals

at all times. While this strategy does reduce competition and some negative effects of social behavior, individual animals are able to more freely determine their intake levels and are often more sensitive to environmental changes, resulting in greater feed intake variation; the carryover of feed also increases waste (Pritchard and Bruns, 2003). Regardless of feeding program, gradual transition to high-concentrate diets is important for long-term stability of feed intake in the feedlot setting (Brown et al., 2006).

2.7.4 Non-Antimicrobial Feed Additives

In attempt to find novel solutions to address liver abscess occurrence, many feed ingredient alternatives have been tested in feedlot cattle. Though generally not as effective as tylosin for liver abscess prevention, direct fed microbials (DFM), essential oils (EO), vitamins, and minerals have been used as feed supplements.

2.7.4.1 Direct-Fed Microbials

Direct fed microbials (a source of live microorganisms) are fed to cattle in an attempt to modify the microbiome to promote efficient fermentation and to stabilize the rumen environment (Krehbiel et al., 2003). Direct fed microbials (either bacteria or fungus) employ various modes of action: competitive attachment (Jones and Rutter, 1972), antimicrobial metabolite production (Gilliland and Speck, 1977), and modulation of host immunity (Erickson and Hubbard, 2000). It is hypothesized that upon inoculation of the rumen with microorganisms that are involved in critical pathways, these microorganisms (DFMs) colonize to make up a greater proportion of the rumen microbiome and enhance the desired pathways. The fermentation products of DFMs included in supplements could also act as substrates to temporarily alter rumen fermentation pathways.

Bacterial DFMs are typically lactate-utilizing bacteria administered as a feed supplement to promote propionate production and reduce acidosis occurrence; common bacteria utilized include *Lactobacillus acidophilus, Propionibacteria spp.,* and *Megasphaera elsdenii* (Krehbiel et al., 2003). In the few studies that have evaluated liver abscess prevalence after supplementing a bacterial DFM, no effect on liver abscess prevalence was observed (Trenkle, 2003).

Fungal DFMs are typically derived from yeasts or yeast extracts. *Saccharomyces cerevisiae* and *Aspergillus oryzae* are among the most common yeast products added to animal feeds; these appear to stimulate lactate utilization by ruminal *Selenomonas ruminantium* (Martin and Nisbet, 1992). While a numerical decrease in liver abscess prevalence was observed when feedlot cattle are fed fungal DFMs by Scott et al. (2017), others have found no reduction in liver abscess prevalence (Swyers et al., 2014). Huebner et al. (2019) reported no effect of a *Saccharomyces cerevisiae* product on liver abscess prevalence or severity in a large-pen randomized complete block study including 4,689 steers. While DFMs may be beneficial for promoting lactate utilization in the rumen, there is not sufficient evidence to indicate that they reduce liver abscess occurrence.

2.7.4.2 Essential Oils

Essential oils are aromatic secondary plant metabolites extracted from volatile plant compounds by steam distillation or organic solvents (Croteau and Ronald, 1983; Patra and Saxena, 2010). Varying antimicrobial properties have been documented for EOs (Hammer et al., 1999). Nazzaro et al. (2013) proposed that the hydrophobic properties of EOs allow them to disrupt the bacterial cell envelope, generally of gram-positive bacteria, thereby gaining entry to interact with intracellular proteins. Thymol (thyme extract), eugenol (clove extract), vanillin (vanilla extract), limonene (lemon extract), anise oil, capsicum oil, cinnamaldehyde (cinnamon

extract), guaiacol (guaiacum extract), linalool (scented herbs), and α-pinene (pine extract) have all been administered to livestock for antimicrobial and fermentation-altering effects (Beauchemin and McGinn, 2006; Cardozo et al., 2006; Fandiño et al., 2008; Meyer et al., 2009; Yang et al., 2010a; Yang et al., 2010b). Khiaosa-ard and Zebeli (2013) found effects on performance and ruminal fermentation to be marginal in a meta-analysis of a wide variety of EOs.

Relative to liver abscesses, *Fusobacterium necrophorum* may be inhibited by EOs. Elwakeel et al. (2013) found that concentrations of 20 or 100 μg/mL of limonene or 100 μg/mL of thymol were sufficient to halt *F. necrophorum* growth in an in vitro study. When Meyer et al. (2009) fed a mixture of five EOs (including limonene and thymol) to feedlot steers at a level of one gram per head per day, liver abscess prevalence was intermediate (16.6%) to the negative control and tylosin treatment (27.2% and 6.5%, respectively).

2.7.4.3 Vitamins and Minerals

Several studies have been performed to test the effects of micronutrients on liver abscess prevalence. When the diet of finishing cattle was supplemented with alpha-tocopherol acetate (Vitamin E) either solely or in combination with ascorbate (Vitamin C), Müller et al. (2018) found that liver abscess prevalence was unaffected. Similarly, when ethoxyquin (an antioxidant used in food preservation) was supplemented to finishing cattle, liver abscess prevalence was not significantly affected (Krumsiek and Owens, 1998). Studies by Van Bibber-Krueger et al. (2015; 2016; 2017a; 2017b) found no effect of supplemental zinc, calcium, or chromium on liver abscess occurrence in feedlot cattle. However, Lundy et al. (2017) found a numerical decrease in liver abscess prevalence when steers were fed a zinc-amino acid complex in a small pen study. While chelation may alter mineral bioavailability (Spears, 1996) and explain the numerically

greater reduction in liver abscess occurrence when a chelated mineral was supplemented compared to a non-chelated mineral, more research is needed to determine the applicability of mineral supplementation to liver abscess prevention.

2.7.5 Vaccine

The development of an effective vaccine to prevent liver abscess formation would likely reduce the use of antibiotics and other supplements in feedlot diets. A liver abscess vaccine would be particularly valuable for natural cattle as they are fed longer than conventional cattle and antimicrobials are prohibited. With well-established virulence factors and major roles in liver abscess pathogenesis (Tan et al., 1994b; Tan et al., 1994c; Narayanan et al., 2002; Tadepalli et al., 2008; Menon et al., 2018), *Fusobacterium necrophorum* would be a natural vaccine target. Several studies have reported discovery of serum antibodies against *F. necrophorum* (W H Feldman et al., 1936; Tan et al., 1994b) and attempts were later made to induce protective immunity a variety of antigenic compounds (Nagaraja and Chengappa, 1998). Researchers used whole-cell cultures, culture supernatants, cytoplasmic fractions, outer membrane proteins, lipopolysaccharides, and leukotoxins in attempts to develop effective vaccines (Nagaraja and Chengappa, 1998). The investigations resulted in commercialization of two products: Centurion™ (formerly Merck Animal Health, Madison, NJ) and Fusoguard® (Elanco Animal Health, Greenfield, IN). Though no longer commercially manufactured, Centurion™ included pyolysin from *Trueperella pyogenes* and the leukotoxin from *Fusobacterium necrophorum* as active ingredients (Nagaraja and Lechtenberg, 2007a). Though typically marketed for foot rot prevention, Fusoguard® is an attenuated vaccine labeled for prevention of liver abscesses as well (Amachawadi and Nagaraja, 2016).

The lack of widespread market establishment of both Centurion™ and Fusoguard® following USDA approval is indicative of the vaccine cost relative to efficacy for liver abscess prevention. Fox et al. (2009) assigned 1,307 naturally managed feedlot cattle to control, Centurion™, or Fusoguard® vaccination treatments. After being finished on a SFC-based diet for nearly 240 days, cattle were harvested. Liver abscess prevalence across all treatments was 56%; no effect of vaccine treatment was observed on liver abscess prevalence or severity (Fox et al., 2009). Checkly et al. (2005) presented similar results, further supporting the idea that current vaccines are ineffective for reduction of liver abscess occurrence in cattle prone to high rates of liver abscess incidence. However, it is possible that vaccination after the timepoint of *Fusobacterium* colonization is ineffective. Wiley et al. (2011) found a numerical benefit to vaccinating cows and calves to reduce infection with pathogenic bacteria. Prevention of *Fusobacterium* colonization in calves prior to feedlot entry could provide a new target for vaccine studies.

2.8 DIRECTION OF FUTURE WORK

The pathology of liver abscesses is generally accepted as infection within the liver caused by inoculation of commensal bacteria from the gastrointestinal tract into portal blood. Though generally associated with rumenitis, the exact site of bacterial entry into the blood is poorly understood. Bacterial entry into portal circulation from the lower gastrointestinal tract might be possible and unresearched. Similarly, little is known regarding the timing of abscess formation and healing; factors that could mitigate bacteria within portal circulation or expedite healing are also unexplored.

In most feedlots, prevention of liver abscess formation is currently limited to diet formulation, roughage inclusion, bunk management, and feeding of tylosin. Current research into

liver abscess occurrence in feedlot cattle focuses on both prevention and detection of liver abscesses. Recently, researchers have concentrated on the development of new feed additives to control liver abscesses that are perceived as more natural than the use of subtherapeutic feeding of antimicrobials. Additionally, research has been aimed toward finding effective methods of detection of liver abscess throughout the feeding period.

Culture-based methods have historically been used to determine the composition of liver abscess purulent material, but advancement in molecular techniques (specifically 16S rRNA sequencing and shotgun metagenomics) enable a broader understanding of the microbiome of the liver abscesses. With greater knowledge of abscess composition, more specific research questions will guide future investigations of abscess pathology and prevention. Advancement in molecular techniques also enable deeper understanding of microbial communities that reside in the bovine gastrointestinal tract. Though microbial interactions are complex and compartmentalized, the establishment and dysbiosis of the ruminal, intestinal, and fecal microbiomes are beginning to be studied. Considering the known link between nutritional stress and liver abscess formation, future liver abscess investigation should be informed by the growing knowledge of microbiomes.

Though most liver abscess research has been conducted following placement of cattle at feedlots, it is likely that many aspects of cow/calf and stocker management affect liver abscess occurrence. Previously, genetic variation (Keele et al., 2016) between individual animals has been used to explain some variation in liver abscess incidence. However, more research is needed to enable appropriate application of the findings. Additionally, calving systems, climate, soil characteristics of range lands and dry lots, creep feeding, and backgrounding systems are all possible influences on microbiome development and predispose responses to dietary changes.

Studies have demonstrated that responses to acidosis are largely influenced by feeding history (Silberberg et al., 2013). To avoid confounding in future large-scale studies and to better understand programming effects of management systems, future research into the rumenitis-liver abscess complex should consider factors that predispose cattle to developing liver abscesses throughout the feeding period.

CHAPTER III: FECAL MICROBIOTA OF FEEDLOT STEERS MANAGED IN NATURAL AND CONVENTIONAL PROGRAMS

3.1 OVERVIEW

The bovine microbiome is becoming better understood in the context of feed efficiency and gastrointestinal disease states; however, changes in the fecal microbiota of feedlot steers in commercial settings has not been investigated throughout the feeding period, particularly in regard to standard step-up feeding programs. The objectives of this observational study were two-fold: i) to characterize the fecal microbiota of steers managed in conventional and natural programs throughout the feeding period with respect to diet changes, and ii) to evaluate the relationship between the diversity of the fecal microbiota and liver abscess prevalence observed at harvest. At placement, pens of steers enrolled in natural (n = 7 pens) and conventional (n = 7 pens) management programs were identified for repeated fecal sampling throughout the finishing period. At 14-day intervals, individual samples were collected from 10 individual fecal pats, and one composited sample including 12 separate fecal pats was collected from freshly voided feces on each pen floor. All steers were fed using a step-up feeding program including receiving, intermediate, and finishing diet formulations. Conventionally managed steers received a hormonal implant and were administered tylosin, monensin, and ractopamine through feed delivery. The V4 region of the 16S rRNA gene was used to characterize the microbiota of fecal samples after placement (T1), after transition to the finishing diet (T2), immediately before the beta-adrenergic agoinst feeding period (T3), and immediately before shipment for harvest (T4). Across all time points, the phyla Firmicutes and Bacteroidetes composed greater than 88% of the fecal microbiota. The relative abundance of Bacteroidetes increased following transition to the

finishing diet, largely driven by increases in Prevotellaceae. A large change in beta diversity (measured as unweighted UniFrac) was observed between T1 and T2 ($P = 0.001$). At T1, fecal microbiotas of naturally managed steers had greater alpha diversity, measured as richness ($P = <0.001$) and Shannon Diversity Index ($P = <0.001$); however, no differences in richness ($P = 0.828$) or Shannon Diversity Index ($P = 0.774$) were observed between management programs at T4. Across both management programs, greater Shannon Diversity within pen-level composited samples at T4 was associated with decreased liver abscess prevalence ($r_s = -0.438$).

3.2 INTRODUCTION

A reduction in cost of metagenomic sequencing has allowed researchers to gain a greater understanding of the microbiota of feedlot cattle (Henderson et al., 2015; Shabat et al., 2016; Clemmons et al., 2019). The microbiota of the hind gut of feedlot cattle has been investigated by shotgun and 16S sequencing (Shanks et al., 2011; Beukers et al., 2015; Chopyk et al., 2016). Through research that has evaluated the fecal microbiota under conditions of nutritional disorders and with respect to starch passage to the hind gut (Shanks et al., 2011; Thompson et al., 2017; Azad et al., 2019), diet has been identified as a determinant of fecal microbial communities. Nonetheless, little is understood about the longitudinal shifts of fecal microbial populations throughout the feeding period of steers adapted to high-concentrate finishing diets.

The fecal microbiota could function independent of the rumen microbiota and serve as an indicator of nutritional stress (Azad et al., 2019; Ogunade et al., 2019). Liver abscess occurrence is commonly identified as a nutrition-related pathology due to association with rumenitis and acidosis (Nagaraja and Lechtenberg, 2007a). Liver abscess formation in feedlot cattle is attributed to *Fusobacterium necrophorum* from the digestive tract being released into the portal circulation by compromised epithelial tissue (Nagaraja and Chengappa, 1998). Since the

microbial communities of the rumen contents and the rumen epithelium have demonstrated changes in response to acidosis (Petri et al., 2013; Ogunade et al., 2019), the development of liver abscesses could also be impacted by these factors. The objectives of this observational study were two-fold: to characterize the fecal microbiota of steers managed in conventional and natural programs throughout the feeding period with respect to diet changes and to evaluate the relationship between the diversity or change in diversity of the fecal microbiota over the feeding period and liver abscess prevalence and severity observed at harvest.

3.3 MATERIALS AND METHODS

3.3.1 Cattle Population

Fourteen pens of yearling steers with an average of 281 steers per pen (range 212 to 323; SD = 38 steers per pen) were identified for observation in a commercial feedlot in the High Plains region. Pens enrolled in a conventional management program (n = 7) and pens enrolled in a natural management program (n = 7) arrived at the feedlot over a 45-day period from late August through early October 2018. Upon arrival, all steers were sorted and vaccinated according to standard feedlot protocol. Conventionally managed cattle received hormonal implants containing trenbolone acetate and estradiol.

All steers were fed using a step-up feeding program including receiving, intermediate, and finishing diet formulations. Conventionally managed steers were fed a fourth diet including Optaflexx (Elanco Animal Health; Greenfield, IN) during the final 28 to 42 days of the finishing period. Rumensin (Elanco Animal Health) and Tylan (Elanco Animal Health) were also fed to conventionally managed steers. Naturally managed cattle were not administered growth-promoting technologies or antibiotics. In the natural pens, if treatment with an antibiotic was necessary, the animal was removed from the pen and consequently, the study population

3.3.2 Fecal Sample Collection

Individual and pen-level composited fecal samples were collected from each pen at 14-day intervals throughout the feeding period. Though not feasible to collect individual fecal samples from the same steers every collection, individual samples were collected to evaluate animal-to-animal variation within the pen. Composited samples were collected to summarize the microbiota present within the pen. Beginning seven days (SD = 2.9 days) after placement, freshly voided and undisturbed individual fecal pats were sampled from pen floors. For all samples, the outermost layer of the fecal pat was removed using a sterile glove to mitigate environmental contamination. Individual samples (n = 10 per pen) consisted of approximately 100 g of feces from distinct fecal pats; each sample was placed in a sterile collection bag and immediately sealed for transportation. Pen-level composited fecal samples were created by combining 30 g of feces from 12 fecal pats (not collected for individual-level sampling) collected from equally spaced locations along crossing diagonals of each pen as previously described (Noyes et al., 2016; Yang et al., 2016). Composited samples were manually homogenized within the sterile collection bag immediately following collection. Fecal samples were placed in insulated containers for transport to the Center for Meat Safety and Quality at Colorado State University (Fort Collins, CO). Upon arrival, feces were manually homogenized within collection bags and aseptically transferred to sterile 50-mL conical tubes (VWR; Radnor, PA). Aliquots of feces were stored at -80 °C until the time of DNA extraction.

3.3.3 Selection of Fecal Samples for Analysis

From the biweekly fecal collection dates, four time points were identified to represent distinct phases throughout the feeding period; time points were selected based on placement, diet

change, and shipment dates. Individual and pen-level samples collected at these four time points were used for fecal microbiota analysis. The first time point (T1) was selected to represent the fecal microbiota at placement. Averaged across all pens, T1 occurred on average of 7 days (SD = 3 days) following placement. The second time point (T2) was selected to represent the fecal microbiota following transition to the finishing diet. Averaged across all pens, T2 occurred on average 10 days (SD = 4 days) following the first day of the pen being fed the finishing diet. Timepoints T1 and T2 were separated by an average of 30 days (SD = 5 days). The third timepoint (T3) was selected to represent the fecal microbiota after adaptation to the finishing diet, but before transition to the period of β-adrenergic agonist feeding for conventionally managed cattle (time point was mimicked for naturally managed cattle). Averaged across all pens, T3 occurred 117 days (SD = 21 days) after T2 and 42 days (SD = 4 days) prior to harvest. The fourth timepoint (T4) was selected to represent the fecal microbiota at shipment for harvest. Averaged across all pens, T4 occurred 9 days (SD = 3 days) prior to shipment and 33 days (SD = 7 days) after T3. From each pen, at each of the four time points described, the pen-level composite sample and a randomly selected subset of individual samples (n = 5) were subject to microbiota analysis.

3.3.4 Liver Evaluation

All steers were marketed without modification to feedlot protocols. After an average of 196 days on feed (SD = 22 days), cattle were shipped to a commercial processing facility for harvest from February through April of 2019. Identities of feedlot pens were maintained through the harvest process to allow for determination of liver abscess occurrence by pen. All livers (N = 3,929) were evaluated for abscess prevalence and severity by trained personnel using the Elanco Liver Check System (Elanco, 2016) immediately after carcass evisceration. Livers without

visible abscesses were scored as normal (0); livers with abscesses were scored as severe (A+) or mild and regressing (A).

3.3.5 DNA Extraction and Sequencing

DNA extraction and library preparation were performed at the Metcalf Laboratory at Colorado State University (Fort Collins, CO). Selected fecal samples were thawed to 4 °C prior to extraction. Fecal sample aliquots were individually sampled in a randomly assigned order with sterile swabs (BD; Franklin Lakes, NJ) and loaded into 96-well plates by cutting the inoculated swab tip into the plate well with flame-sterilized scissors. Cross contamination was controlled by covering all inactive wells with tape. Forty negative controls and six positive controls (ZymoBIOMICS Microbial Community Standard 6300; Zymo Research; Irvine, CA) were included. Twenty technical replicates were included. The loaded plate was stored at -20 °C until the time of DNA extraction.

DNA was extracted using the DNEasy PowerSoil HTP 96 Kit (Qiagen; Hilden, Germany) following the manufacturer's protocol; however, vortex procedures were replaced with manual pipetting to avoid cross contamination. The extraction product was amplified with barcoded primers targeting the V4 region of the 16S RNA gene. Primer constructs included the Illumina MiSeq adaptor (Illumina; San Diego, CA), Golay barcode, spacer, and primer. Earth Microbiome Project (EMP) primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) were used for amplification (Caporaso et al., 2011; Caporaso et al., 2012; Apprill et al., 2015; Parada et al., 2016).

Polymerase chain reaction (PCR) was conducted in duplicate using an Eppendorf Vapo.Protect MasterCycler Pro-S thermocycler (Eppendorf; Hauppauge, NY). For the initial PCR, 25 µL of reaction mix was prepared by combining 1 µL of template DNA, 1 µL of each

barcoded primer (10 μM), 12 μL of molecular-grade water, and 10 μL of Platinum Hot Start PCR Master Mix (Thermo Fisher Scientific; Waltham, MA). PCR conditions followed EMP protocols and included initial denaturation at 94 °C for 3 min; 30 cycles of denaturation (94 °C, 45 s), annealing (50 °C, 60 s) and elongation (72 °C, 90 s); and a final 10-min extension at 72 °C. PCR products were visually evaluated for effective amplification by agarose gel electrophoresis with expected band size of approximately 300 to 350 bp. Similarly, negative controls were visually evaluated for lack of banding pattern. When plates were confirmed of acceptable quality and purity, the second PCR process was completed using the same reaction conditions as described above; 50 μL of reaction mix was prepared by combining 2 μL template DNA, 2 μL of each barcoded primer (10 μM), 24 μL of molecular-grade water, and 20 μL of PCR Master Mix. Agarose gel evaluation of the product was performed as described above.

Duplicate PCR products were pooled and subjected to agarose gel electrophoresis to visualize correct sizes of PCR products and the absence of signal from negative controls. The concentration of amplicon products was determined by Quant-IT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) read on a Fluoroskan (Thermo Fisher Scientific) plate reader. Pico assay concentration results were qualitatively verified by comparison to agarose gel banding patterns. Amplicons were pooled by plate with a target amplicon inclusion of 300 ng of DNA per sample. No more than 50 μL from a single sample were added to the amplicon pool to maintain a reasonable volume. Pooled amplicons were cleaned using MinElute PCR Purification Kit (Qiagen) following manufacturer protocols. The cleaned plate pool was evaluated for DNA concentration by NanoDrop Lite spectrophotometer (Thermo Fisher Scientific) and pooled in equimolar concentrations to form the final sequencing library.

The amplicon library was diluted to a loading concentration of 8 pM and combined with 15% PhiX control library. Paired-end sequencing (2 × 250 bp) was performed using the 500 cycle MiSeq Reagent Kit v2 (Illumina; San Diego, CA) on the Illumina MiSeq platform at the Next Generation Sequencing Core Laboratory at Colorado State University.

3.3.6 Bioinformatics and Statistical Analysis

All amplicon sequence data were bioinformatically processed in QIIME2 version 2019.4 (Bolyen et al., 2018). Imported and demultiplexed paired-end sequences were denoised with DADA2 (Callahan et al., 2016) with both forward and reverse reads trimmed to 248 bp. Feature tables and representative sequences from separate sequencing runs were merged using the q2-feature-table plugin. Taxonomy was assigned with the q2-feature-classifier plugin (Bokulich et al., 2018) using a pretrained naive Bayes Greengenes 13_8 classifier (DeSantis et al., 2006; McDonald et al., 2012), a pretrained naive Bayes Silva 132 classifier, and a Silva 132 (Quast et al., 2013) classifier trained specifically for the primer set used for amplification. The pretrained Silva classifier was selected for downstream analysis as it exhibited the fewest unclassified amplicon sequence variants. Reads classified as mitochondria and chloroplast were removed from the data set; controls and technical replicates were also removed. Amplicon sequence variants were assigned phylogeny using SEPP methodology to construct an insertion tree with q2-fragment-insertion (Janssen et al., 2018). Adequate sampling depth was justified by constructing a rarefaction curve with diversity metrics. Sampling depth was standardized for diversity analysis by subsampling without replacement (Weiss et al., 2017) to 9,367 sequences per sample using q2-diversity.

Individual samples were analyzed within time point and compared across management programs, cattle sources, and pen assignments. Alpha diversity was measured by richness (the

number of observed taxonomic units) and Shannon Diversity Index (Shannon, 1948). Differences in richness and Shannon Diversity were evaluated between management programs and pens with Kruskal-Wallis testing (Kruskal and Wallis, 1952) and visualized in R version 3.4.1 (R Core Team, 2017) using ggplot2 (Wickham, 2009). Beta diversity was measured with unweighted UniFrac (Lozupone and Knight, 2005) and weighted UniFrac (Lozupone et al., 2007). Group significance for management program and pen was evaluated for beta diversity with PERMANOVA testing (Anderson, 2017). Principal Coordinate Analysis (PCoA) was used to spatially visualize samples (Vázquez-Baeza et al., 2013). PCoA plots generated in QIIME2 were exported for visualization in Vega version 5.4.0 (Satyanarayan et al., 2016). Taxa differential abundance was evaluated by ANCOM testing at both the phylum and family level (Mandal et al., 2015). Significance for differential abundance was evaluated as a W value indicating log-fold change against a model-determined threshold based on a bimodal distribution. Rarefied abundance data was exported from QIIME2 as relative abundance and visualized in R version 3.4.1 (R Core Team, 2017) using ggplot2 (Wickham, 2009) to characterize the composition of the microbiota.

Composited samples were analyzed as described above and using the q2-longitudinal plugin (Bokulich et al., 2017). Pairwise difference comparisons for richness and Shannon Index were performed within management program for composited samples between T1 and T4 using the Wilcoxon-Signed-Rank test (Wilcoxon, 1945) corrected for FDR (Benjamini and Hochberg, 1995). Differences in change between management programs were evaluated with Mann-Whitney U (Mann and Whitney, 1947). Non-parametric microbial interdependence testing (NMIT) was performed to determine longitudinal sample similarity as a function of temporal microbial composition (Zhang et al., 2017); PERMANOVA testing (Anderson, 2017) was used

to test distances produced from a pairwise correlation matrix of each microbial feature's relative abundance. NMIT of family-level taxa was conducted to compare management programs over the four selected timepoints of the feeding period.

The sequencing depth of each negative control was evaluated to ensure cleanliness of extraction and library preparation; the number of reads generated by each control well before and after denoising were recorded. The sequencing depth of positive controls was similarly recorded. Additionally, the taxa relative abundance of each positive control was exported and visualized in R version 3.4.1 (R Core Team, 2017) using ggplot2 (Wickham, 2009) and compared to the known composition of the mock community for qualitative evaluation. Technical replicates were qualitatively evaluated in pairs by taxa relative abundance to ensure consistency of taxa relative abundance between separate extraction plates.

Liver abscess presence was recorded for all carcasses and summarized as pen-level prevalence. Prevalence was calculated as the number of abscessed livers observed divided by the total number of livers evaluated per pen. Liver abscess occurrence (both overall and severe) was analyzed using logistic regression in R version 3.4.1 (R Core Team, 2017) using lme4 (Bates et al., 2015) and emmeans (Searle et al., 1980). A mixed effects model was fit using management program as a fixed effect and pen assignment as a random effect. Spearman correlations (Spearman, 1904) were calculated between liver abscess prevalence and alpha diversity and change in diversity over the feeding period in R version 3.4.1 (R Core Team, 2017). A predetermined alpha level of 0.05 was used for all comparisons in the observational study.

3.4 RESULTS AND DISCUSSION

3.4.1 DNA Sequencing Data

A total of 9,178,093 sequence reads were generated by Illumina MiSeq sequencing for fecal samples, technical replicates, and controls. Fecal samples and replicates (n = 520) averaged 26,783 sequences per sample (range: 7,788 to 69,584; SD = 7,667). Negative controls (n = 40) averaged 76 sequences per sample (range: 1 to 644; SD = 132). Positive controls (n = 6) averaged 24,827 sequences per sample (range: 14,918 to 39,812; SD = 8,198).

Following denoising, filtering for sequencing depth, and removing controls, replicates, chloroplasts, and mitochondria, a total of 6,673,232 reads mapped to 7,825 unique features were included for analysis. Rarefying to 9,367 sequences per sample resulted in retaining 279 of 280 individual fecal samples and 55 of 56 composited fecal samples. After denoising, negative controls (n = 40) averaged 28 sequences per sample (range: 0 to 309; SD = 62) and positive controls (n = 6) averaged 17,165 sequences per sample (range: 12,552 to 23,773; SD = 3,889).

3.4.2 Alpha Diversity Within Fecal Microbial Communities

3.4.2.1 Feeding Period Overview

Based on individual fecal samples averaged across all timepoints, the fecal microbial communities of steers managed in the natural program had greater richness compared to those of steers managed in the conventional program ($P = <0.001$). Fecal microbial communities from steers managed in the natural program contained an average of 348 OTUs (SD = 79), and fecal microbial communities from steers managed in the conventional program contained an average of 299 OTUs (SD = 91). Richness of the fecal microbial communities among pens did not differ within natural ($P = 0.571$) and conventional ($P = 0.498$) management programs, indicating that variation in fecal richness is greater between management programs than between pens under

similar management. Shanks et al. (2011) found that cattle raised in the same location but under different management practices had divergent microbiotas, and differences were attributed to variation in feed rations, antibiotic exposure, and supplements. Relative to the current study, antibiotic treatment is most relevant as naturally managed steers were not exposed to antimicrobial medications.

Based on individual fecal samples averaged across all timepoints, the fecal microbial communities of steers managed in the natural program had greater Shannon Diversity compared to the microbial communities of steers managed in the conventional program (P = <0.001). Fecal microbial communities from steers managed in the natural program had an average Shannon Diversity of 7.022 (SD = 0.516), and fecal microbial communities from steers managed in the conventional program had an average Shannon Diversity of 6.665 (SD = 0.783). These findings of Shannon Diversity are comparable to those found by Xu et al. (2014) and Durso et al. (2012) in fecal samples from feedlot cattle consuming a high-concentrate diet and numerically lower than those found by Azad et al. (2019) in fecal samples from steers grazing legume pasture. Similar to richness, Shannon Diversity of the fecal microbial communities among individual pens within natural and conventional management programs did not differ (P = 0.823 and P = 0.658, respectively).

Pen-level composited fecal samples were statistically analyzed as repeated measures under the assumption that the composited fecal sample was representative of the individual fecal pats within the pen. The increase in richness between T1 and T4 averaged 31 OTUs for fecal samples from naturally managed pens (SD = 187, $P = 0.612$) and 75 OTUs for fecal samples from conventionally managed pens (SD = 42, $P = 0.036$). The magnitude of change in richness from T1 to T4 did not differ between management programs ($P = 0.201$). The increase in

Shannon Diversity between T1 and T4 averaged 0.317 for fecal samples from naturally managed pens (SD = 0.600, $P = 0.176$) and 0.535 for fecal samples from conventionally managed pens (SD = 0.432, $P = 0.085$). The magnitude of change in Shannon Diversity Index from T1 to T4 did not differ between management programs ($P = 0.307$). These results suggest that alpha diversity of fecal microbiota was not static throughout the feeding period, but levels of change in microbial diversity throughout the feeding period were similar between the two management programs in the study. This indicates that fecal microbial diversity was largely a product of the environment and general management (confinement in large-pen settings and step-up to high concentrate diets), rather than the specific management program characteristics (including variation in cattle source, growth technologies, and antimicrobial exposure). Previously, diet and increasing age have been identified as having large influences on both the human (Mariat et al., 2009; Singh et al., 2017) and ruminant gastrointestinal microbiota (Henderson et al., 2015; Dias et al., 2018).

3.4.2.2 Longitudinal Comparisons

Across the four timepoints, differences in microbial richness based on individual fecal samples were observed within natural (P <0.001) and conventional (P <0.001) management programs (Table 3.1). The richness of the fecal microbiota of steers managed in the conventional program was similar between T1 and T2 ($P = 0.406$), increased between T2 and T3 (P = <0.001), and remained consistent between T3 and T4 ($P = 0.787$). The richness of the fecal microbiota of steers managed in the natural program numerically decreased between T1 and T2 ($P = 0.129$), increased between T2 and T3 (P = <0.001), and was similar between T3 and T4 ($P = 0.137$). The decrease in richness among naturally managed steers from T1 to T2 (a transition from the receiving to finishing diet) could be explained by the absence of tylosin and monensin; these

additives are known to reduce variations in feed intake and assist with more gradual diet transitions by impeding rapid microbial changes (Stock et al., 1995).

The richness of the fecal microbial communities of steers managed in the natural program was greater than that of steers managed in the conventional program at T1 (P = <0.001) and T2 (P = <0.001) and similar between programs at T3 (P = 0.497) and T4 (P = 0.828). This suggests that natural management program procedures prior to feedlot entry and at processing promote greater alpha diversity in comparison the conventional management techniques. Namely, antimicrobial exposure through either treatment for illness in calfhood or metaphylaxis upon feedlot arrival could reduce fecal alpha diversity of conventionally managed steers early in the feeding period. Across the U.S., greater than 30% of incoming feedlot cattle (particularly high risk and light-weight cattle) receive an injectable antibiotic treatment at initial processing targeted for prevention of BRD (USDA, 2011). For the cattle feeding industry metaphylactic treatment is worth greater than $530 million for disease treatment and prevention (Dennis et al., 2018), but its long-term effects on the microbiome are not well known. Previously, metaphylaxis has been associated with differences in the fecal microbiota between control and treated animals until at least 12 days following antimicrobial injection; specifically, a reduction in alpha diversity was observed when cattle were treated (Holman et al., 2019). For the current study, all samples collected for T1 were within 12 days of cattle placement at the feedlot. Metaphylactic treatment could explain a portion of the difference in alpha diversity between management programs early in the feeding period.

Differences in Shannon Diversity of individual fecal samples were observed within natural (P <0.001) and conventional (P <0.001) management programs over the course of the feeding period (Table 3.2). The Shannon Diversity of the fecal microbiota of steers managed in

the natural program remained consistent between T1 and T2 (P = 0.458), increased between T2 and T3 (P = <0.001), and remained consistent between T3 and T4 (P = 0.764). The Shannon Diversity Index of the fecal microbiota of steers managed in the conventional program remained consistent between T1 and T2 (P = 0.856), increased between T2 and T3 (P = <0.001), and remained consistent between T3 and T4 (P = 0.205). The fecal microbial communities of steers managed in the natural program had a greater Shannon Diversity than those of steers managed in the conventional program at T1 (P = <0.001) and T2 (P = <0.001) and had similar Shannon Diversity at T3 (P = 0.240) and T4 (P = 0.774). Similar to previously discussed results for richness, this difference could potentially be explained by antimicrobial exposure. Visually, the variation in individual-level Shannon Diversity among fecal microbial communities of naturally managed steers was less than that of conventionally managed steers; however, variation in Shannon Diversity among fecal microbial communities of conventionally managed steers decreased from T1 to T4 (Figure 3.1B).

3.4.3 Beta Diversity Between Fecal Microbial Communities

Qualitative beta diversity differences can be identified by testing phylogenetic UniFrac distance matrices without regard to relative abundance of individual taxa (Lozupone and Knight, 2005). By unweighted UniFrac, differences between fecal microbial communities were observed when compared by management program (P = 0.001) and time point (P = 0.001). Qualitatively, time point in the feeding period seems to explain the greatest amount of variation in fecal microbiotas (Axis 2 of Figure 3.2). Based on principal coordinate analysis (PCoA), T1 appears distinct from other time points. Considering diet transitions, the clustering of T1 away from other time points is reasonable; at T1, all steers were being fed a receiving diet with moderate grain inclusion whereas steers were fed a finishing diet high in concentrate feeds at all later time points

(T2 through T4). By incorporating phylogeny into the PCoA construction, functional differences between distinct clusters are assumed (Díaz et al., 2013). Since feedstuffs entering the gastrointestinal tract are the primary microbial substrates, greater proportions of amylolytic bacteria are needed to ferment feeds once steers are adapted to the finishing diet (Firkins and Yu, 2015). It appears that this functional transition is captured by beta diversity analysis and confirmed by taxonomic analysis.

Using unweighted UniFrac analysis, fecal microbial communities of naturally managed steers differed between T1 and T2 ($P = 0.001$) and T2 and T3 ($P = 0.001$), but were similar between T3 and T4 ($P = 0.082$), demonstrating microbial stability and acclimation to the finishing diet late in the feeding period. Fecal microbial communities of conventionally managed steers differed between T1 and T2 ($P = 0.001$), T2 and T3 ($P = 0.001$), and T3 and T4 ($P = 0.015$). The larger p-value for the comparison between T3 and T4 in comparison to previous intervals indicates greater similarity between fecal microbial communities late in the finishing period; however, a difference was still observed between T3 and T4 whereas no difference was observed in the same interval among fecal microbial communities of naturally managed steers. Between T3 and T4, conventionally managed steers were exposed to several diet changes: inclusion of the beta-adrenergic agonist ractopamine hydrochloride, discontinuation of tylosin, and increased roughage, among others. Conversely, naturally managed steers experienced fewer dietary changes during the same period; the difference in diet consistency could explain the different levels of significance identified across management programs between T3 and T4. Differences were observed between management programs at all four time points ($P = 0.001$ for each pairwise comparison). Minor dietary differences and prolonged selection pressure of feed

additives, along with differences in cattle source, are likely causes of these differences (Henderson et al., 2015).

Differences in unweighted UniFrac distances were not observed between pens within the natural program ($P = 0.070$) or the conventional program ($P = 0.561$). The numerically smaller p-value calculated for differences in beta diversity between pens within the natural program is likely indicative of greater variation among individual steers within the natural program. Cattle ranch of origin has a probable effect on development of rare taxa within the microbiota. It is unlikely that differences in rare taxa are promoted within the common feedlot environment; instead, it is more likely that variation in rare taxa is reduced upon entry into the feedlot, especially within the conventional management program with antimicrobial exposure (Shanks et al., 2011). In the current study, variation in source was considered as an intrinsic variable in pen-level analysis. Rare taxa related to cattle source could explain greater beta diversity among the fecal microbiotas of pens of naturally managed steers, especially when measured by unweighted UniFrac (Wong et al., 2016).

Quantitative differences between microbial communities can be identified by testing phylogenetic UniFrac distance matrices with regard to relative abundance of individual taxa (Lozupone et al., 2007). Using weighted UniFrac, differences between fecal microbial communities were observed when compared by management program ($P = 0.001$) and time point ($P = 0.001$). Similar to unweighted UniFrac, time point in the feeding period seems to explain the greatest amount of variation between fecal microbiotas (Figure 3.3). Based on PCoA, T1 and T2 appear distinct from other time points while T3 and T4 seem similar. As previously discussed, the unique T1 diet likely explains differences between T1 and other timepoints. Unlike the results of unweighted UniFrac, a considerable difference was qualitatively observed

between T2 and T3/T4. The x axis of the weighted UniFrac PCoA plot accounts for greater than 39% of variation between microbial communities. Microbial communities at T1 represent the minimum x values while that of T2 represent the maximum x values with T3 and T4 intermediate. This indicates a large shift in microbiota between T1 and T2 followed by a gradual regression to an intermediate community at T3 and T4. The seemingly overcorrected T2 community is likely indicative of microbial dysbiosis associated with acclimation to the finishing diet (Petersen and Round, 2014).

Results of weighted UniFrac analysis showed that fecal microbial communities of naturally managed steers differed between T1 and T2 ($P = 0.001$) and T2 and T3 ($P = 0.001$) but were similar between T3 and T4 ($P = 0.279$). Fecal microbial communities of conventionally managed steers differed between T1 and T2 ($P = 0.001$), T2 and T3 ($P = 0.001$), and T3 and T4 ($P = 0.001$). As previously explained for unweighted UniFrac results, the diet change between T3 and T4 as part of the conventional management program is a probable factor associated with the change in fecal microbial communities between T3 and T4 among conventionally managed steers; this diet change did not occur for naturally managed steers. Differences weighted UniFrac distances were observed between management programs at T1 ($P = 0.005$), T2 ($P = 0.001$), T3 ($P = 0.001$), and T4 ($P = 0.003$). Differences were not observed between pens within the natural program ($P = 0.058$) or the conventional program ($P = 0.933$). Similar to unweighted UniFrac, the numerically smaller p-value calculated for differences in beta diversity between pens within the natural program is likely indicative of greater variation between fecal microbiota of naturally managed steer.

3.4.4 Fecal Microbiota Composition

3.4.4.1 Observed Taxa

Throughout the feeding period, 23 phyla, 34 classes, 63 orders, and 138 families were observed in the fecal microbial communities of steers managed in both the natural and conventional program. The phyla identified in greater than 1% relative abundance across both management programs over the entire feeding period were Firmicutes (48.67%), Bacteroidetes (40.37%), Spirochaetes (4.87%), Actinobacteria (2.02%), Tenericutes (1.94%), and Proteobacteria (1.34%). Firmicutes and Bacteroidetes are commonly cited as the predominant phyla composing the fecal microbiota (Dowd et al., 2008; Callaway et al., 2010; Shanks et al., 2011; Durso et al., 2012; Rice et al., 2012; Petri et al., 2013; Myer et al., 2015; Liu et al., 2016; Huebner et al., 2019). Spirochaetes, Tenericutes, and Proteobacteria have previously been identified as predominant features of fecal samples from feedlot cattle managed in natural programs (Huebner et al., 2019). Actinobacteria has previously been identified in feces of feedlot cattle at placement and at shipment for harvest (Yang et al., 2016).

Thirteen families were identified in greater than 1% relative abundance across both management programs over the entire feeding period. Families belonging to the phylum Firmicutes included Ruminococcaceae (29.74%), Lachnospiraceae (9.63%), Peptostreptococcaceae (2.03%), Clostridiaceae 1 (1.47%), Erysipelotrichaceae (1.44%), Christensenellaceae (1.40%), and Acidaminococcaceae (1.27%). Families belonging to Bacteroidetes included Prevotellaceae (18.26%), Rikenellaceae (10.38%), Bacteroidaceae (5.12%), and Muribaculaceae (4.20%). Spirochaetaceae (4.87%) of the phylum Spirochaetes and Bifidobacteriaceae (2.01%) of the phylum Actinobacteria were the only families identified in greater than 1% relative abundance aside from the Firmicutes and Bacteroidetes phyla.

3.4.4.2 Microbial Trends Over the Entire Feeding Period

At the phylum level, Firmicutes and Bacteroidetes dominated the fecal microbial communities of steers managed in natural and conventional programs. Within fecal microbiotas of naturally managed steers, the relative abundance of Firmicutes was consistently higher than that of Bacteroidetes across all time points (Table 3.3). Within fecal microbiotas of conventionally managed steers, the relative abundance of Firmicutes was higher than that of Bacteroidetes at T1 and T4; Bacteroidetes relative abundance exceeded that of Firmicutes at T2 and T3 (Table 3.4). The intestinal relative abundances of Firmicutes and Bacteroides have been discussed extensively relative to human metabolic disease (Johnson et al., 2017; Koliada et al., 2017). The ratio of the two phyla has been associated with age (Mariat et al., 2009) and obesity (Castaner et al., 2018) among humans. Generally, a greater proportion of Firmicutes is associated with greater fat deposition in humans (Ley et al., 2005; Ley et al., 2006; Johnson et al., 2017; Castaner et al., 2018). Theoretically, applied to feedlot steers, a higher relative abundance of Firmicutes could accelerate fat deposition and improve carcass quality grade at the detriment of carcass cutability (Bruns et al., 2004).

Across both management programs, the relative abundance of Firmicutes was greatest at T1 and least at T2. On the family level, similar changes were observed between management programs (Tables 3.5 and 3.6). The Ruminococcaceae family remained a common member of fecal microbial communities of steers in both management programs throughout the feeding period and is known to exhibit high relative abundance in cattle fed processed grains (Shanks et al., 2011). Ruminococcaceae bacteria *Ruminococcus flavefaciens* and *Ruminococcus albus* have been associated with greater roughage inclusion and are expected to perform cellulolytic functions (White et al., 1993; Deusch et al., 2017). Increases in the Ruminococcaceae family

among conventionally managed cattle late in the feeding period suggests a more thorough fermentation of fiber could be occurring in the hindgut as time from T2 increases.

The relative abundance of Firmicutes families Peptostreptococcaceae, Clostridiaceae 1, Erysipelotrichaceae, and Christensenellaceae decreased from T1 to T2 then increased to the end of the feeding period. Peptostreptococcaceae bacteria have previously been demonstrated to catabolize amino acids within ruminants but are sensitive to ionophore inclusion (Flythe and Andries, 2009). Clostridiaceae bacteria have been associated with mucin secretion in the hind gut (Wlodarska et al., 2015; McCormack et al., 2017). Presence of the Clostridiaceae genus *Clostridium sensu stricto 1* has been associated with longitudinal homeostasis of the gastrointestinal microbiota (Lopetuso et al., 2013). Erysipelotrichaceae includes the genera *Turicibacter*, *Sharpea,* and *Kandleria*, known to produce lactate from a variety of sugar substrates and is associated with high rates of rumen content turnover and reduced methane production (Morita et al., 2008; Kittelmann et al., 2014; Kamke et al., 2016). Christensenellaceae has been associated with reduced ruminal pH in dairy heifers (De Nardi et al., 2016), but research of its role in gastrointestinal fermentation is limited (Derakhshani et al., 2016).

The relative abundance of Firmicutes families Acidaminococcaceae and Veillonellaceae peaked at T2 then generally declined to the end of the feeding period. Acidaminococcaceae and its prominent genus *Succiniclasticum* has been identified more frequently when cattle are fed corn silage and is associated with fermentation of succinate to propionate (Yost et al., 1977; Vanswyk, 1995; Deusch et al., 2017). Veillonellaceae includes the genera *Megasphaera* and *Selenomas* that are known to convert lactate and succinate into butyrate and propionate (Marounek et al., 1989; Nisbet and Martin, 1990). Lachnospiraceae was observed in greater relative abundance early in the feeding period (T1 and T2) compared to late in the feeding period

(T3 and T4). High abundance of Lachnospiraceae has been associated with high-producing dairy cows (Tong et al., 2018), and the Lachnospiraceae genus *Butyrivibrio* is known to form butyrate with proteolytic and fibrinolytic degradation (Cotta and Hespell, 1986; Kelly et al., 2010).

Across both management programs, the relative abundance of Bacteroidetes peaked at T2. Shanks et al. (2011) found that fecal starch content was positively associated with relative abundance of Bacteroidetes. Upon transitioning steers to high-concentrate finishing diets, steers are exposed to greater quantities of starch. By increasing rate of passage or reducing extent of digestion, starch could reach the hindgut and promote growth of Bacteroidetes bacteria. At T2, the increase in Bacteroidetes relative abundance was driven largely by an increase in the Prevotellaceae family (Tables 3.5 and 3.6). *Prevotella* bacteria are known for amylolytic function and their relative abundance is often inversely related with the Ruminococcaceae family (Derakhshani et al., 2016).

Numerically smaller increases in the relative abundance of Bacteroidaceae, Muribaculaceae, and Tannerellaceae were also consistent from T1 to T2 across both management programs. *Bacteroides* bacteria of the Bacteroidaceae family have been found in higher concentrations when ruminants are fed grains (Gylswyk and Toorn, 1986). Muribaculaceae (formerly the family S24–7; Lagkouvardos et al., 2017) is an uncultured taxon of the rumen with genomic-based projected function of production of acetate, propionate, and succinate (Ormerod et al., 2016). Muribaculaceae abundance has demonstrated positive correlation with average daily gain (Paz et al., 2018). The genera *Macellibacteroides* and *Parabacteroides* of the Tannerellaceae family observed in this study have not been well-described within ruminants, though *Parabacteroides* has been identified in the gastrointestinal tract of calves (Dias et al., 2018). Interestingly, the lowest relative abundance of the

Bacteroidetes family Rikenellaceae was observed at T2, followed by gradual increases in relative abundance to the end of the feeding period across both management programs. Rikenellaceae has previously been identified in the gastrointestinal microbiota of cattle (Schären et al., 2018), and a decreasing relative abundance has been associated with greater grain inclusion and acidotic conditions (Petri et al., 2013). Rikenellaceae's increasing relative abundance at the end of the feeding period suggests stabilization of the hindgut.

The relative abundance of Proteobacteria peaked at T2 and decreased to the end of the feeding period, largely driven by changes in the family Succinovibrionaceae. The genera *Ruminobacter* and *Succinovibrio* were present as members of the Succinovibrionaceae family which is known to reduce methane emissions by producing succinate as opposed to acetate resulting in capture of metabolic hydrogen rather than release as hydrogen gas (Wallace et al., 2015). Though found in small amounts, the relative abundance of Proteobacteria family Enterobacteriaceae decreased throughout the feeding period. Particularly in the early feeding period, the predominant genus identified was *Escherichia*, which is a known human pathogen (Callaway et al., 2009). It is favorable for human food safety that a decreased relative abundance of Enterobacteriaceae was identified late in the feeding period closer to shipment for harvest. Previous research indicates that Enterobacteriaceae abundance is increased when cattle are fed high-grain diets, but Enterobacteriaceae abundance has also been documented to decrease during the winter compared to summer (Callaway et al., 2009). Limited detection of Enterobacteriaceae late in the feeding period could be the result of a seasonal effect.

Across both management programs, fecal relative abundance of Spirochaetes increased from T1 to T2 and remained similar from T2 to T4. The family Spirochaetaceae has been associated with decreasing feed efficiency (Paz et al., 2018) and its genus *Treponema* is known

to ferment pectin to acetate and formate (Ziołecki, 1979). Similarly, Fibrobacteres relative abundance generally increased throughout the feeding period. The family Fibrobacteraceae and genus *Fibrobacter* are associated with cellulose digestion (Hungate, 1950; Neumann et al., 2017). Cellulose availability within the fecal microbial community would seemingly drive increases in abundance of *Fibrobacter* by creating a more favorable environment for growth (Martiny et al., 2006). This would lead to greater passage of cellulose to the hindgut; higher feed intakes late in the feeding period increase rate of passage from the rumen and decrease extent of digestion (Okine and Mathison, 1991).

The relative abundance of Actinobacteria, namely the gram-positive family Bifidobacteriaceae, decreased from 4.04% in fecal communities of naturally managed steers and 11.23% in fecal communities of conventionally managed steers at T1 to roughly 0.10% at T4 in fecal microbiotas of steers managed in both programs. The Bifidobacteriaceae family has been associated with greater production of butyrate in the rumen (Schären et al., 2018) and has been associated with increased daily feed intake (Paz et al., 2018). Notable higher relative abundance of Bifidobacteriaceae has been reported for cattle fed diets high in roughage compared to those consuming diets high in concentrates (Deusch et al., 2017) with inferred function of digesting complex plant carbohydrates (Pokusaeva et al., 2011). *Bifidobacteria* are regarded in human medicine as favorable for overall health (Arboleya et al., 2016) and are noted in both infants and milk-fed calves (Vlková et al., 2006). Feeding of milk by-products or high roughage diets (as fed at T1) could promote *Bifidobacteria* abundance.

Fusobacteria were most frequently observed at T1 across both management programs. The family Fusobacteriaceae was observed in only 16 fecal samples, 12 of which originated from naturally managed steers and 4 of which originated from conventionally managed steers. Most

widely investigated as an etiologic agent in liver abscess pathogenesis (Nagaraja and Chengappa, 1998), the genus *Fusobacterium* is known to rapidly increase in ruminal abundance during acclimation to high concentrate diets (Nagaraja and Lechtenberg, 2007a). Based on the lack of Fusobacteriaceae in the feces, it appears that *Fusobacterium* and its role in liver abscess development is localized in the rumen, rumen lining, or intestinal lining rather than the contents of the hindgut.

3.4.4.3 Microbial Composition of T1

The phylum Firmicutes dominated the fecal microbiota at T1, accounting for more than half of the fecal microbial communities of naturally and conventionally managed steers. Bacteroidetes was the second-most commonly identified constituent of the fecal microbiota. Together, the phyla Firmicutes and Bacteroidetes composed greater than 85% of the fecal microbiota (Figure 3.4). The fecal microbiota of naturally managed steers exhibited greater relative abundance of Spirochaetes, Proteobacteria, and Tenericutes compared to that of conventionally managed steers. Actinobacteria relative abundance was greater in the fecal microbial communities of conventionally managed steers (11.23%) compared to naturally managed steers (4.04%; W = 20).

Actinobacteria abundance was dictated by the family Bifidobacteriaceae which accounted for 4.40% of the fecal microbiota of naturally managed steers and 11.23% of the fecal microbiota of conventionally managed steers. Numerically higher levels of Lachnospiraceae were observed from the fecal communities of conventionally managed steers (16.48%) compared to that of naturally managed steers (10.72%), while higher levels of Rikenellaceae were observed from the fecal communities of naturally managed steers (16.14 %) compared to that of conventionally managed steers (9.67%). Anaeroplasmataceae was the only family identified as differentially

abundant (W = 126) between naturally managed steers (0.02%) and conventionally managed steers (0.29%).

3.4.4.4 Microbial Composition of T2

The transition from T1 (receiving diet) to T2 (finishing diet) was accompanied by similar changes in the fecal microbiota for naturally and conventionally managed steers. Among the 20 phyla observed in the fecal microbiotas of naturally managed steers between T1 and T2, Actinobacteria (W = 18), Spirochaetes (W = 18), Proteobacteria (W = 16), Fibrobacteres (W = 16), Bacteroidetes (W = 15), and Firmicutes (W = 15) were identified as differentially abundant. Among the 19 phyla observed in the fecal microbiotas of conventionally managed steers between T1 and T2, Actinobacteria (W = 19), Euryarchaeota (W = 16), Proteobacteria (W = 16), Bacteroidetes (W = 15), and Verrucomicrobia (W = 15) were identified as differentially abundant. Increases in Bacteroidetes, Spirochaetes, and Proteobacteria were similar between management programs; decreases in Firmicutes and Actinobacteria were similar between management programs.

On the family level (Table 3.7), similar trends were observed between the fecal microbiotas of steers across management programs. Observed increases in Bacteroidetes were driven by increases in Prevotellaceae and Muribaculaceae. Prevotellaceae within fecal microbiotas increased from T1 to T2 among naturally managed steers by 12.57 percentage points (W = 102) and among conventionally managed steers by 29.74 percentage points (W = 89). The relative abundance of Muribaculaceae within fecal microbiotas increased among naturally managed steers and conventionally managed steers by 3.73 percentage points (W = 99) and 3.06 percentage points (W = 88), respectively. Numerically, the relative abundance of Peptostreptococcaceae and Clostridiaceae 1 (of the phylum Firmicutes) decreased within fecal

microbiotas of both naturally and conventionally managed steers. Christensenellaceae relative abundance decreased within conventionally managed cattle (W = 97) and numerically but not significantly decreased within naturally managed cattle. Together, these families appear to mark a relative increase of Bacteroidetes in relation to Firmicutes during the transition from T1 to T2. Additionally, the relative abundance of Bifidobacteriaceae decreased among naturally managed steers (W = 111) and conventionally managed steers (W = 101) to T2 levels less that were one tenth of that observed at T1. Relative abundance of Spirochaetaceae, Veilonellaceae, and Acidaminococcaceae numerically increased within both management programs to T2; relative abundance of Enterobacteriaceae, Erysipelotrichaceae, Peptostreptococcaceae numerically decreased within both management programs to T2 from T1.

Though changes between T1 and T2 were generally similar between management programs, not all taxa were uniformly abundant across management programs at T2. Relative abundance of Firmicutes was lower in fecal communities of conventionally managed steers compared to that of naturally managed steers (W = 11); thus, a larger portion of the fecal microbiota of conventionally managed steers was composed of Bacteroidetes. On the family level, Bacteroidales RF 16 was enriched in fecal communities of naturally managed steers (W = 63) and Prevotellaceae was enriched in fecal communities of conventionally managed steers (W = 63) and accompanied by a numerical decrease of Ruminococcaceae. Like observed at T1, Anaeroplasmataceae was identified as differentially abundant (W = 56) between naturally managed steers and conventionally managed steers at T2.

3.4.4.5 Microbial Composition of T3

The transition from T2 to T3 was characterized by less change in the fecal microbiota than was observed between T1 and T2. Among the 20 phyla observed in the fecal microbiotas of

naturally managed steers between T2 and T3, only Proteobacteria (W = 18) was identified as differentially abundant. Among the 16 phyla observed in the fecal microbiotas of conventionally managed steers between T2 and T3, only Verrucomicrobia (W = 15) was identified as differentially abundant.

On the family level (Table 3.8), a greater number of differentially abundant taxa were observed within conventionally managed steers (n = 9) than natural managed steers (n = 2) from T2 to T3. Relative abundance of Prevotellaceae was lower in fecal microbial communities from conventionally managed steers (W = 53). Ruminococcaceae relative abundance numerically increased within fecal microbial communities of conventionally managed cattle. Relative abundance of Christensenellaceae, Clostridiaceae 1, and Peptostreptococcaceae increased (W = 64, 52, and 51, respectively) within conventionally managed steers and numerically increased within naturally managed steers. Veilonellaceae relative abundance decreased (W = 54) within conventionally managed steers and numerically decreased within naturally managed steers. Relative abundance of Rikenellaceae and Bacteroidaceae numerically increased in fecal microbial communities from steers managed in natural and conventional programs. Relative abundance of Lachnospiraceae, Bifidobacteraceae, and Enterobacteriaceae numerically decreased in fecal microbial communities from steers managed in natural and conventional programs.

As observed between T1 and T2, differences between T2 and T3 were generally similar between management programs, but not all taxa were uniformly abundant across management programs at T3. Relative abundance of Verrucomicrobia was higher in fecal communities of conventionally managed steers compared to that of naturally managed steers (W = 15). On the family level, Bacteroidales RF 16 was again found in higher relative abundance in fecal

communities of naturally managed steers (W = 90). Paludibacteraceae was also enriched in fecal communities of naturally managed steers (W = 86).

3.4.4.6 Microbial Composition of T4

The transition from T3 to T4 was characterized by the least amount of change in the fecal microbiota across all consecutive time point comparisons. Among the 16 phyla observed in the fecal microbiotas of naturally managed steers between T3 and T4, no phyla were identified as differentially abundant. Among the 16 phyla observed in the fecal microbiotas of conventionally managed steers between T3 and T4, only Proteobacteria (W = 15) was identified as differentially abundant.

As with the the phylum level, few families (Table 3.9) were differentially abundant. Between T3 and T4, no families were identified as differentially abundant within fecal microbial communities of naturally managed steers. The relative abundance of the Proteobacteria families Succinovibrionaceae and Burkholderiaceae decreased (W =81 and 78, respectively) within conventionally managed cattle. Relative abundance of Prevotellaceae, Acidaminococcaceae, and Bacteroidaceae numerically decreased in fecal microbial communities within both management programs. Relative abundance of Rikenellaceae, Christensenellaceae, Clostridiaceae 1, Peptostreptococcaceae, Muribaculaceae, and Fibrobacteraceae numerically increased in fecal microbial communities within both management programs.

Similar to previously discussed timepoints, differences between T3 and T4 were generally similar between management programs, but not all taxa were uniformly abundant across management programs at T3. Relative abundance of Verrucomicrobia remained greater in fecal communities of conventionally managed steers compared to that of naturally managed steers (W = 15). On the family level, Bacteroidales RF 16 was again found in higher relative

abundance in fecal communities of naturally managed steers (W = 94). Akkermansiaceae was enriched in fecal communities of conventionally managed steers compared to naturally managed steers (W = 94). Akkermansiaceae, a mucin-degrading bacterial family, has been linked with obesity in humans (Everard et al., 2013; Schneeberger et al., 2015).

3.4.4.7 Microbial Interdependence

Among the fecal microbiotas of naturally managed steers compared across the entire feeding period, 22 families were identified as differentially abundant between all timepoints. Among the fecal microbiotas of conventionally managed steers compared across the entire feeding period, 38 families were identified as differentially abundant between all timepoints. Between management programs compared across all time points, only seven families were identified as differentially abundant, four of which belonged to the order Bacteriodales and were enriched in fecal microbiotas of naturally managed steers. This indicates that consistent and systematic changes in fecal microbial relative abundance in feedlot cattle are likely attributable to feeding period progression regardless of management program.

A non-parametric microbial interdependence test was used to identify community-level differences in associations between family-level taxa. No differences were observed in microbial interdependence between management programs ($P = 0.089$) or time points throughout the feeding period ($P = 0.105$). It appears that similar community dynamics exist within fecal microbiotas of steers even though relative abundance changes in response to diet shifts as previously discussed. Previously, microbial interdependence has been demonstrated in vitro with ruminal bacteria; substrate availability and cross feeding were identified as driving factors to changes within the microbial ecosystem (Miura et al., 1980). This may explain the lack of difference found in temporal patterns of family-level taxa relationships within the fecal

microbiota; systematic changes in microbial relative abundance are likely caused by the interaction between metabolite concentrations in the gastrointestinal tract and the metabolic pathways of individual bacteria.

3.4.5 Liver Abscess Occurrence

Livers (N = 3,929) from all steers in the study population were evaluated for abscesses at the time of harvest using the Elanco scoring system (Elanco, 2016). Of the 1,777 livers evaluated from naturally managed steers, 657 (36.97%) were identified as abscessed (score of A or A+); 296 (16.66%) livers were identified as severely abscessed (A+). The prevalence and severity of liver abscesses among naturally managed steers was similar to findings of Huebner et al. (2019) who reported the adjusted risk of liver abscess occurrence was 38.9% and that of severe liver abscess occurrence was 15.2%. Of the 2,152 livers evaluated from conventionally managed steers, 564 livers (26.21%) were identified as abscessed (score of A or A+); 269 livers (12.51%) were identified as severely abscessed (A+). This represents a slightly greater but comparable liver abscess prevalence compared to industry-wide reported prevalence near 20% (Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007a; Rezac et al., 2014; Reinhardt and Hubbert, 2015; Eastwood et al., 2017; Holland et al., 2018; Walter et al., 2018). When accounting for the effect of live management pen, naturally managed steers had increased overall probability of liver abscess occurrence compared to conventionally managed steers ($P = 0.012$; Table 3.10). Naturally managed steers exhibited a numerically higher adjusted probability of severe liver abscess incidence compared to conventionally managed steers as well ($P = 0.183$). Maxwell et al. (2014) previously reported that naturally managed cattle exhibited greater prevalence of liver abscesses compared to that of conventionally managed steers. Additionally, Holland et al. (2018) demonstrated that cattle not fed tylosin have a greater prevalence of severe liver abscesses.

Spearman correlations were used to evaluate the relationship between alpha diversity of the pen-level composited fecal sample and pen-level liver abscess prevalence and severity. Higher alpha diversity, measured as richness or Shannon Diversity, at T4 was associated with reduced total liver abscess occurrence (r_s = -0.384 and r_s = -0.438, respectively). Negative correlations of lesser magnitude between richness/Shannon Diversity and total liver abscess occurrence were observed at T3 (r_s = -0.090 and r_s = -0.093, respectively). Larger increases of richness or Shannon Diversity between T1 and T4 were associated with lower total liver abscess occurrence (r_s = -0.335 and r_s = -0.390, respectively). When similar correlations were evaluated between alpha diversity and prevalence of only severe liver abscesses, no correlation greater in magnitude than 0.16 was observed. Future studies are needed to better understand correlations between fecal microbial diversity and liver abscess prevalence and severity.

3.5 SUMMARY

As expected, the fecal microbiota of feedlot steers changed throughout the feeding period, most notably immediately following the transition to the finishing diet. More stable communities were observed later in the finishing period. While fecal microbiota alpha diversity of conventionally managed steers was lower compared to that of naturally managed steers early in the finishing period, fecal microbiota alpha diversity of steers was similar across management programs closer to shipment for harvest. The greatest differences in fecal microbiota beta diversity were observed between placement, while steers were consuming the receiving diet, and transition to the finishing diet. Fewer differences were observed between management programs than longitudinally within management program; this suggests similar fecal microbial interdependencies. The fecal microbiotas of steers managed in natural and conventional management programs demonstrate differences early in the feeding period; however, all steers

experienced microbiota shifts once transitioned to the finishing diet, resulting in similar stable fecal microbiotas among both management programs late in the finishing period. Generally, increased fecal alpha diversity over the feeding period and greater fecal alpha diversity late in the feeding period were inversely related to liver abscess prevalence. Together, these results indicate that the fecal microbiota becomes similar among cattle managed in a single feedlot regardless or management program and that fecal microbiota could be useful as an indicator of nutritionally related pathologies in feedlot cattle.

3.6 TABLES AND FIGURES

Table 3.1. Mean observed OTUs within individual fecal pats collected from pen floors housing steers managed in natural and conventional programs at four timepoints throughout the feeding period.

	Mean Observed OTUs (n)				
Management Program	T1[1]	T2[2]	T3[3]	T4[4]	*P* value[5]
Natural	342[ab] (35)	309[b] (35)	381[a] (35)	362[a] (35)	<0.001
Conventional	237[b] (35)	249[b] (35)	353[a] (34)	358[a] (35)	<0.001

[1] Time point 1: 7 days (SD = 3 days) following placement.
[2] Time point 2: 10 days (SD = 4 days) following complete transition the finishing diet.
[3] Time point 3: 42 days (SD = 4 days) prior to shipment for harvest and before the feeding of β-adrenergic agonist to conventional steers.
[4] Time point 4: 9 days (SD = 3 days) prior to shipment for harvest.
[5] *P* value for the overall Kruskal-Wallis test with Benjamini-Hochberg multiple testing correction to control the false discovery rate.
[a-b] Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by pairwise Kruskal-Wallis testing with Benjamini-Hochberg multiple testing correction to control the false discovery rate.

Table 3.2. Mean Shannon Diversity Index of the microbial communities of individual fecal pats collected from pen floors housing steers managed in natural and conventional programs at four timepoints throughout the feeding period.

	Mean Shannon Diversity Index (n)				
Management Program	T1[1]	T2[2]	T3[3]	T4[4]	*P* value[5]
Natural	6.83[b] (35)	6.79[b] (35)	7.25[a] (35)	7.22[a] (35)	<0.001
Conventional	6.10[b] (35)	6.20[b] (35)	7.12[a] (34)	7.25[a] (35)	<0.001

[1] Time point 1: 7 days (SD = 3 days) following placement.
[2] Time point 2: 10 days (SD = 4 days) following complete transition the finishing diet.
[3] Time point 3: 42 days (SD = 4 days) prior to shipment for harvest and before the feeding of β-adrenergic agonist to conventional steers.
[4] Time point 4: 9 days (SD = 3 days) prior to shipment for harvest.
[5] P value for the overall Kruskal-Wallis test with Benjamini-Hochberg multiple testing correction to control the false discovery rate.
a-b Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by pairwise Kruskal-Wallis testing with Benjamini-Hochberg multiple testing correction to control the false discovery rate

Table 3.3. Mean relative abundance of microbial phyla throughout the feeding period from individual fecal pats collected from pen floors housing steers managed in the natural program with differential abundance determined by ANCOM[1] testing across 22 observed phyla.

	Mean Relative Abundance, %				
Phylum	T1[2]	T2[3]	T3[4]	T4[5]	W[6]
Firmicutes	55.32	50.10	50.23	52.12	NA[7]
Bacteroidetes	33.31	39.73	38.90	37.51	17
Spirochaetes	2.58	5.86	6.85	5.98	19
Tenericutes	2.25	1.92	2.32	2.51	NA[7]
Proteobacteria	1.39	1.77	1.13	1.10	21
Actinobacteria	4.16	0.40	0.09	0.11	21
Euryarchaeota	0.42	0.09	0.21	0.25	19
Fibrobacteres	0.05	0.08	0.14	0.31	19
Verrucomicrobia	0.24	0.02	0.07	0.05	20
Cyanobacteria	0.16	0.00	0.00	0.00	17
Planctomycetes	0.05	0.00	0.00	0.01	16
Fusobacteria	0.02	0.00	0.00	0.00	17

[1] Analysis of composition of microbiomes.
[2] Time point 1: 7 days (SD = 3 days) following placement.
[3] Time point 2: 10 days (SD = 4 days) following complete transition the finishing diet.
[4] Time point 3: 42 days (SD = 4 days) prior to shipment for harvest and before the feeding of β-adrenergic agonist to conventional steers.
[5] Time point 4: 9 days (SD = 3 days) prior to shipment for harvest.
[6] W value for ANCOM test result indicating significance of differential abundance for a single phylum by the number of log-ratio comparisons to other phyla that were significant by pairwise one-way ANOVA testing.
[7] Phylum not identified as differentially abundant. Included for reference only.

Table 3.4. Mean relative abundance of microbial phyla throughout the feeding period from individual fecal pats collected from pen floors housing steers managed in the conventional program with differential abundance determined by ANCOM[1] testing across 19 observed phyla.

	Mean Relative Abundance, %				
Phylum	T1[2]	T2[3]	T3[4]	T4[5]	W[6]
Firmicutes	55.79	32.06	44.32	49.33	18
Bacteroidetes	29.69	57.35	45.83	40.83	18
Spirochaetes	1.03	5.99	5.07	5.62	18
Actinobacteria	11.34	0.05	0.03	0.14	18
Tenericutes	1.23	1.32	1.85	2.14	15
Proteobacteria	0.62	2.65	1.73	0.77	18
Fibrobacteres	0.01	0.28	0.33	0.51	18
Verrucomicrobia	0.07	0.09	0.48	0.42	18
Euryarchaeota	0.18	0.16	0.27	0.19	NA[7]
Lentisphaerae	0.00	0.01	0.02	0.01	14
Fusobacteria	0.01	0.00	0.00	0.00	15
Epsilonbacteraeota	0.01	0.01	0.00	0.00	18

[1] Analysis of composition of microbiomes.
[2] Time point 1: 7 days (SD = 3 days) following placement.
[3] Time point 2: 10 days (SD = 4 days) following complete transition the finishing diet.
[4] Time point 3: 42 days (SD = 4 days) prior to shipment for harvest and before the feeding of β-adrenergic agonist to conventional steers.
[5] Time point 4: 9 days (SD = 3 days) prior to shipment for harvest.
[6] W value for ANCOM test result indicating significance of differential abundance for a single phylum by the number of log-ratio comparisons to other phyla that were significant by pairwise one-way ANOVA testing.
[7] Phylum not identified as differentially abundant. Included for reference only.

Table 3.5. Mean relative abundance of select microbial families throughout the feeding period from individual fecal pats collected from pen floors housing steers managed in the natural program with differential abundance determined by ANCOM[1] testing across 126 observed families.

		Mean Relative Abundance, %				
Phylum	Family	T1[2]	T2[3]	T3[4]	T4[5]	W[6]
Firmicutes	Ruminococcaceae	31.28	30.66	32.20	32.45	NA[7]
	Lachnospiraceae	10.72	11.84	8.25	8.71	NA[7]
	Peptostreptococcaceae	4.89	1.60	2.21	2.76	NA[7]
	Clostridiaceae 1	1.76	0.72	1.90	2.36	102
	Erysipelotrichaceae	2.49	1.16	1.34	1.56	NA
	Christensenellaceae	2.36	0.75	1.20	1.45	103
	Acidaminococcaceae	0.26	1.60	1.22	1.14	121
	Veillonellaceae	0.02	0.25	0.16	0.24	118
Bacteroidetes	Prevotellaceae	7.16	19.73	14.38	13.08	110
	Rikenellaceae	16.14	8.94	12.79	13.60	NA[7]
	Bacteroidaceae	3.55	4.61	4.99	4.27	NA[7]
	Muribaculaceae	1.72	4.45	3.93	4.14	110
	Tannerellaceae	0.18	0.59	0.36	0.44	111
Spirochaetes	Spirochaetaceae	2.58	5.86	6.85	5.98	NA[7]
Actinobacteria	Bifidobacteriaceae	4.04	0.39	0.07	0.10	124
Proteobacteria	Succinivibrionaceae	0.93	0.92	0.76	0.89	105
	Enterobacteriaceae	0.36	0.07	0.01	0.02	124
Fibrobacteres	Fibrobacteraceae	0.05	0.08	0.14	0.31	NA[7]
Verrucomicrobia	Akkermansiaceae	0.24	0.02	0.07	0.05	101

[1] Analysis of composition of microbiomes.
[2] Time point 1: 7 days (SD = 3 days) following placement.
[3] Time point 2: 10 days (SD = 4 days) following complete transition the finishing diet.
[4] Time point 3: 42 days (SD = 4 days) prior to shipment for harvest and before the feeding of β-adrenergic agonist to conventional steers.
[5] Time point 4: 9 days (SD = 3 days) prior to shipment for harvest.
[6] W value for ANCOM test result indicating significance of differential abundance for a single family by the number of log-ratio comparisons to other families that were significant by pairwise one-way ANOVA testing.
[7] Phylum not identified as differentially abundant. Included for reference only.

Table 3.6. Mean relative abundance of select microbial families throughout the feeding period from individual fecal pats collected from pen floors housing steers managed in the conventional program with differential abundance determined by ANCOM[1] testing across 112 observed families.

		Mean Relative Abundance, %				
Phylum	Family	T1[2]	T2[3]	T3[4]	T4[5]	W[6]
Firmicutes	Ruminococcaceae	30.33	18.42	29.36	33.18	99
	Lachnospiraceae	16.48	7.80	6.66	6.50	93
	Peptostreptococcaceae	1.90	0.56	1.24	1.60	92
	Clostridiaceae 1	1.25	0.47	1.31	2.00	99
	Erysipelotrichaceae	2.09	0.84	0.91	1.15	NA[7]
	Christensenellaceae	2.14	0.36	1.17	1.78	111
	Acidaminococcaceae	0.17	2.19	2.08	1.47	110
	Veillonellaceae	0.04	0.40	0.17	0.15	110
Bacteroidetes	Prevotellaceae	11.01	40.75	24.24	15.93	110
	Rikenellaceae	9.67	5.35	6.71	9.72	39
	Bacteroidaceae	4.82	5.06	7.19	6.54	NA[7]
	Muribaculaceae	1.94	5.00	5.89	6.55	105
	Tannerellaceae	0.18	0.86	0.95	0.72	105
Spirochaetes	Spirochaetaceae	1.03	5.99	5.07	5.62	109
Actinobacteria	Bifidobacteriaceae	11.23	0.03	0.02	0.12	111
Proteobacteria	Succinivibrionaceae	0.19	1.80	1.19	0.52	110
	Enterobacteriaceae	0.29	0.06	0.01	0.01	100
Fibrobacteres	Fibrobacteraceae	0.01	0.28	0.33	0.51	104
Verrucomicrobia	Akkermansiaceae	0.07	0.09	0.47	0.42	108

[1] Analysis of composition of microbiomes.
[2] Time point 1: 7 days (SD = 3 days) following placement.
[3] Time point 2: 10 days (SD = 4 days) following complete transition the finishing diet.
[4] Time point 3: 42 days (SD = 4 days) prior to shipment for harvest and before the feeding of β-adrenergic agonist to conventional steers.
[5] Time point 4: 9 days (SD = 3 days) prior to shipment for harvest.
[6] W value for ANCOM test result indicating significance of differential abundance for a single family by the number of log-ratio comparisons to other families that were significant by pairwise one-way ANOVA testing.
[7] Phylum not identified as differentially abundant. Included for reference only.

Table 3.7. Mean percent relative abundance of differentially abundant microbial families between at T1 and T2 from individual fecal pats collected from pen floors housing steers managed in natural and conventional programs as determined by ANCOM[1] testing within management program.

Management Program (Families Observed)	Phylum	Family	Mean Relative Abundance, % T1[2]	T2[3]	W[4]
Natural (119)	Actinobacteria	Bifidobacteriaceae	4.04	0.39	111
	Bacteroidetes	Prevotellaceae	7.16	19.73	102
		Tannerellaceae	0.18	0.59	101
		Muribaculaceae	1.72	4.45	99
		Bacteroidales p-2534-18B5	0.53	0.00	98
		Bacteroidales F082	0.28	0.02	97
	Firmicutes	Veillonellaceae	0.02	0.25	116
		Acidaminococcaceae	0.26	1.60	116
		Peptococcaceae	0.15	0.50	100
	Proteobacteria	Burkholderiaceae	0.01	0.60	118
		Succinivibrionaceae	0.93	0.92	101
		Rhodospirillales (uncultured)	0.06	0.15	92
	Verrucomicrobia	Akkermansiaceae	0.24	0.02	97
Conventional (102)	Actinobacteria	Bifidobacteriaceae	11.23	0.03	101
	Bacteroidetes	Tannerellaceae	0.18	0.86	90
		Prevotellaceae	11.01	40.75	89
		Muribaculaceae	1.94	5.00	88
		Barnesiellaceae	0.28	0.05	85
	Fibrobacteres	Fibrobacteraceae	0.01	0.28	85
	Firmicutes	Acidaminococcaceae	0.17	2.19	97
		Christensenellaceae	2.36	0.75	97
		Veillonellaceae	0.04	0.40	93
		Peptococcaceae	0.11	0.27	85
	Proteobacteria	Succinivibrionaceae	0.19	1.80	96
		Burkholderiaceae	0.07	0.58	95
		Rhodospirillales (uncultured)	0.02	0.16	91
	Spirochaetes	Spirochaetaceae	1.03	5.99	94
	Tenericutes	Mollicutes RF39 (uncultured)	0.39	0.62	79

[1] Analysis of composition of microbiomes.
[2] Time point 1: 7 days (SD = 3 days) following placement.
[3] Time point 2: 10 days (SD = 4 days) following complete transition the finishing diet.
[4] W value for ANCOM test result indicating significance of differential abundance for a single family by the number of log-ratio comparisons to other families that were significant by pairwise one-way ANOVA testing.

Table 3.8. Mean percent relative abundance of differentially abundant microbial families between at T2 and T3 from individual fecal pats collected from pen floors housing steers managed in natural and conventional programs as determined by ANCOM[1] testing within management program.

Management Program (Families Observed)	Phylum	Family	Mean Relative Abundance, %		
			T2[2]	T3[3]	W[4]
Natural (95)	Bacteroidetes	Paludibacteraceae	0.15	0.44	89
	Proteobacteria	Burkholderiaceae	0.60	0.23	84
Conventional (106)	Bacteroidetes	Prevotellaceae	40.75	24.24	53
	Firmicutes	Christensenellaceae	0.36	1.17	64
		Veillonellaceae	0.40	0.17	54
		Clostridiaceae 1	0.47	1.31	52
		Peptostreptococcaceae	0.56	1.24	51
		Clostridiales (uncultured)	0.03	0.12	48
	Protoebacteria	Burkholderiaceae	0.58	0.32	48
	Tenericutes	Mollicutes RF39 (uncultured)	0.03	0.11	63
	Verrucomicrobia	Akkermansiaceae	0.09	0.47	67

[1] Analysis of composition of microbiomes.
[2] Time point 2: 10 days (SD = 4 days) following complete transition the finishing diet.
[3] Time point 3: 42 days (SD = 4 days) prior to shipment for harvest and before the feeding of β-adrenergic agonist to conventional steers.
[4] W value for ANCOM test result indicating significance of differential abundance for a single family by the number of log-ratio comparisons to other families that were significant by pairwise one-way ANOVA testing.

Table 3.9. Mean percent relative abundance of differentially abundant microbial families between at T3 and T4 from individual fecal pats collected from pen floors housing steers managed in natural and conventional programs as determined by ANCOM[1] testing within management program.

Management Program (Families Observed)	Phylum	Family	Mean Relative Abundance, %		
			T3[2]	T4[3]	W[4]
Natural (94)	NA[5]	NA	NA	NA	NA
Conventional (84)	Proteobacteria	Succinivibrionaceae	1.19	0.52	81
		Burkholderiaceae	0.32	0.09	78

[1] Analysis of composition of microbiomes.
[2] Time point 2: 10 days (SD = 4 days) following complete transition the finishing diet.
[3] Time point 3: 42 days (SD = 4 days) prior to shipment for harvest and before the feeding of β-adrenergic agonist to conventional steers.
[4] W value for ANCOM test result indicating significance of differential abundance for a single family by the number of log-ratio comparisons to other families that were significant by pairwise one-way ANOVA testing.
[3] No families were identified as differentially abundant between T3 and T4 in fecal samples collected from naturally managed steers.

Table 3.10. Adjusted probability of liver abscess incidence of an individual steer managed in a natural or conventional program within a single feedlot.[1]

	Conventional		Natural		
Liver Abscess Score	Probability of Incidence	SE	Probability of Incidence	SE	*P* value[2]
A	12.9%	0.018	19.9%	0.025	0.018
A +	11.1%	0.021	15.8%	0.028	0.183
Total Abscess	25.4%	0.029	36.8%	0.035	0.012

[1] Adjusted probability was generated by transforming the odds estimated by logistic regression to a probability scale.
[2] P value for the test of the log odds ratio between natural and conventional management programs.

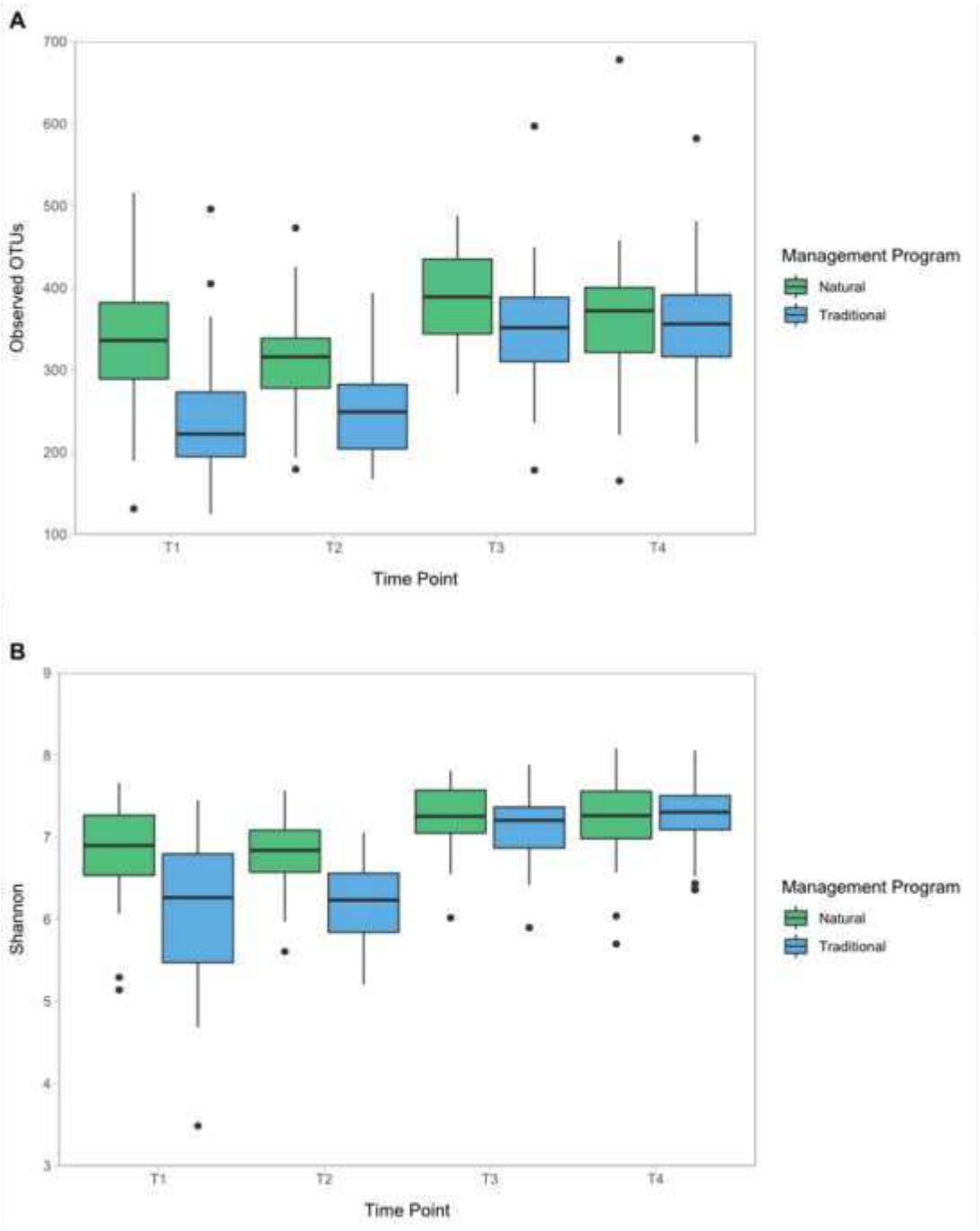

Figure 3.1. Alpha diversity box plots for richness (A) and Shannon Diversity Index (B) of the fecal microbial communities from individual fecal pats collected from pen floors housing steers in natural and conventional management programs within a single feedlot following placement (T1), following transition to the finishing diet (T2), before the feeding of β-adrenergic agonist to conventionally managed steers (T3; mimicked for naturally managed steers), and prior to shipment for harvest (T4).

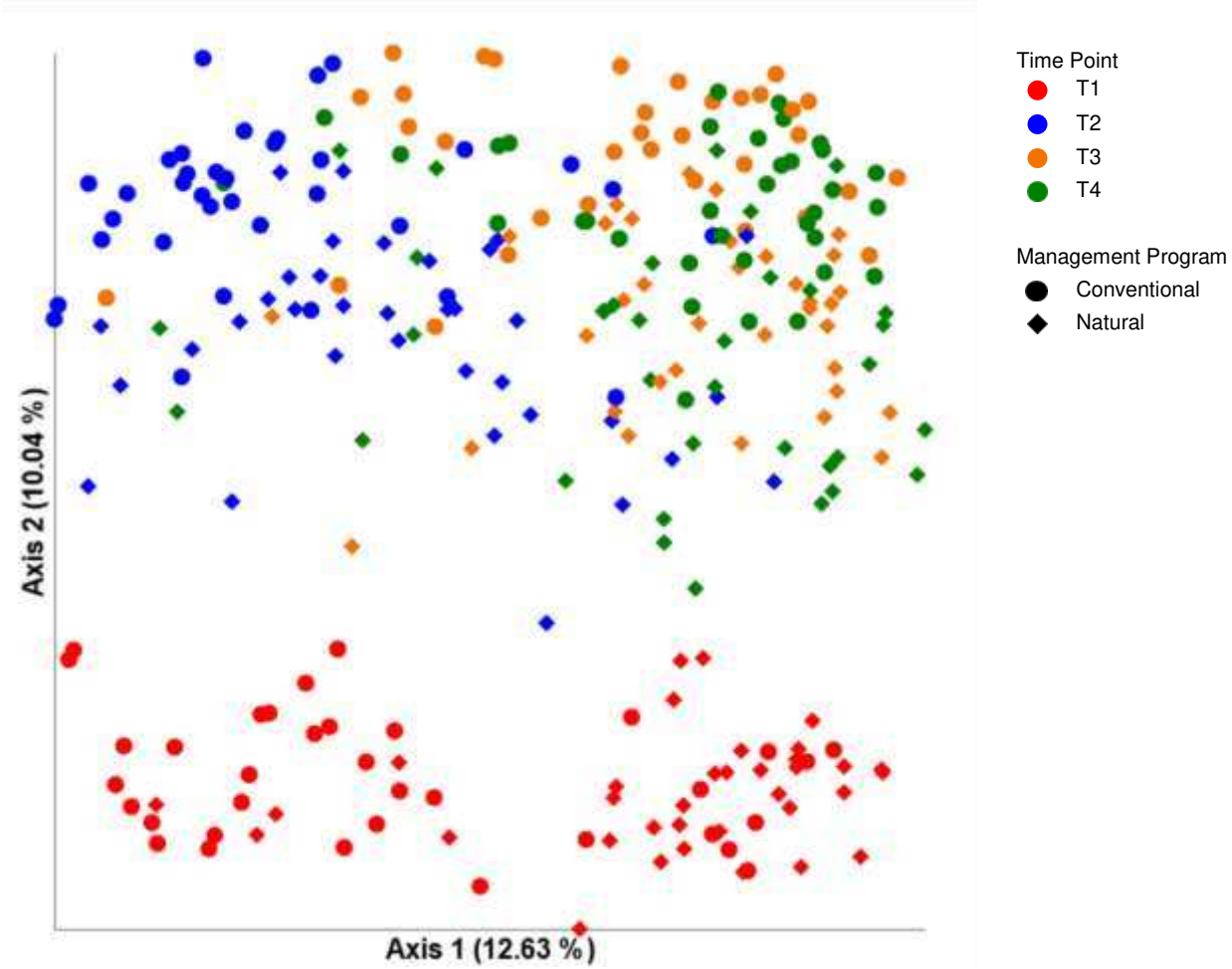

Figure 3.2. Principal coordinate analysis (PCoA) of unweighted UniFrac distance matrices of the fecal microbial communities from individual fecal pats collected from pen floors housing steers in natural and conventional management programs within a single feedlot following placement (T1), following transition to the finishing diet (T2), before the feeding of β-adrenergic agonist to conventionally managed steers (T3; mimicked for naturally managed steers), and prior to shipment for harvest (T4).

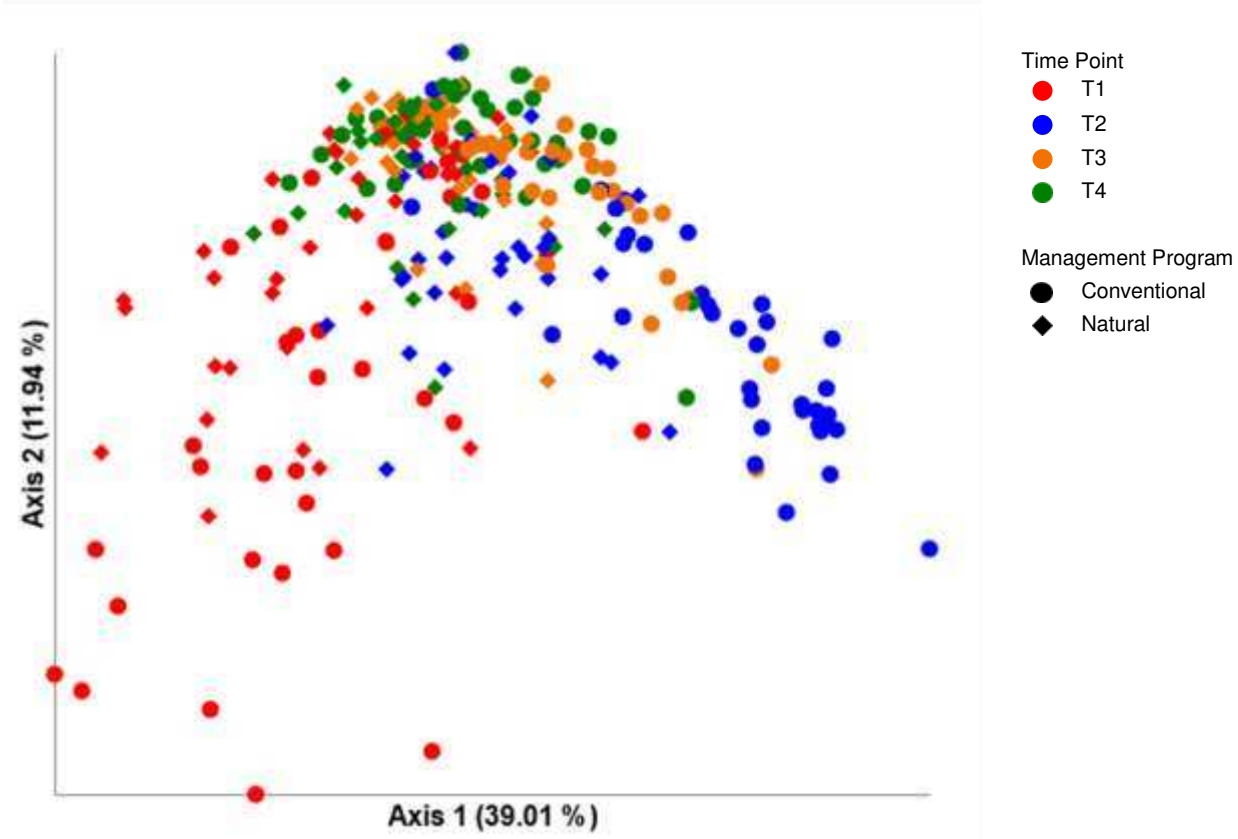

Figure 3.3. Principal coordinate analysis (PCoA) of weighted UniFrac distance matrices of the fecal microbial communities from individual fecal pats collected from pen floors housing steers in natural and conventional management programs within a single feedlot following placement (T1), following transition to the finishing diet (T2), before the feeding of β-adrenergic agonist to conventionally managed steers (T3; mimicked for naturally managed steers), and prior to shipment for harvest (T4).

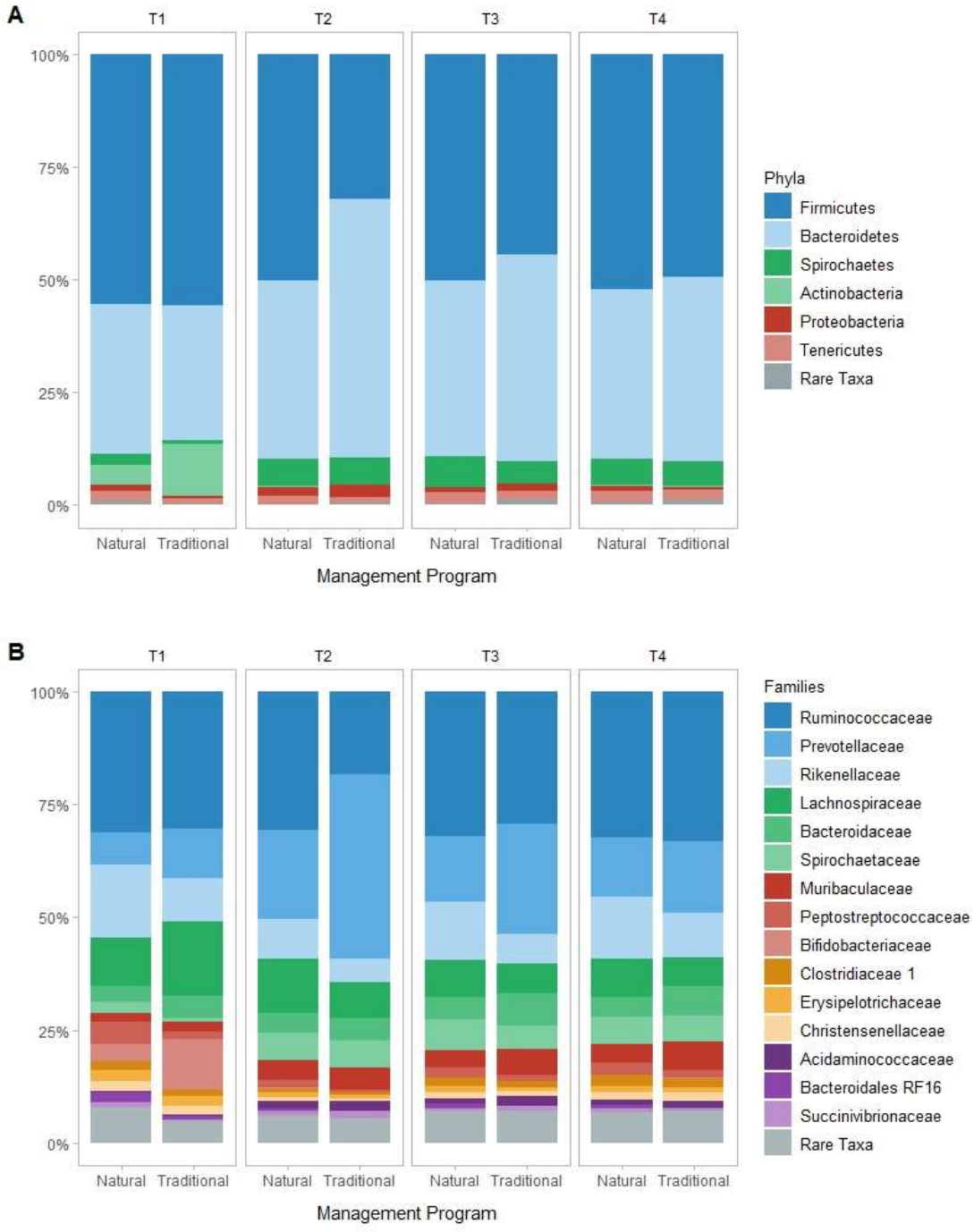

Figure 3.4. Phylum-level (A) and family-level (B) average taxonomic composition of the fecal microbial communities from individual fecal pats collected from pen floors housing steers in natural and conventional management programs within a single feedlot following placement (T1), following transition to the finishing diet (T2), before the feeding of β-adrenergic agonist to conventionally managed steers (T3; mimicked for naturally managed steers), and prior to shipment for harvest (T4).

CHAPTER IV: LIVER ABSCESS MICROBIOTA OF FED STEERS MANAGED IN NATURAL AND CONVENTIONAL PROGRAMS

4.1 OVERVIEW

Liver abscesses are an economic burden to cattle feeders and beef packers in the United States and raise concerns of animal welfare and food safety. While the etiology of liver abscesses generally involves escape of bacteria from the digestive tract to form a polymicrobial abscess within or on the external surface of the liver, little is known about the effects of various production systems on the microbial composition of the liver abscess purulent material. The objective of this study was to use 16S rRNA gene sequencing to compare the microbial community of liver abscess purulent material from steers managed in a conventional program (which includes tylosin supplementation) to those managed in a natural program (without tylosin supplementation) within a single feedlot. Liver abscesses were collected at the time of harvest. The purulent material of liver abscesses from conventionally managed steers (n = 53 abscesses) and naturally managed steers (n = 52 abscesses) was charcaterized using the V4 region of the 16S rRNA gene. Two phyla and three genera were found in greater than 1% relative abundance across all abscesses. The genus *Fusobacterium* was identified in all liver abscess samples and accounted for 64.18% of sequencing reads. *Bacteroides* and *Porphyromonas* genera accounted for 33.59% and 1.25% of reads, respectively. *Trueperella* was more likely to be found in the liver abscesses of naturally manged steers than conventionally manged steers ($P = 0.022$) and was the only taxa found to be differentially abundant by ANCOM testing (W = 6). Over 99% of the genus-level bacterial sequences observed across all liver abscess belonged to gram-negative genera. Bacteria known to colonize both the rumen and hindgut were identified as rare taxa

within liver abscesses. No differences in alpha diversity or beta diversity were detected between liver abscess communities (between the two management programs or individual pens) when tested as richness, Shannon Diversity Index, or weighted UniFrac distances ($P > 0.05$). A Spearman correlation of -0.886 was identified between the relative abundances of *Fusobacterium* and *Bacteroides*. These results are consistent with previous identification of *Fusobacterium necrophorum* as the primary etiologic agent of liver abscesses and emphasize the relationship between the gastrointestinal microbiota and liver abscess formation. Additionally, the microbiota of the liver abscess purulent material appears to be similar among steers from the same feedlot.

4.2 INTRODUCTION

Occurrence of liver abscess in fed steers is associated with a reduction in live performance, hot carcass weight, and visceral value (Brink et al., 1990; Brown and Lawrence, 2010). Collectively, these reductions are thought to cost the fed beef industry a conservative estimate of $60 million each year (Herrick et al., 2018). Prevalence of liver abscesses among fed cattle has risen from the earliest reports of 5.3% (Smith, 1940) to 17.8% reported in the results of most recent National Beef Quality Audit (Eastwood et al., 2017). Feeding antibiotics to livestock is increasingly scrutinized (Aarestrup, 1999; Haskell et al., 2018; Chen et al., 2019), but tylosin phosphate supplementation remains the most effective method of control for liver abscesses when sound bunk management is practiced (Brown et al., 1975; Nagaraja and Lechtenberg, 2007a).

Fusobacterium necroporum has historically been implicated as the primary etiologic agent in liver abscess formation (Nagaraja and Lechtenberg, 2007a). The use of culture-based techniques has consistently demonstrated *F. necroporum* presence within polymicrobial abscesses, but results from these methods may oversimplify the microbial community of liver

abscess purulent material as uncluturable microbes are not assessed (Nagaraja and Chengappa, 1998). Differences in the microbial community of liver abscesses have been attributed to cattle source (ranch of origin, backgrounding location, etc.), breed compostion, feedlot location, and inclusion of tylosin phosphate in the diet (Amachawadi et al., 2017; Weinroth et al., 2017). Limited studies have utilized metagenomics to assess the microbiota within liver abcsesses (Amachawadi et al., 2016; Weinroth et al., 2017). Therfore, the objective of this study was to use 16S rRNA gene sequencing to compare the microbial community of liver abscess purulent material from steers managed in a conventional program (which includes tylosin supplementation) to those managed in a natural program (without tylosin supplementation) within a single feedlot.

4.3 MATERIALS AND METHODS

4.3.1 Cattle Population

Fourteen pens of yearling steers with an average of 281 steers per pen (range 212 to 323; SD = 38 steers per pen) were identified for observation in a commercial feedlot in the High Plains region. Pens enrolled in a conventional management program (n = 7) and pens enrolled in a natural management program (n = 7) arrived at the feedlot over a 45-day period from late August through early October 2018. Upon arrival, all steers were sorted and vaccinated according to standard feedlot protocol. Conventionally managed cattle received hormonal implants containing trenbolone acetate and estradiol.

All steers were fed using a step-up feeding program including receiving, intermediate, and finishing diet formulations. Conventionally managed steers were fed a fourth diet including Optaflexx (Elanco Animal Health; Greenfield, IN) during the final 28 to 42 days of the finishing period. Rumensin (Elanco Animal Health) and Tylan (Elanco Animal Health) were also fed to

conventionally managed steers. Naturally managed cattle were not administered growth-promoting technologies or antibiotics. When treated for illness with antibiotic medication, naturally managed cattle were removed from natural pens and, consequently, removed from the study population.

4.3.2 Liver Evaluation and Liver Abscess Collection

Cattle were transported to a commercial processing facility for harvest from February through April of 2019. Identities of feedlot pens were maintained through the harvest process to allow for collection of liver abscesses from each pen. Livers identified as inedible (for abscess, adherence to internal tissues, cirrhosis, flukes, telangiectasias, or contamination) were removed from the production line and evaluated by trained personnel for abscess presence. Using sterile gloves, livers were palpated to identify abscesses that harbored purulent material. Abscesses and surrounding liver tissue were extracted with sterile scalpels. When the liver abscess capsule was compromised during tissue removal, the entire abscess sample was discarded. Abscesses from individual livers (n = 40 per pen) were placed in sterile collection bags (VWR; Radnor, PA), sealed, and transported in disinfected insulated containers to the Center for Meat Safety and Quality at Colorado State University (Fort Collins, CO).

4.3.3 Liver Abscess Processing

On the same date as sample collection, liver abscess samples were processed and prepared for storage. Ethanol was used to flame sterilize the abscess capsule or external surface of the liver tissue. Abscess capsules were opened with sterile scalpels and purulent material was transferred to sterile 50-mL conical tubes (VWR) with sterile tongue depressors. Aliquots of liver abscess purulent material were stored at -80 °C until the time of DNA extraction. All liver abscesses were qualitatively described using a standard methodology. Abscess visibility

(superficial or deep), number (single or multiple), and adherence of other tissues to capsule were recorded for each liver, along with color, texture, and viscosity of the purulent material.

4.3.4 DNA Extraction and Sequencing

DNA extraction and library preparation were performed at the Metcalf Laboratory at Colorado State University (Fort Collins, CO). A randomly selected subset of the liver abscess purulent material aliquots (n = 10 per pen) was thawed to 4 °C prior to extraction. Purulent material aliquots were individually sampled with sterile swabs (Becton, Dickinson and Company; Franklin Lakes, NJ) and loaded into a 96-well plate in a randomly assigned order by cutting the inoculated swab tip into the plate well with flame-sterilized scissors. Cross contamination was prevented by covering all inactive wells with tape. Sixteen negative controls and two positive controls (ZymoBIOMICS Microbial Community Standard 6300; Zymo Research; Irvine, CA) were included. Ten technical replicates were also included. The loaded plate was stored at -20 °C until the time of DNA extraction.

DNA was extracted using the DNEasy PowerSoil HTP 96 Kit (Qiagen; Hilden, Germany) following the manufacturer's protocol; however, vortex procedures were replaced with manual pipetting to avoid cross contamination. The extraction product was amplified with barcoded primers targeting the V4 region of the 16S RNA gene. Primer constructs included the Illumina MiSeq adaptor (Illumina; San Diego, CA), Golay barcode, spacer, and primer. Earth Microbiome Project (EMP) primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) were used for amplification (Caporaso et al., 2011; Caporaso et al., 2012; Apprill et al., 2015; Parada et al., 2016).

Polymerase chain reaction (PCR) was conducted in duplicate using an Eppendorf Vapo.Protect MasterCycler Pro-S thermocycler (Eppendorf; Hauppauge, NY). For the initial

PCR, 25 μL of reaction mix was prepared by combining 1 μL of template DNA, 1 μL of each barcoded primer (10 μM), 12 μL of molecular-grade water, and 10 μL of Platinum Hot Start PCR Master Mix (Thermo Fisher Scientific; Waltham, MA). PCR conditions followed EMP protocols and included initial denaturation at 94 °C for 3 min; 30 cycles of denaturation (94 °C, 45 s), annealing (50 °C, 60 s) and elongation (72 °C, 90 s); and a final 10-min extension at 72 °C. PCR products were visually evaluated for effective amplification by agarose gel electrophoresis with expected band size of approximately 300 to 350 bp. Similarly, negative controls were visually evaluated for lack of banding pattern. When plates were confirmed of acceptable quality and purity, the second PCR process was completed using the same reaction conditions as described above; 50 μL of reaction mix was prepared by combining 2 μL template DNA, 2 μL of each barcoded primer (10 μM), 24 μL of molecular-grade water, and 20 μL of PCR Master Mix. Agarose gel evaluation of the product was performed as described above.

Duplicate PCR products were pooled and subjected to agarose gel electrophoresis to visualize correct sizes of PCR products and the absence of signal from negative controls. Concentration of amplicon products was determined by Quant-IT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) read on a Fluoroskan (Thermo Fisher Scientific) plate reader. Pico assay concentration results were qualitatively verified by comparison to agarose gel banding patterns. Amplicons were pooled by plate with a target amplicon inclusion of 300 ng of DNA per sample. No more than 50 μL from a single sample were added to the amplicon pool to maintain a reasonable volume. Pooled amplicons were cleaned using MinElute PCR Purification Kit (Qiagen) following manufacturer protocols. The cleaned plate pools were evaluated for DNA concentration by NanoDrop Lite spectrophotometer (Thermo Fisher Scientific) and pooled in equimolar concentrations to form the final sequencing library.

The amplicon library was diluted to a loading concentration of 8 pM and combined with 15% PhiX control library. Paired-end sequencing (2 × 250 bp) was performed using the 500 cycle MiSeq Reagent Kit v2 (Illumina; San Diego, CA) on the Illumina MiSeq platform at the Next Generation Sequencing Core Laboratory at Colorado State University.

4.3.5 Bioinformatics and Statistical Analysis

All amplicon sequence data were bioinformatically processed in QIIME2 version 2019.4 (Bolyen et al., 2018). Imported and demultiplexed paired-end sequences were denoised with DADA2 (Callahan et al., 2016) with both forward and reverse reads trimmed to 250 bp. Taxonomy was assigned with the q2-feature-classifier plugin (Bokulich et al., 2018) using a pretrained naive Bayes Greengenes 13_8 classifier (DeSantis et al., 2006; McDonald et al., 2012), a pretrained naive Bayes Silva 132 classifier, and a Silva 132 (Quast et al., 2013) classifier trained specifically for the primer set used for amplification. The pretrained Silva classifier was selected for downstream analysis due to a fewer operational taxonomic units (OTUs) being defined as unclassified. Reads classified as mitochondria and chloroplast were removed from the data set; controls and technical replicates were also removed. Amplicon sequence variants were assigned phylogeny using SEPP methodology to construct an insertion tree using q2-fragment-insertion (Janssen et al., 2018). Adequate sampling depth was justified by constructing a rarefaction curve with diversity metrics. Sampling depth was standardized for diversity analysis by subsampling without replacement (Weiss et al., 2017) to 10,049 sequences per sample using q2-diversity.

Alpha diversity was measured by richness (the number of observed OTUs) and Shannon Diversity Index (Shannon, 1948). Differences in richness and Shannon Diversity were evaluated between management programs and pen assignments with Kruskal-Wallis testing (Kruskal and

Wallis, 1952) and visualized in R version 3.4.1 (R Core Team, 2017) using ggplot2 (Wickham, 2009). Beta diversity was measured with unweighted UniFrac (Lozupone and Knight, 2005) and weighted UniFrac (Lozupone et al., 2007). Differences in beta diversity between management programs and pen assignments were evaluated with PERMANOVA testing (Anderson, 2017). Principal Coordinate Analysis (PCoA) was used to spatially visualize samples (Vázquez-Baeza et al., 2013). PCoA plots generated in QIIME2 were exported for visualization in Vega version 5.4.0 (Satyanarayan et al., 2016). Taxa differential abundance was evaluated by ANCOM testing at both the phylum and genus level (Mandal et al., 2015). Significance for differential abundance was evaluated as a W value indicating log-fold change against a model-determined threshold based on a bimodal distribution. Rarefied abundance data was exported from QIIME2 as relative abundance and visualized in R version 3.4.1 (R Core Team, 2017) to characterize the composition of the microbiota.

The sequencing depth of each negative control was evaluated to ensure cleanliness of extraction and library preparation; the number of reads generated by each control well before and after denoising were recorded. The sequencing depth of positive controls was similarly recorded. Additionally, the taxa relative abundance of each positive control was exported and visualized in R version 3.4.1 (R Core Team, 2017) using ggplot2 (Wickham, 2009) and compared to the known composition of the mock community for qualitative evaluation. Technical replicates were qualitatively evaluated in pairs by taxa relative abundance to ensure consistency of taxa relative abundance between separate extraction plates.

For each individual liver abscess microbial community, the presence or absence of *Fusobacterium*, *Bacteroides*, and *Trueperella* was determined from rarefied taxonomy tables. Bacterial presence was compared between management programs by logistic regression in R

version 3.4.1 (R Core Team, 2017) using lme4 (Bates et al., 2015) and emmeans (Searle et al., 1980). A mixed effects model was fit using management program as a fixed effect and pen assignment as a random effect. A predetermined alpha level of 0.05 was used for all comparisons in the observational study.

4.4 RESULTS AND DISCUSSION

4.4.1 DNA Sequencing Data

A total of 3,437,552 sequence reads were generated by Illumina MiSeq sequencing for liver abscess purulent material samples, technical replicates, and controls. Liver abscess purulent material samples and replicates (n = 160) averaged 21,037 sequences per sample (range: 18 to 43,192; SD = 11,673). Negative controls (n = 16) averaged 146 sequences per sample (range: 7 to 547; SD = 189). Positive controls (n = 2) averaged 34,636 sequences per sample (range: 31,915 to 37,356; SD = 3,847).

Following denoising, filtering for sequencing depth, and removing controls, replicates, chloroplasts, and mitochondria, a total of 1,055,145 reads mapped to 69 unique features were included for analysis. Rarefying to 10,049 sequences per sample resulted in retaining 53 of 70 liver abscess purulent material samples from steers managed in a conventional program and 52 of 70 liver abscess purulent material samples from steers managed in a natural program. After denoising, negative controls (n = 16) averaged 32 sequences per sample (range: 0 to 311; SD = 77) and positive controls (n = 2) averaged 24,943 sequences per sample (range: 22,272 to 27,614; SD = 3,777).

4.4.2 Bacteria of Interest

Fusobacterium necrophorum is considered the primary etiological agent of liver abscesses in fed cattle (Jensen et al., 1954b; Nagaraja and Chengappa, 1998). In agreement with

previous studies, the genus *Fusobacterium* was identfied in all liver samples from both conventionally and naturally managed steers (Table 4.1). Nagaraja et al. (1999a) and Amachawadi and Nagaraja (2016) found *Fusobacterium* to be present in all liver abscess samples collected from fed catle by culture-based techniques. Others have identified *Fusobacterium* within nearly all liver abscess samples collected from commercial processing facilities using culture-based microbiological techniques (Newsom, 1938; Simon and Stovell, 1971). Weinroth et al. (2017) found *Fusobacterium* to be present in all liver abscess samples collected from fed cattle using similar molecular techniques.

Trueperella pyogenes has previously been observed as the second most frequntly isolated pathogen from liver absces purulent material (Berg and Scanlan, 1982; Nagaraja et al., 1996). *T. pyogenes* is an oppourtunistic Gram-positive bacteria known to inhabit the ruminant mucosal membranes and gastrointestinal tract (Ribeiro et al., 2015). In the current study, the genus *Trueperella* was found more frequently within liver abscesses from naturally managed cattle (14 of 52; 26.92%) than conventionally managed cattle (3 of 53; 5.66%; Table 1). ANCOM testing at the genus level showed *Trueperella* to be the only bacteria in liver abscesses that was differentially abundant between steers manged in conventional and natural programs (W = 6). Conventionally managed cattle received tylosin phosphate, a macrolide antibiotic, during the feeding period. Macrolide antibiotics typically act as a bacteriostat against Gram-positive bacteria (Hof, 1994), seeming to justify a reduction in *Trueperella* prevalence within liver abscesses from conventionally managed cattle. However, in a previous study, *T. pyogenes* was isolated more often in liver abscesses of cattle fed tylosin compared to cattle not fed tylosin (Nagaraja et al., 1999a). Others have identifed *T. pyogenes* in all liver abscesses sampled by molecuar techniques, regardless of cattle management program (Weinroth et al., 2017). Across

the catte feeding industry, the occurrence of *Trueperella* within liver abscesses seems to be variable and not solely dependent on the inclusion of tylosin in the diet. In the current study, *Trueperella* comprised only 0.13% of all sequences identified, indicatung a limited presence of *Trueperella* within liver abscesses from the study population.

4.4.3 Core Microbial Composition

Taxonomic classification with the pretrained naive Bayes Silva 132 classifier identified 2 phyla and 3 genera of bacteria in greater than 1% relative abundance of all reads (Figure 4.1), representing a much simpler microbial community than was reported by Weinroth et al. (2017). However, the purulent material analyzed in this observational study originated from steers housed in a single feedlot over the same time period while the purulent material analyzed by Weinroth et al. (2017) originated from five different feedlots. The dominant phyla included *Fusobacteria* (64.42% of reads) and *Bacteroidetes* (34.87% of reads). The genera identified in greater than 1% relative abundance of all reads included *Fusobacterium* (64.18% of reads; 100% of samples), *Bacteroides* (33.59% of reads; 93.33% of samples), and *Porphyromonas* (1.25% of reads; 7.62% of samples). Bacteroides is a Gram-negative bacterium found in the gastrointestinal tracts of cattle (Miura et al., 1980; Wetzels et al., 2017; Ozbayram et al., 2018) that has been previously described within liver abscesses (Simon and Stovell, 1971; Kanoe et al., 1979; Scanlan and Hathcock, 1983; Nagaraja and Lechtenberg, 2007a). Bacteroides has been demonstrated to increase in relative abundance on the rumen epithelium during acidosis challenge (Wetzels et al., 2017). *Porphyromonas* is a ruminal bacterium closely related to *Bacteroides* (Summanen et al., 2005) and has been previously found in bovine liver abscesses (Scanlan and Hathcock, 1983; Nagaraja and Lechtenberg, 2007a; Weinroth et al., 2017)

Bovine liver abscesses have often been described as polymicrobial infections dominated by Gram-negative bacteria (Newsom, 1938; Calkins et al., 1968; Scanlan and Hathcock, 1983; Nagaraja et al., 1996; Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007a). In the current study, over 99% of the bacterial sequences observed across all liver abscesses belonged to genera previously classified as Gram-negative by Langworth (1977), Hofstad (1984), and Bostanci and Belibasakis (2012). *Fusobacterium* was the sole microbial genera in only 2 of the 105 purulent material samples (1.90%). All other samples (98.2%) were identified as mixed cultures of *Fusobacterium* with other bacteria.

4.4.4 Rare Taxa

Taxa observed at a frequency of less than 1% of all reads were considered rare taxa. Families *Ruminococcaceae* and *Prevotellaceae* were identified in 25.71% and 2.86% of all liver abscesses, respectively. *Ruminococcus* and *Prevotella* genera are commonly identified as constituents of the rumen microbiota (Firkins and Yu, 2015; Henderson et al., 2015; Ozbayram et al., 2018; Holman and Gzyl, 2019). The genus *Treponema* was identified in 8.57% of liver abscesses and has been demonstrated to be present in the rumen, specifically on the rumen epithelium (Stanton and Canale-Parola, 1980; Liu et al., 2016). Generally, bacteria known to inhabit and dominate the microbial communities of the rumen epithelium were found more frequently within liver abscesses than bacteria known to strictly inhabit the rumen contents. This supports the proposed etiology of bovine liver abscesses with respect to the rumenitis-liver abscess complex described by Jensen et al. (1954b) and the hypothesis that bacteria escape the rumen by damage to epithelial tissues (Nagaraja et al., 1996).

However, many bacteria found in the rumen are also found in the lower gastrointestinal tract and feces. *Ruminococcaceae* and *Bacteroidaceae* are commonly found in greater relative

abundance in the feces compared to the rumen (Ozbayram et al., 2018; Holman and Gzyl, 2019). *Turicibacter*, a dominant genus of fecal microbial communities (Liu et al., 2016), was identified in 13.33% of liver abscesses. Additionally, *Romboutsia* and *Clostridium sensu stricto 1*, bacteria known to colonize the hindgut in ruminants as early as immediately following birth (Alipour et al., 2018), were identified in 9.52% and 11.43% of liver abscesses, respectively. Generally, bacteria known to inhabit and dominate the microbial communities of the hind gut were found more frequently within liver abscesses than bacteria known to strictly inhabit the rumen. These findings suggest possible entry of bacteria into the portal circulation originating from the hindgut. In theory, intestinal epithelial damage from hindgut acidosis could allow passage of microbes into the portal blood (Oba and Wertz-Lutz, 2011). Gressley et al. (2011) suggested that hindgut epithelium might be more susceptible to damage caused by excess fermentation compared with ruminal epithelium, due to the lack of salivary flow, limited protozoa, and reduced epithelial layers. Hindgut acidosis in feedlot cattle fed high-concentrate diets could contribute to liver abscess formation.

Relevant to food safety and concern of cross contamination, *Campylobacter* was found within ten liver abscesses and composed 0.28% of all reads. *Campylobacter* has been previously identified in bovine liver abscesses (Weinroth et al., 2017) and has been found to be more prevalent on rumen epithelium than in rumen contents or feces (Liu et al., 2016). Similar to findings of Weinroth et al. (2017) *Pseudomonas*, *Parvimonas*, and *Atopobium* were found within liver abscesses as rare taxa. Findings of *Streptococcus* and *Peptostreptococcus* within liver abscesses mirrored previous reports by Scanlan and Berg (1983) and Kanoe et al. (1984), respectively.

4.4.5 Alpha Diversity Within Liver Abscess Microbial Communities

Richness did not differ between liver abscess purulent material from steers managed in conventional and natural programs ($P = 0.488$; Figure 4.2A). Numerically, a slightly larger mean number of OTUs was found in liver abscesses from naturally managed steers in comparison to those of conventionally managed steers. Richness of liver abscess communities between pens was also similar ($P = 0.855$) with a range of mean richness values per pen between 5.13 and 7.22 OTUs. Richness observed in the liver abscess purulent material in this study was considerably lower than values previously observed for rumen or fecal samples (Shanks et al., 2011; Azad et al., 2019).

Shannon Diversity Index was not statistically different between liver abscess purulent material from steers managed in conventional and natural programs ($P = 0.356$; Figure 4.2B). Numerically, a slightly larger average Shannon Diversity Index value was found in liver abscesses from naturally managed steers in comparison to those of conventionally managed steers. Shannon Diversity Index of liver abscess communities was also similar ($P = 0.495$) between pens with a range of mean Shannon Diversity Index values per pen between 0.83 and 1.30. These observed Shannon Diversity Index values were considerably lower than values previously observed for rumen or fecal samples (Yang et al., 2016; Azad et al., 2019) and indicated a relatively simple microbial community.

4.4.6 Beta Diversity Between Liver Abscess Microbial Communities

Qualitative beta diversity differences can be identified by testing phylogenetic UniFrac distance matrices without regard to relative abundance of individual taxa (Lozupone and Knight, 2005). Using unweighted UniFrac for analysis, differences between purulent material microbial communities were not observed when compared by management program ($P = 0.169$; Figure 4.3). However, differences in purulent material communities were observed between individual

pens ($P = 0.013$). These results indicated that there was more variation in abscess communities between pens than between feedlot management programs. Variation between pens was expected due to the intrinsic pen-specific factors including steers' ranch of origin, contemporary environment, and feeding behaviors. Unweighted UniFrac is sensitive to presence and absence of individual taxa (Lozupone and Knight, 2005). Since previously discussed findings of this study indicated that the liver abscess purulent material microbial community was relatively simple, the differences in beta diversity between pens are likely driven by the presence of rare taxa within individual liver abscesses and are not practically important.

Quantitative differences between microbial communities can be identified by testing phylogenetic UniFrac distance matrices with regard to relative abundance of individual taxa (Lozupone et al., 2007). Using weighted UniFrac for analysis, differences between purulent material microbial communities were not observed when compared by management program ($P = 0.799$; Figure 4.4A). Similarly, differences in purulent material communities were not observed between individual pens ($P = 0.067$). Weighted UniFrac analysis (Lozupone et al., 2007) accounts for the ecological mass-ratio hypothesis which implies that community dynamics are largely influenced by dominant species and are insensitive to the abundance of rare taxa (Grime, 1998). Applied to the context of the findings of this study, the abundance of *Fusobacteria* and *Bacteroides* (combined average relative abundance of 97.77%) would seem to characterize major differences in microbial communities. This is reflected by the horizontal axis of the PCoA plot (Figure 4.4) of weighted UniFrac distances explaining over 95% of the variation between liver abscess microbial communities.

Much of the difference between microbial communities in liver abscess purulent material appeared to be driven by the relative abundances of *Fusobacteria* and *Bacteroides*. The dominant

genus for a given sample was defined as any genus comprising greater than half of the reads within that sample. If no genus comprised over 50% of the reads within a single sample, no dominant genus was declared for the sample. *Fusobacteria* was the dominant genus in 59 liver abscess samples (30 natural steers, 29 conventional steers). *Bacteroides* was the dominant genus in 43 liver abscess samples (20 natural steers, 23 conventional steers). Using unweighted UniFrac, differences among purulent material microbial communities were observed between abscesses dominated by *Fusobacteria* and *Bacteroides* ($P = 0.001$). Principle coordinate analysis demonstrates a general clustering of abscess communities by dominant bacterial genus (Figure 4.3). This indicates that a large portion of the variation between the microbial communities can be explained by two bacteria. However, not all samples were represented by a dominant genus and samples with a greater proportion of rare taxa appear to segregate from expected clustering by dominant genus.

To account for relative abundance of rare taxa, unweighted UniFrac distance matrices were tested to identify differences between communities dominated by differing genera. Using weighted UniFrac, differences in purulent material microbial communities were observed between abscesses dominated by *Fusobacteria* and abscesses dominated by *Bacteroides* ($P = 0.001$). A clear separation of communities dominated by *Fusobacteria* and those dominated by *Bacteroides* was observed when visualized by PCoA (Figure 4.4A). When colored by *Fusobacteria* relative abundance (Figure 4.4B), PCoA appears to show a nearly linear gradient from communities low in *Fusobacteria* (left) to communities high in *Fusobacteria* (right). A Spearman correlation of -0.886 was observed between *Fusobacteria* relative abundance and *Bacteroides* relative abundance (Figure 4.5). The strength of this relationship is likely an artifact of the simplicity of the liver abscess community and the method of expressing abundance in

relative terms. Differences in abscess characteristics, including color, texture, viscosity, size and adhesion to other internal tissues were not explained by the presence of a specific dominant genus.

4.5 SUMMARY

In this observational study, *Fusobacterium* and *Bacteroides* appeared to dominate the microbial communities of liver abscess purulent material by relative abundance. The microbial communities of liver abscess purulent material from this study were much simpler than previously published studies, emphasizing the need for further investigation. Though management program did not have an effect on the beta diversity of the microbiota of the liver abscess purulent material, *Trueperella* was found more frequently within liver abscesses from steers managed in the natural program (without exposure to tylosin). Rare taxa identified suggest a link between the microbiota of the ruminant gastrointestinal tract and the microbiota of the liver abscess purulent material. Together, these results indicate that steers managed within the same feedlot have similar liver abscess microbiota. While exposure to tylosin could affect the liver abscess microbiota, the rare taxa identified within liver abscesses confirm the link between nutritional stress and liver abscess formation.

4.6 TABLES AND FIGURES

Table 4.1. Adjusted probability[1] of an individual abscess from a steer managed in a natural[2] or conventional[3] program to harbor *Fusobacterium*, *Bacteroides*, or *Trueperella*.

	Natural		Conventional		
Bacterial genus	Probability of Presence	SE	Probability of Presence	SE	*P* value[4]
Fusobacterium[5]	100.00%	NA	100.00%	NA	NA
Bacteroides	95.56%	3.422	94.71%	4.326	0.850
Trueperella	23.82%	8.676	4.71%	3.316	0.022

[1] Adjusted probability was generated by transforming the odds estimated by logistic regression to a probability scale.
[2] Program included no growth-promoting technologies or antimicrobials.
[3] Program included hormonal implants, tylosin, monensin, and ractopamine.
[4] *P* value for the test of the log odds ratio between natural and conventional management programs.
[5] Given *Fusobacterium* presence in all liver abscesses, no logistic regression analysis was performed.

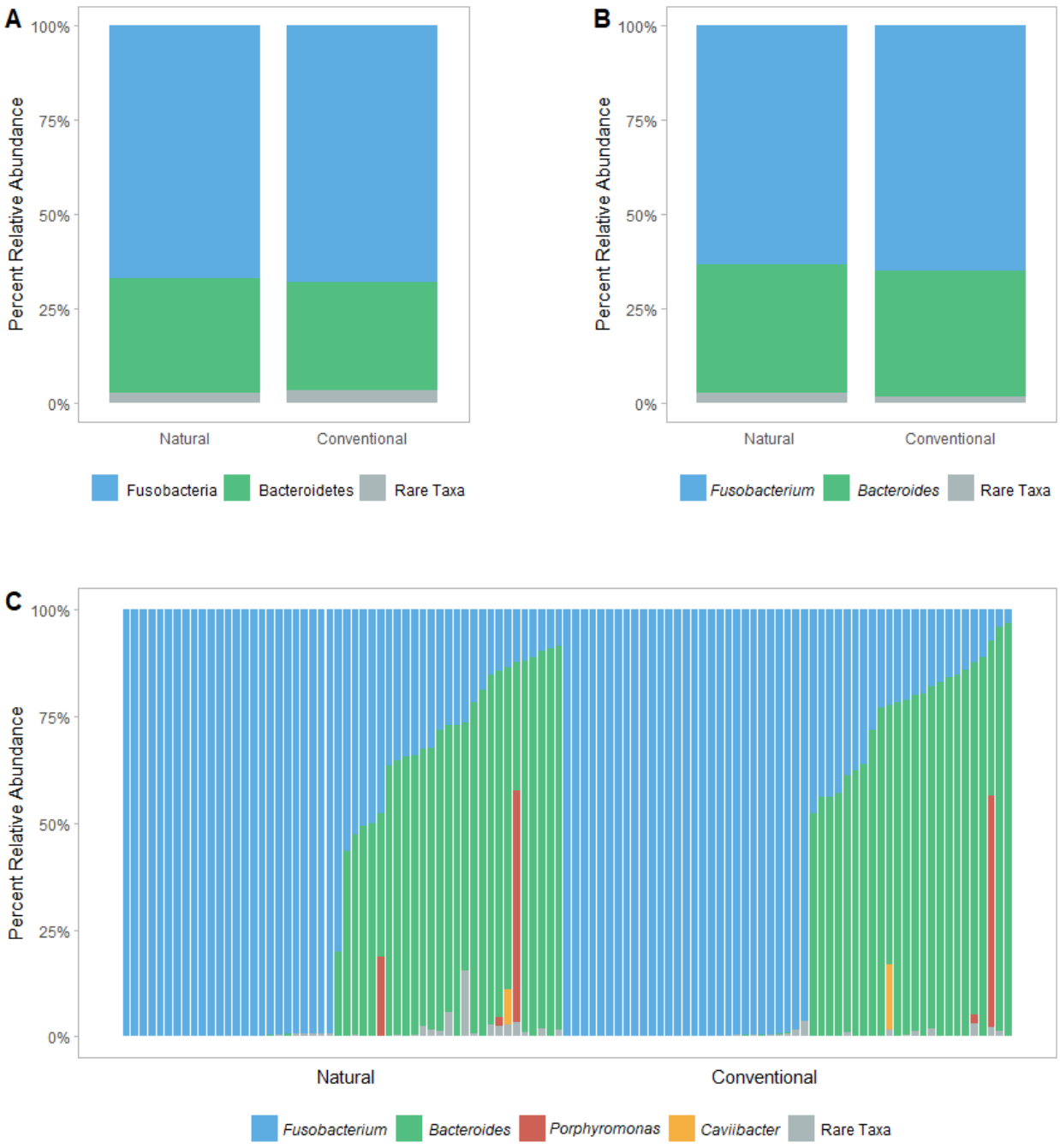

Figure 4.1. Average taxonomic composition of the bacterial communities within liver abscesses from steers in a single feedlot under natural (n = 52) and conventional (n = 53) management programs by averaged over management program at the phylum-level (A) and genus-level (B) and by individual sample (C).

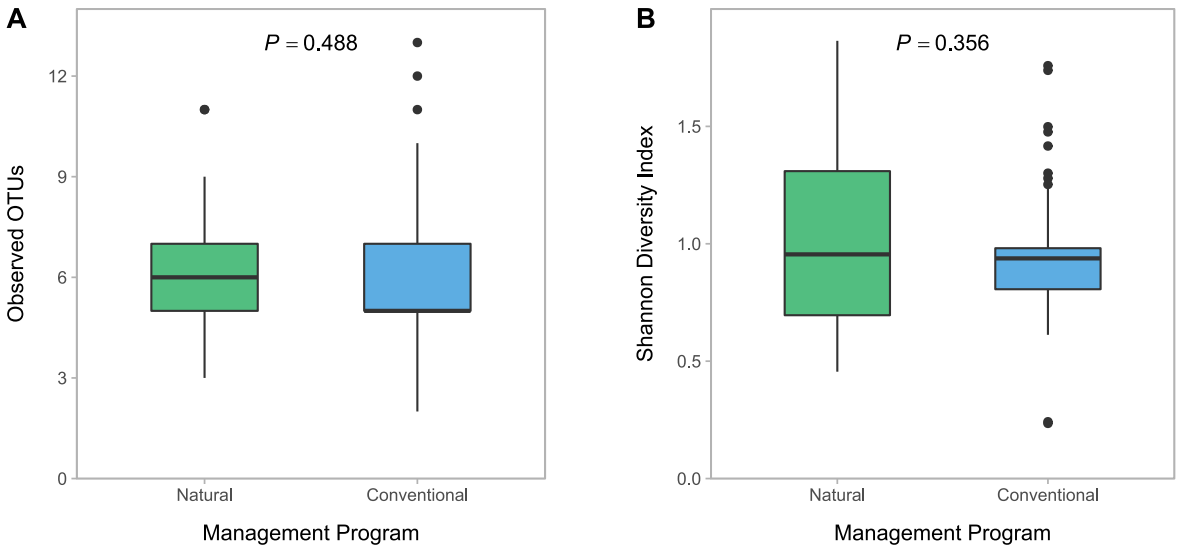

Figure 4.2. Alpha diversity, depicted as richness (A) and Shannon Diversity Index (B), of the bacterial communities within liver abscess purulent material from steers in a single feedlot within natural (n = 52) and conventional (n = 53) management programs.

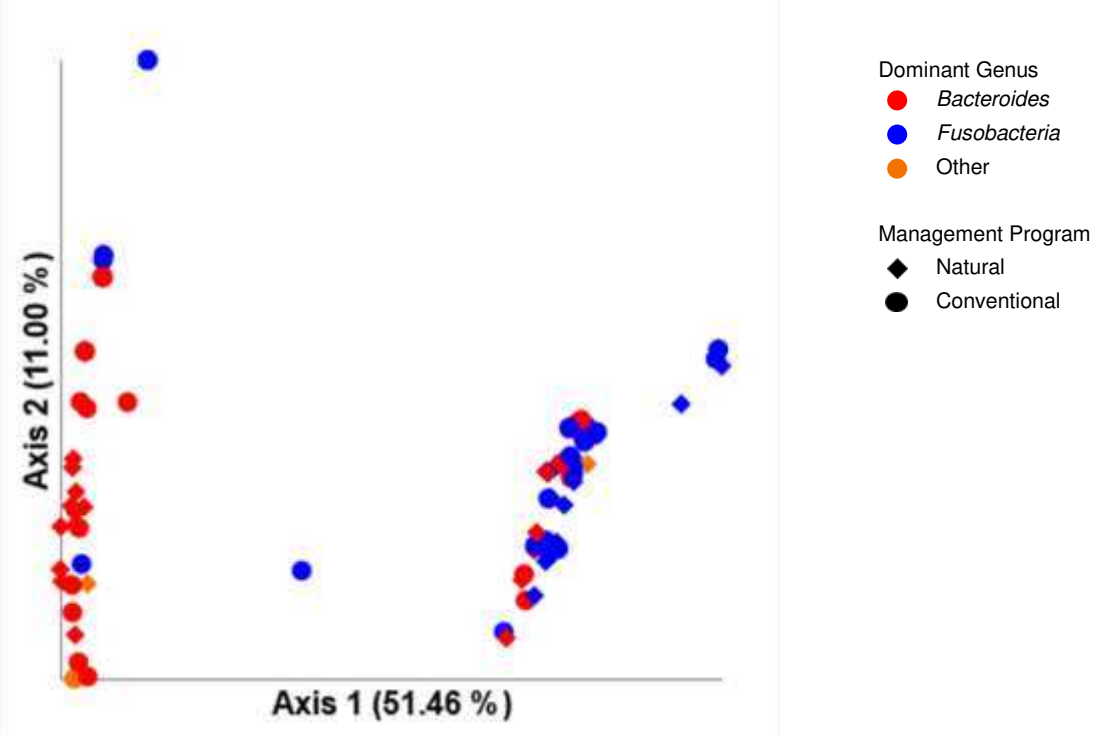

Figure 4.3. Principal coordinate analysis (PCoA) of unweighted UniFrac distance matrices of the bacterial communities within liver abscesses from steers managed in a single feedlot.

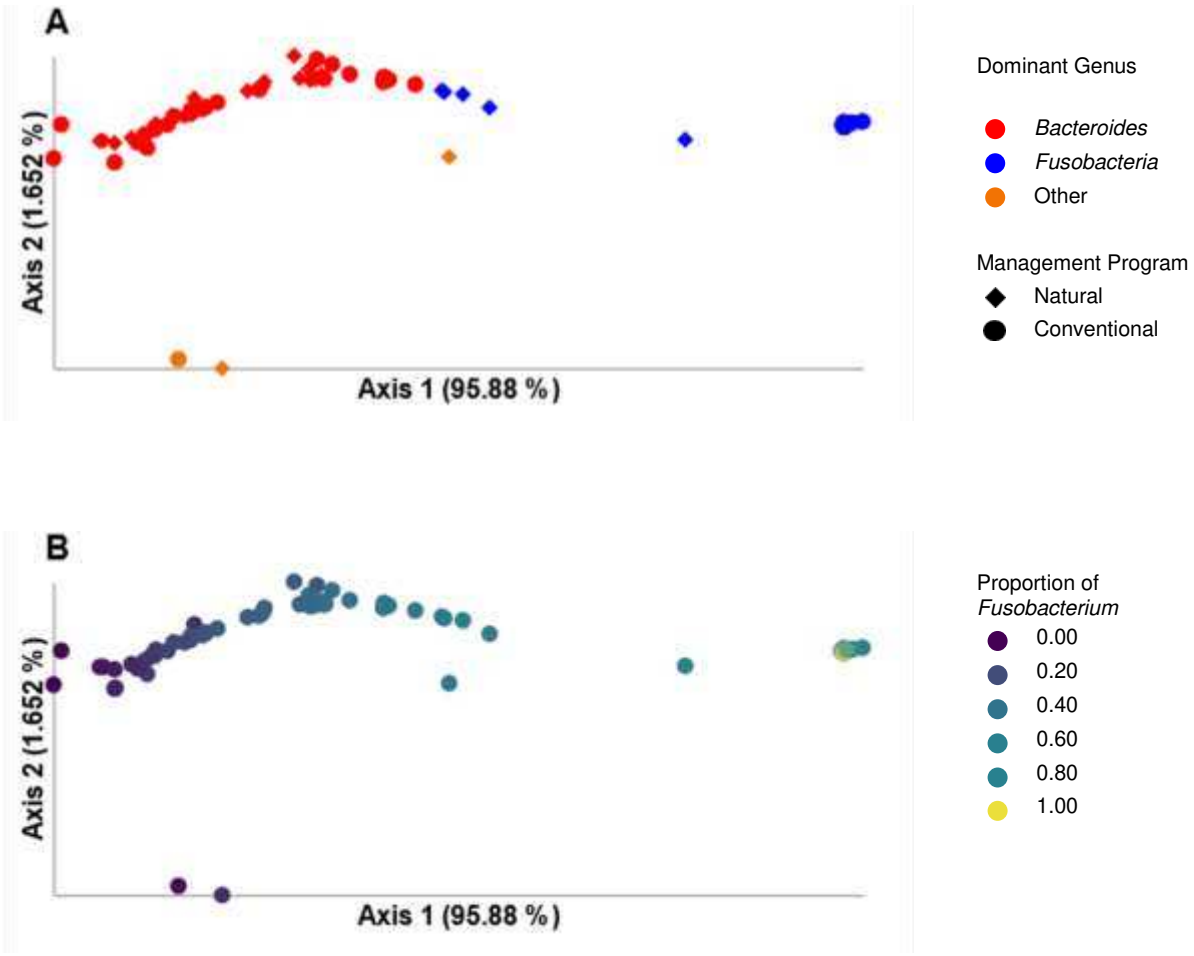

Figure 4.4. Principal coordinate analysis (PCoA) of weighted UniFrac distance matrices of the bacterial communities within liver abscesses from steers managed in a single feedlot by the management program of the live steer corresponding to each abscess and the dominant bacterial genus of the abscess microbiota (A) and the relative abundance of the genus *Fusobacteria* (B).

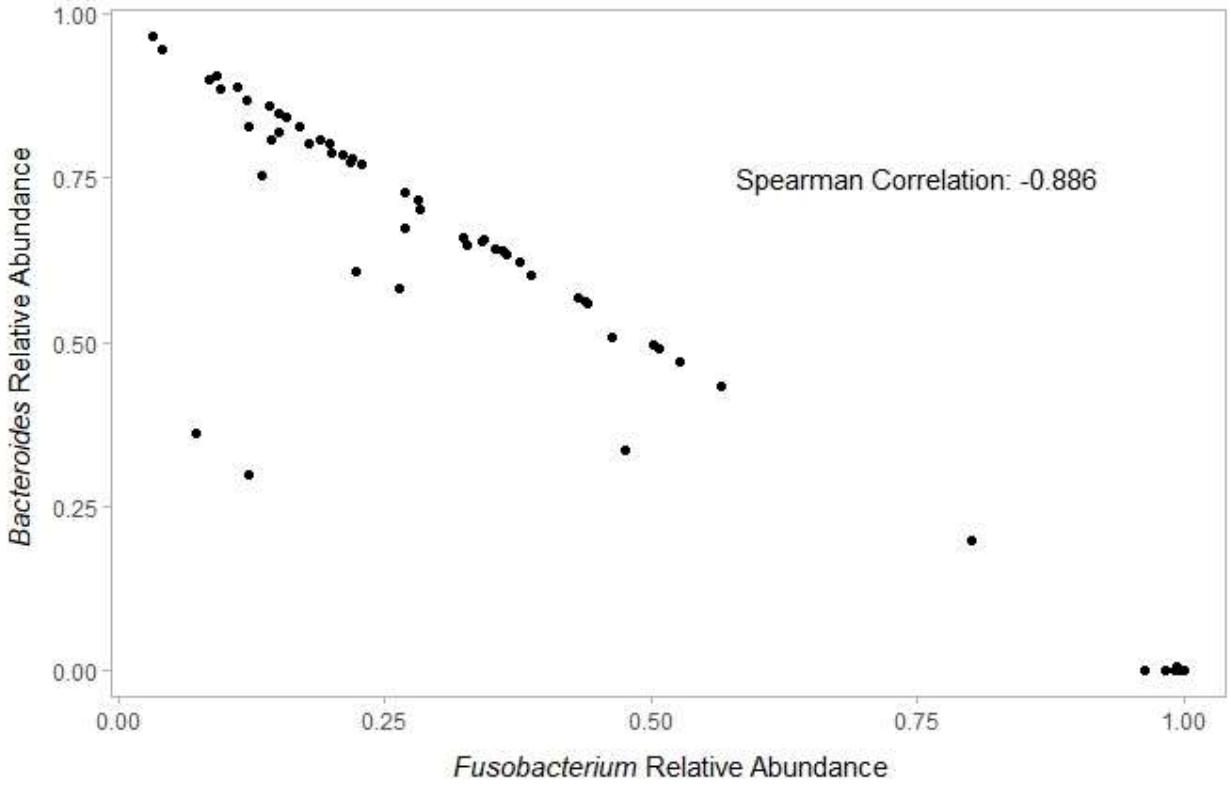

Figure 4.5. Scatter plot of the relative abundances of Fusobacterium and Bacteroides found within the liver abscesses of steers managed in a single feedlot.

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APPENDIX A: FECAL ANALYSIS CODE

FECAL QIIME2 ANALYSIS CODE

```
################################################################################
##Project: Feedlot Microbiota: Fecal
##Version: QIIME2-2019.4
##Sequencing Dates:  6/10/19
                     6/28/19
################################################################################

################################################################################
##IMPORT DEMUX

#!/bin/sh

#SBATCH --job-name=r2-demux-import
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

qiime tools import \
  --type 'SampleData[PairedEndSequencesWithQuality]' \
  --input-path /scratch/summit/fuerniss@colostate.edu/feedlot-microbiome/r2-manifest.txt \
  --output-path r2-demux.qza \
  --input-format PairedEndFastqManifestPhred33V2

#Summarize
qiime demux summarize \
  --i-data r2-demux.qza \
  --o-visualization r2-demux.qzv
```

```
################################################################################
##DENOISE

#!/bin/sh

#SBATCH --job-name=r2-dada2
#SBATCH --nodes=1
#SBATCH --ntasks=2
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#dada2
qiime dada2 denoise-paired \
  --i-demultiplexed-seqs r2-demux.qza \
  --p-trunc-len-f 248 \
  --p-trunc-len-r 248 \
  --p-n-threads 2 \
  --o-table r2-table.qza \
  --o-representative-sequences r2-rep-seqs.qza \
  --o-denoising-stats r2-denoising-stats.qza

#Visualize
qiime metadata tabulate \
  --m-input-file r2-denoising-stats.qza \
  --o-visualization r2-denoising-stats.qzv

qiime feature-table summarize \
  --i-table r2-table.qza \
  --o-visualization r2-table.qzv \
  --m-sample-metadata-file r2-metadata.txt

qiime feature-table tabulate-seqs \
  --i-data r2-rep-seqs.qza \
  --o-visualization r2-rep-seqs.qzv
```

```
################################################################################
##MERGE RUN 1 AND RUN 2

#!/bin/sh

#SBATCH --job-name=merge
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=00:20:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Merge Feature Tables
qiime feature-table merge \
  --i-tables r1-table.qza \
  --i-tables r2-table.qza \
  --o-merged-table merged-table.qza

#Merge Rep Seqs
qiime feature-table merge-seqs \
  --i-data r1-rep-seqs.qza \
  --i-data r2-rep-seqs.qza \
  --o-merged-data merged-rep-seqs.qza

################################################################################
##FILTER FOR ONLY FECAL

sinteractive --time=00:30:00

#Activate Qiime2
source activate qiime2-2019.4

#Filter
qiime feature-table filter-samples \
  --i-table merged-table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_analysis_control='yes'" \
  --o-filtered-table complete-fecal-table.qza
```

```
#Visualize
qiime feature-table summarize \
  --i-table complete-fecal-table.qza \
  --m-sample-metadata-file metadata.txt \
  --o-visualization complete-fecal-table.qzv

################################################################################
##TAXONOMY

#Run pretrained Greengenes
#Train and run SILVA
#Compare results

################################################################################
##DOWNLOAD DATABASES

#download pretrained Greengenes database
wget https://data.qiime2.org/2019.4/common/gg-13-8-99-515-806-nb-classifier.qza

#download pretrained Silva database
wget https://data.qiime2.org/2019.4/common/silva-132-99-515-806-nb-classifier.qza

#download untrained SILVA
wget https://www.arb-silva.de/fileadmin/silva_databases/qiime/Silva_132_release.zip

################################################################################
##FECAL GG TAXONOMY

#!/bin/sh

#SBATCH --job-name=fecal-taxonomy-gg
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=00:30:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Run GG Taxonomy
qiime feature-classifier classify-sklearn \
  --i-classifier gg-13-8-99-515-806-nb-classifier.qza \
  --i-reads merged-rep-seqs.qza \
  --o-classification fecal-taxonomy-gg.qza
```

```
#Visualize GG Taxonomy
qiime metadata tabulate \
  --m-input-file fecal-taxonomy-gg.qza \
  --o-visualization fecal-taxonomy-gg.qzv

#Taxa GG Barplot
qiime taxa barplot \
  --i-table complete-fecal-table.qza \
  --i-taxonomy fecal-taxonomy-gg.qza \
  --m-metadata-file metadata.txt \
  --o-visualization fecal-taxa-bar-plots-gg.qzv

################################################################################
##TRAIN SILVA CLASSIFIER

sinteractive --time=00:30:00

#Activate Qiime2
source activate qiime2-2019.4

#Unzip Silva
unzip Silva_132_release.zip

#Import Sequences
qiime tools import \
  --type 'FeatureData[Sequence]' \
  --input-path SILVA_132_QIIME_release/rep_set/rep_set_16S_only/99/silva_132_99_16S.fna \
  --output-path silva_132_99_16S.qza

#Import Taxonomy
qiime tools import \
  --type 'FeatureData[Taxonomy]' \
  --input-format HeaderlessTSVTaxonomyFormat \
  --input-path SILVA_132_QIIME_release/taxonomy/16S_only/99/taxonomy_all_levels.txt \
  --output-path 16S-all-levels-99-ref-taxonomy.qza
```

```
#Train Classifier Using Script

#!/bin/sh

#SBATCH --job-name=silva-classifier
#SBATCH --nodes=1
#SBATCH --partition=smem
#SBATCH --ntasks=1
#SBATCH --time=12:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Extract Reference Reads
qiime feature-classifier extract-reads \
  --i-sequences silva_132_99_16S.qza \
  --p-f-primer GTGYCAGCMGCCGCGGTAA \
  --p-r-primer GGACTACNVGGGTWTCTAAT \
  --o-reads ref-seqs.qza

#Train Classifier
qiime feature-classifier fit-classifier-naive-bayes \
  --i-reference-reads ref-seqs.qza \
  --i-reference-taxonomy 16S-all-levels-99-ref-taxonomy.qza \
  --o-classifier silva-99-classifier.qza

##############################################################################
#TEST CLASSIFIER
#tested on Moving Pictures Tutorial Rep Seqs

#!/bin/sh

#SBATCH --job-name=silva-test
#SBATCH --nodes=1
#SBATCH --partition=smem
#SBATCH --ntasks=1
#SBATCH --time=02:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4
```

```
qiime feature-classifier classify-sklearn \
  --i-classifier silva-99-classifier.qza \
  --i-reads mp-rep-seqs.qza \
  --o-classification test-taxonomy.qza

qiime metadata tabulate \
  --m-input-file test-taxonomy.qza \
  --o-visualization test-taxonomy.qzv

################################################################################
##FECAL HAND-TRAINED SILVA TAXONOMY

#!/bin/sh

#SBATCH --job-name=liver-taxonomy-silva-self
#SBATCH --nodes=1
#SBATCH --partition=smem
#SBATCH --ntasks=1
#SBATCH --time=06:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Run Silva Taxonomy
qiime feature-classifier classify-sklearn \
  --i-reads merged-rep-seqs.qza \
  --i-classifier silva-99-classifier.qza \
  --o-classification fecal-taxonomy-silva-s.qza

#Visualize Silva Taxonomy
qiime metadata tabulate \
  --m-input-file fecal-taxonomy-silva-s.qza \
  --o-visualization fecal-taxonomy-silva-s.qzv

#Taxa Silva Barplot
qiime taxa barplot \
  --i-table complete-fecal-table.qza \
  --i-taxonomy fecal-taxonomy-silva-s.qza \
  --m-metadata-file metadata.txt \
  --o-visualization fecal-taxa-bar-plots-silva-s.qzv
```

```
#############################################################################
##FECAL PRETRAINED SILVA TAXONOMY
#script

#!/bin/sh

#SBATCH --job-name=fecal-taxonomy-silva-7
#SBATCH --nodes=1
#SBATCH --partition=smem
#SBATCH --ntasks=1
#SBATCH --time=6:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Run Silva Taxonomy
qiime feature-classifier classify-sklearn \
  --i-classifier silva-132-99-515-806-nb-classifier.qza \
  --i-reads merged-rep-seqs.qza \
  --o-classification fecal-taxonomy-silva-7.qza

#Visualize Silva Taxonomy
qiime metadata tabulate \
  --m-input-file fecal-taxonomy-silva-7.qza \
  --o-visualization fecal-taxonomy-silva-7.qzv

#Taxa Silva Barplot
qiime taxa barplot \
  --i-table complete-fecal-table.qza \
  --i-taxonomy fecal-taxonomy-silva-7.qza \
  --m-metadata-file metadata.txt \
  --o-visualization fecal-taxa-bar-plots-silva-7.qzv

#############################################################################
##SELECT BEST CLASSIFIER

#fecal-taxonomy-silva-s.qzv --> QIIME2VIEW
#fecal-taxa-bar-plots-silva-s.qzv --> QIIME2VIEW

#fecal-taxonomy-gg.qzv --> QIIME2VIEW
#fecal-taxa-bar-plots-gg.qzv --> QIIME2VIEW

#fecal-taxonomy-silva-7.qzv --> QIIME2VIEW
#fecal-taxa-bar-plots-silva-7.qzv --> QIIME2VIEW
```

```
#Compare proportion of unclassified taxa
#Pretrained SILVA selected

################################################################################
##FILTER CHLOROPLASTS AND MITOCHONDRIA
##FILTER SAMPLES, CONTROLS, AND REPLICATES

#!/bin/sh
#SBATCH --job-name=fecal-filtering
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=00:10:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Remove Spaces from Taxonomy
qiime tools export \
  --input-path fecal-taxonomy-silva-7.qza \
  --output-path fecal-taxonomy-silva-7-with-spaces
qiime metadata tabulate \
  --m-input-file fecal-taxonomy-silva-7-with-spaces/taxonomy.tsv  \
  --o-visualization fecal-taxonomy-silva-7-as-metadata.qzv
qiime tools export \
  --input-path fecal-taxonomy-silva-7-as-metadata.qzv \
  --output-path fecal-taxonomy-silva-7-as-metadata
qiime tools import \
  --type 'FeatureData[Taxonomy]' \
  --input-path fecal-taxonomy-silva-7-as-metadata/metadata.tsv \
  --output-path fecal-taxonomy-silva-7-without-spaces.qza


#Remove Mitochondria and Chloroplasts
qiime taxa filter-table \
  --i-table complete-fecal-table.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-exclude mitochondria,chloroplast \
  --o-filtered-table fecal-table-no-mito-chlo-silva-7.qza
```

```
#************************************************************
#Filter for All Samples
qiime feature-table filter-samples \
  --i-table fecal-table-no-mito-chlo-silva-7.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_analysis IN ('yes')" \
  --o-filtered-table fecal-table-samples.qza

#Visualize Samples
qiime feature-table summarize \
  --i-table fecal-table-samples.qza \
  --o-visualization fecal-table-samples.qzv \
  --m-sample-metadata-file metadata.txt

#Samples Taxa Barplot
qiime taxa barplot \
  --i-table fecal-table-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --m-metadata-file metadata.txt \
  --o-visualization fecal-samples-taxa-bar-plots.qzv

#************************************************************
#Filter for Individual Samples
qiime feature-table filter-samples \
  --i-table fecal-table-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "individual_composite IN ('individual')" \
  --o-filtered-table fecal-table-ind-samples.qza

#Visualize Samples
qiime feature-table summarize \
  --i-table fecal-table-ind-samples.qza \
  --o-visualization fecal-table-ind-samples.qzv \
  --m-sample-metadata-file metadata.txt

#Samples Taxa Barplot
qiime taxa barplot \
  --i-table fecal-table-ind-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --m-metadata-file metadata.txt \
  --o-visualization fecal-ind-samples-taxa-bar-plots.qzv
```

```
#************************************************************
# Filter for Composite Samples
qiime feature-table filter-samples \
  --i-table fecal-table-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "individual_composite IN ('composite')" \
  --o-filtered-table fecal-table-comp-samples.qza

#Visualize Samples
qiime feature-table summarize \
  --i-table fecal-table-comp-samples.qza \
  --o-visualization fecal-table-comp-samples.qzv \
  --m-sample-metadata-file metadata.txt

#Samples Taxa Barplot
qiime taxa barplot \
  --i-table fecal-table-comp-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --m-metadata-file metadata.txt \
  --o-visualization fecal-comp-samples-taxa-bar-plots.qzv

#************************************************************
# Filter for Negative Controls
qiime feature-table filter-samples \
  --i-table fecal-table-no-mito-chlo-silva-7.qza \
  --m-metadata-file metadata.txt \
  --p-where "control_type IN ('negative')" \
  --o-filtered-table fecal-table-neg-con.qza

#Visualize Negative Controls
qiime feature-table summarize \
  --i-table fecal-table-neg-con.qza \
  --o-visualization fecal-table-neg-con.qzv \
  --m-sample-metadata-file metadata.txt

#Neg Con Taxa Barplot
qiime taxa barplot \
  --i-table fecal-table-neg-con.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --m-metadata-file metadata.txt \
  --o-visualization fecal-neg-con-taxa-bar-plots.qzv
```

```
#************************************************************
# Filter for Positive Controls
qiime feature-table filter-samples \
  --i-table fecal-table-no-mito-chlo-silva-7.qza \
  --m-metadata-file metadata.txt \
  --p-where "control_type IN ('positive')" \
  --o-filtered-table fecal-table-pos-con.qza

#Visualize Positive Controls
qiime feature-table summarize \
  --i-table fecal-table-pos-con.qza \
  --o-visualization fecal-table-pos-con.qzv \
  --m-sample-metadata-file metadata.txt

#Pos Con Taxa Barplot
qiime taxa barplot \
  --i-table fecal-table-pos-con.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --m-metadata-file metadata.txt \
  --o-visualization fecal-pos-con-taxa-bar-plots.qzv

#************************************************************
#Technical Reps
qiime feature-table filter-samples \
  --i-table fecal-table-no-mito-chlo-silva-7.qza \
  --m-metadata-file metadata.txt \
  --p-where "tech_rep_pairs IN ('pair', 'rep')" \
  --o-filtered-table fecal-table-tech-reps.qza

#Visualize Technical Reps
qiime feature-table summarize \
  --i-table fecal-table-tech-reps.qza \
  --o-visualization fecal-table-tech-reps.qzv \
  --m-sample-metadata-file metadata.txt

#Tech reps Taxa Barplot
qiime taxa barplot \
  --i-table fecal-table-tech-reps.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --m-metadata-file metadata.txt \
  --o-visualization fecal-tech-reps-taxa-bar-plots.qzv
```

```
################################################################################
##Phylogeny

#!/bin/sh

#SBATCH --job-name=fecal-sepp
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=06:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Filter Rep Seqs for Only Samples
qiime feature-table filter-seqs \
  --i-data merged-rep-seqs.qza \
  --i-table fecal-table-samples.qza \
  --o-filtered-data fecal-samples-rep-seqs.qza

#Build Sepp Tree
qiime fragment-insertion sepp \
  --i-representative-sequences fecal-samples-rep-seqs.qza \
  --o-tree fecal-insertion-tree.qza \
  --o-placements fecal-insertion-placements.qza

################################################################################
##ALPHA REREFACTION

sinteractive --time=00:20:00

#Activate Qiime2
source activate qiime2-2019.4

qiime diversity alpha-rarefaction \
  --i-table fecal-table-samples.qza \
  --p-max-depth 45000 \
  --m-metadata-file metadata.txt \
  --o-visualization fecal-alpha-rarefaction.qzv
```

```
################################################################################
##CORE METRICS: Entire Feeding Period Comparisons

#!/bin/sh

#SBATCH --job-name=fecal-core-metrics
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Individual Samples
qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-ind-samples.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-ind

#Composite Samples
qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-comp-samples.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-comp

#All Samples: For Visualization, Not Comparison
qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-samples.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-all
```

```
################################################################################
##CORE METRICS: Entire Feeding Period Comparisons Within Program

#!/bin/sh

#SBATCH --job-name=fecal-core-metrics
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4


#Filter for Natural
qiime feature-table filter-samples \
  --i-table fecal-table-ind-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "feed_program IN ('natural')" \
  --o-filtered-table fecal-table-ind-nat.qza

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-ind-nat.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-ind-nat

#*************************************************************
#Filter for Conventional
qiime feature-table filter-samples \
  --i-table fecal-table-ind-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "feed_program IN ('conventional')" \
  --o-filtered-table fecal-table-ind-trad.qza

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-ind-trad.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-ind-trad
```

```
#################################################################################
##CORE METRICS: Within Timepoint

#!/bin/sh

#SBATCH --job-name=fecal-core-metrics
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=02:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Timepoint 1 Individuals
qiime feature-table filter-samples \
  --i-table fecal-table-ind-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('A')" \
  --o-filtered-table fecal-table-ind-1.qza

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-ind-1.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-ind-1

#Timepoint 1 Comp
qiime feature-table filter-samples \
  --i-table fecal-table-comp-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('A')" \
  --o-filtered-table fecal-table-comp-1.qza

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-comp-1.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-comp-1
```

```
#************************************************************
#Timepoint 2 Individuals
qiime feature-table filter-samples \
  --i-table fecal-table-ind-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('B')" \
  --o-filtered-table fecal-table-ind-2.qza

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-ind-2.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-ind-2

#Timepoint 2 Comp
qiime feature-table filter-samples \
  --i-table fecal-table-comp-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('B')" \
  --o-filtered-table fecal-table-comp-2.qza

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-comp-2.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-comp-2

#************************************************************
#Timepoint 3 Individuals
qiime feature-table filter-samples \
  --i-table fecal-table-ind-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('C')" \
  --o-filtered-table fecal-table-ind-3.qza

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-ind-3.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-ind-3
```

```
#Timepoint 3 Comp
qiime feature-table filter-samples \
  --i-table fecal-table-comp-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('C')" \
  --o-filtered-table fecal-table-comp-3.qza

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-comp-3.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-comp-3

#************************************************************
#Timepoint 4 Individuals
qiime feature-table filter-samples \
  --i-table fecal-table-ind-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('D')" \
  --o-filtered-table fecal-table-ind-4.qza

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-ind-4.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-ind-4

#Timepoint 4 Comp
qiime feature-table filter-samples \
  --i-table fecal-table-comp-samples.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('D')" \
  --o-filtered-table fecal-table-comp-4.qza

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny fecal-insertion-tree.qza \
  --i-table fecal-table-comp-4.qza \
  --p-sampling-depth 9367 \
  --m-metadata-file metadata.txt \
  --output-dir core-metrics-fecal-comp-4
```

```
#############################################################################
##ALPHA AND BETA DIVERSITY: Composite and Individual Entire Period by Program

#!/bin/sh

#SBATCH --job-name=fecal-group-sig
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Individual

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-ind/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-ind/weighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind/weighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-ind/unweighted-unifrac-feed-program-significance.qzv \
  --p-pairwise
```

```
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-ind/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind/shannon-group-significance.qzv

#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind/observed_otus_significance.qzv

#**********************************************************
#Composite

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-comp/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-comp/weighted-unifrac-source-significance.qzv \
  --p-pairwise
```

```
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-comp/weighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-comp/unweighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-comp/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-comp/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-comp/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-comp/shannon-group-significance.qzv

#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-comp/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-comp/observed_otus_significance.qzv
```

```
#############################################################################
#ALPHA AND BETA DIVERSITY: Composite and Individual Entire Period by Timepoint

#!/bin/sh

#SBATCH --job-name=fecal-group-sig2
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Individual

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization core-metrics-fecal-ind/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization core-metrics-fecal-ind/unweighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

#***********************************************************
#Composite
#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization core-metrics-fecal-comp/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise
```

```
#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization core-metrics-fecal-comp/unweighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

################################################################################
##ALPHA AND BETA DIVERSITY: Composite and Individual TP1 by Program, Pen, and
Source

#!/bin/sh

#SBATCH --job-name=fecal-group-sig-TP1
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Individual
#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-1/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-ind-1/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-1/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-ind-1/weighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-1/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-1/weighted-unifrac-pen-significance.qzv \
  --p-pairwise
```

```
#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-1/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-ind-1/unweighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-1/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-ind-1/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-1/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-1/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-1/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-1/shannon-group-significance.qzv

#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-1/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-1/observed_otus_significance.qzv

#************************************************************
#Composite

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-1/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-comp-1/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise
```

```
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-1/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-comp-1/weighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-1/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-comp-1/weighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-1/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-comp-1/unweighted-unifrac-feed-program-significance.qzv
\
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-1/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-comp-1/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-1/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-comp-1/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-comp-1/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-comp-1/shannon-group-significance.qzv
```

```
#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-comp-1/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-comp-1/observed_otus_significance.qzv

################################################################################
##ALPHA AND BETA DIVERSITY: Composite and Individual TP2 by Program, Pen, and
Source

#!/bin/sh

#SBATCH --job-name=fecal-group-sig-TP2
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Individual

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-2/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-ind-2/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-2/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-ind-2/weighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-2/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-2/weighted-unifrac-pen-significance.qzv \
  --p-pairwise
```

```
#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-2/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-ind-2/unweighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-2/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-ind-2/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-2/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-2/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-2/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-2/shannon-group-significance.qzv

#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-2/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-2/observed_otus_significance.qzv

#**********************************************************
#Composite

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-2/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-comp-2/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise
```

```
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-2/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-comp-2/weighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-2/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-comp-2/weighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-2/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-comp-2/unweighted-unifrac-feed-program-significance.qzv
\
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-2/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-comp-2/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-2/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-comp-2/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-comp-2/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-comp-2/shannon-group-significance.qzv
```

```
#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-comp-2/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-comp-2/observed_otus_significance.qzv

################################################################################
##ALPHA AND BETA DIVERSITY: Composite and Individual TP3 by Program, Pen, and

#!/bin/sh

#SBATCH --job-name=fecal-group-sig-TP3
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Individual

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-3/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-ind-3/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-3/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-ind-3/weighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-3/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-3/weighted-unifrac-pen-significance.qzv \
  --p-pairwise
```

```
#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-3/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-ind-3/unweighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-3/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-ind-3/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-3/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-3/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-3/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-3/shannon-group-significance.qzv

#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-3/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-3/observed_otus_significance.qzv

#**********************************************************
#Composite

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-3/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-comp-3/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise
```

```
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-3/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-comp-3/weighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-3/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-comp-3/weighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-3/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-comp-3/unweighted-unifrac-feed-program-significance.qzv
\
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-3/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-comp-3/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-3/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-comp-3/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-comp-3/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-comp-3/shannon-group-significance.qzv
```

```
#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-comp-3/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-comp-3/observed_otus_significance.qzv

################################################################################
##ALPHA AND BETA DIVERSITY: Composite and Individual TP4 by Program, Pen, and

#!/bin/sh

#SBATCH --job-name=fecal-group-sig-TP4
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Individual

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-4/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-ind-4/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-4/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-ind-4/weighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-4/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-4/weighted-unifrac-pen-significance.qzv \
  --p-pairwise
```

```
#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-4/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-ind-4/unweighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-4/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-ind-4/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-4/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-4/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-4/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-4/shannon-group-significance.qzv

#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-4/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-4/observed_otus_significance.qzv

#**********************************************************
#Composite

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-4/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-comp-4/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise
```

```
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-4/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-comp-4/weighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-4/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-comp-4/weighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-4/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-fecal-comp-4/unweighted-unifrac-feed-program-significance.qzv
\
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-4/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-fecal-comp-4/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-comp-4/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-comp-4/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-comp-4/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-comp-4/shannon-group-significance.qzv
```

```
#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-comp-4/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-comp-4/observed_otus_significance.qzv

################################################################################
##ALPHA AND BETA DIVERSITY: By Time and Pen Within Porgram

#!/bin/sh

#SBATCH --job-name=fecal-group-sig-3
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Individual

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-nat/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization core-metrics-fecal-ind-nat/weighted-unifrac-time-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-nat/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-nat/weighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-nat/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization core-metrics-fecal-ind-nat/unweighted-unifrac-time-significance.qzv \
  --p-pairwise
```

```
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-nat/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-nat/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-nat/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-nat/shannon-group-significance.qzv

#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-nat/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-nat/observed_otus_significance.qzv

#**********************************************************
#Conventional

#Weighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-trad/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization core-metrics-fecal-ind-trad/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-trad/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-trad/weighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Unweighted UniFrac
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-trad/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization core-metrics-fecal-ind-trad/unweighted-unifrac-feed-program-significance.qzv
\
  --p-pairwise
```

```
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-fecal-ind-trad/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-fecal-ind-trad/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-trad/shannon_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-trad/shannon-group-significance.qzv

#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-fecal-ind-trad/observed_otus_vector.qza \
  --m-metadata-file metadata.txt \
  --o-visualization core-metrics-fecal-ind-trad/observed_otus_significance.qzv


################################################################################
##ANCOM: Both Programs Together by Time

#!/bin/sh

#SBATCH --job-name=fecal-ancom-timepoints
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=00:15:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

LC_ALL=en_US
export LC_ALL

#Activate qiime
source activate qiime2-2019.4

qiime tools export \
  --input-path fecal-taxonomy-silva-7-as-metadata.qzv \
  --output-path fecal-taxonomy-silva-7-as-metadata
qiime tools import \
  --type 'FeatureData[Taxonomy]' \
  --input-path fecal-taxonomy-silva-7-as-metadata/metadata.tsv \
  --output-path fecal-taxonomy-silva-7-without-spaces.qza
```

```
#------------------------------------------------------------
#All Timepoints

#Family Level
qiime taxa collapse \
  --i-table ind_rarefied_table.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-collapsed-table5.qza \
  --o-composition-table fecal-table-comp5.qza

#Run Family ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization fecal-ancom-feed_program5.qzv

#Run Family ANCOM: Timepoint
qiime composition ancom \
  --i-table fecal-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-ancom-time5.qzv

#Run Family ANCOM: Source
qiime composition ancom \
  --i-table fecal-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization fecal-ancom-source5.qzv

#Run Family ANCOM: Pen
qiime composition ancom \
  --i-table fecal-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization fecal-ancom-pen5.qzv
```

```
#Phylum Level
qiime taxa collapse \
  --i-table ind_rarefied_table.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-collapsed-table2.qza \
  --o-composition-table fecal-table-comp2.qza

#Run Phylum ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization fecal-ancom-feed_program2.qzv

#Run Phylum ANCOM: Timepoint
qiime composition ancom \
  --i-table fecal-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-ancom-time2.qzv

#Run Phylum ANCOM: Source
qiime composition ancom \
  --i-table fecal-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization fecal-ancom-source2.qzv

#Run Phylum ANCOM: Pen
qiime composition ancom \
  --i-table fecal-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization fecal-ancom-pen2.qzv
```

```
#------------------------------------------------------------
#Timepoint 1

#Filter for Timepoint 1
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('A')" \
  --o-filtered-table fecal-table-ind-1-samples.qza

#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-1-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-1-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-1-collapsed-table5.qza \
  --o-composition-table fecal-1-table-comp5.qza

#Run Family ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-1-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization fecal-1-ancom-feed_program5.qzv

#Run Family ANCOM: Source
qiime composition ancom \
  --i-table fecal-1-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization fecal-1-ancom-source5.qzv

#Run Family ANCOM: Pen
qiime composition ancom \
  --i-table fecal-1-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization fecal-1-ancom-pen5.qzv
```

```
#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-1-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-1-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-1-collapsed-table2.qza \
  --o-composition-table fecal-1-table-comp2.qza

#Run Phylum ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-1-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization fecal-1-ancom-feed_program2.qzv

#Run Phylum ANCOM: Source
qiime composition ancom \
  --i-table fecal-1-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization fecal-1-ancom-source2.qzv

#Run Phylum ANCOM: Pen
qiime composition ancom \
  --i-table fecal-1-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization fecal-1-ancom-pen2.qzv

#------------------------------------------------------------
#Timepoint 2

#Filter for Timepoint 2
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('B')" \
  --o-filtered-table fecal-table-ind-2-samples.qza
```

```
#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-2-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-2-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-2-collapsed-table5.qza \
  --o-composition-table fecal-2-table-comp5.qza

#Run Family ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-2-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization fecal-2-ancom-feed_program5.qzv

#Run Family ANCOM: Source
qiime composition ancom \
  --i-table fecal-2-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization fecal-2-ancom-source5.qzv

#Run Family ANCOM: Pen
qiime composition ancom \
  --i-table fecal-2-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization fecal-2-ancom-pen5.qzv

#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-2-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-2-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-2-collapsed-table2.qza \
  --o-composition-table fecal-2-table-comp2.qza
```

```
#Run Phylum ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-2-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization fecal-2-ancom-feed_program2.qzv

#Run Phylum ANCOM: Source
qiime composition ancom \
  --i-table fecal-2-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization fecal-2-ancom-source2.qzv

#Run Phylum ANCOM: Pen
qiime composition ancom \
  --i-table fecal-2-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization fecal-2-ancom-pen2.qzv

#------------------------------------------------------------
#Timepoint 3

#Filter for Timepoint 3
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('C')" \
  --o-filtered-table fecal-table-ind-3-samples.qza

#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-3-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-3-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-3-collapsed-table5.qza \
  --o-composition-table fecal-3-table-comp5.qza
```

```
#Run Family ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-3-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization fecal-3-ancom-feed_program5.qzv

#Run Family ANCOM: Source
qiime composition ancom \
  --i-table fecal-3-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization fecal-3-ancom-source5.qzv

#Run Family ANCOM: Pen
qiime composition ancom \
  --i-table fecal-3-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization fecal-3-ancom-pen5.qzv

#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-3-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-3-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-3-collapsed-table2.qza \
  --o-composition-table fecal-3-table-comp2.qza

#Run Phylum ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-3-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization fecal-3-ancom-feed_program2.qzv

#Run Phylum ANCOM: Source
qiime composition ancom \
  --i-table fecal-3-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization fecal-3-ancom-source2.qzv
```

```
#Run Phylum ANCOM: Pen
qiime composition ancom \
  --i-table fecal-3-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization fecal-3-ancom-pen2.qzv

#------------------------------------------------------------
#Timepoint 4

#Filter for Timepoint 4
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('D')" \
  --o-filtered-table fecal-table-ind-4-samples.qza

#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-4-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-4-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-4-collapsed-table5.qza \
  --o-composition-table fecal-4-table-comp5.qza

#Run Family ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-4-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization fecal-4-ancom-feed_program5.qzv

#Run Family ANCOM: Source
qiime composition ancom \
  --i-table fecal-4-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization fecal-4-ancom-source5.qzv
```

```
#Run Family ANCOM: Pen
qiime composition ancom \
  --i-table fecal-4-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization fecal-4-ancom-pen5.qzv

#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-4-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-4-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-4-collapsed-table2.qza \
  --o-composition-table fecal-4-table-comp2.qza

#Run Phylum ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-4-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization fecal-4-ancom-feed_program2.qzv

#Run Phylum ANCOM: Source
qiime composition ancom \
  --i-table fecal-4-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization fecal-4-ancom-source2.qzv

#Run Phylum ANCOM: Pen
qiime composition ancom \
  --i-table fecal-4-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization fecal-4-ancom-pen2.qzv
```

```
#############################################################################
#ANCOM: Both Programs Together by Time

#!/bin/sh

#SBATCH --job-name=fecal-ancom-pairs
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=00:15:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#AB-------------------------------------------------------------------------------

#Filter for Timepoint AB
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('A', 'B')" \
  --o-filtered-table fecal-table-ind-AB-samples.qza

#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-AB-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-AB-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-AB_collapsed-table5.qza \
  --o-composition-table fecal-AB-table-comp5.qza

#Run Family ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-AB-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-AB-ancom-fecal_timepoint5.qzv
```

```
#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-AB-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-AB-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-AB-collapsed-table2.qza \
  --o-composition-table fecal-AB-table-comp2.qza

#Run Phylum ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-AB-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-AB-ancom-fecal_timepoint2.qzv

#BC--------------------------------------------------------------------------------

#Filter for Timepoint BC
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('B', 'C')" \
  --o-filtered-table fecal-table-ind-BC-samples.qza

#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-BC-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-BC-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-BC_collapsed-table5.qza \
  --o-composition-table fecal-BC-table-comp5.qza

#Run Family ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-BC-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-BC-ancom-fecal_timepoint5.qzv
```

```
#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-BC-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-BC-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-BC-collapsed-table2.qza \
  --o-composition-table fecal-BC-table-comp2.qza

#Run Phylum ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-BC-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-BC-ancom-fecal_timepoint2.qzv

#CD--------------------------------------------------------------------------------

#Filter for Timepoint CD
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('C', 'D')" \
  --o-filtered-table fecal-table-ind-CD-samples.qza

#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-CD-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-CD-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-CD_collapsed-table5.qza \
  --o-composition-table fecal-CD-table-comp5.qza

#Run Family ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-CD-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-CD-ancom-fecal_timepoint5.qzv
```

```
#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-CD-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-CD-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-CD-collapsed-table2.qza \
  --o-composition-table fecal-CD-table-comp2.qza

#Run Phylum ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-CD-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-CD-ancom-fecal_timepoint2.qzv


################################################################################
#ANCOM: Natural by Time

#!/bin/sh

#SBATCH --job-name=fecal-ancom-pairs-2
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=04:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#AB--------------------------------------------------------------------------------

#Filter for Timepoint AB
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('A', 'B') AND feed_program='natural'" \
  --o-filtered-table fecal-table-ind-nat-AB-samples.qza
```

```
#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-nat-AB-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-nat-AB-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-nat-AB-collapsed-table5.qza \
  --o-composition-table fecal-nat-AB-table-comp5.qza

#Run Family ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-nat-AB-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-nat-AB-ancom-fecal_timepoint5.qzv

#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-nat-AB-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-nat-AB-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-nat-AB-collapsed-table2.qza \
  --o-composition-table fecal-nat-AB-table-comp2.qza

#Run Phylum ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-nat-AB-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-nat-AB-ancom-fecal_timepoint2.qzv

#BC--------------------------------------------------------------------------------

#Filter for Timepoint BC
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('B', 'C') AND feed_program='natural'" \
  --o-filtered-table fecal-table-ind-nat-BC-samples.qza
```

```
#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-nat-BC-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-nat-BC-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-nat-BC-collapsed-table5.qza \
  --o-composition-table fecal-nat-BC-table-comp5.qza

#Run Family ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-nat-BC-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-nat-BC-ancom-fecal_timepoint5.qzv

#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-nat-BC-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-nat-BC-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-nat-BC-collapsed-table2.qza \
  --o-composition-table fecal-nat-BC-table-comp2.qza

#Run Phylum ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-nat-BC-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-nat-BC-ancom-fecal_timepoint2.qzv

#CD--------------------------------------------------------------------------------

#Filter for Timepoint CD
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('C', 'D') AND feed_program='natural'" \
  --o-filtered-table fecal-table-ind-nat-CD-samples.qza
```

```
#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-nat-CD-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-nat-CD-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-nat-CD-collapsed-table5.qza \
  --o-composition-table fecal-nat-CD-table-comp5.qza

#Run Family ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-nat-CD-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-nat-CD-ancom-fecal_timepoint5.qzv


#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-nat-CD-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-nat-CD-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-nat-CD-collapsed-table2.qza \
  --o-composition-table fecal-nat-CD-table-comp2.qza

#Run Phylum ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-nat-CD-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-nat-CD-ancom-fecal_timepoint2.qzv
```

```
################################################################################
#ANCOM: Conventional by Time

nano

#!/bin/sh
#SBATCH --job-name=fecal-ancom-pairs-4
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=04:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#AB--------------------------------------------------------------------------------

#Filter for Timepoint AB
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('A', 'B') AND feed_program='conventional'" \
  --o-filtered-table fecal-table-ind-conv-AB-samples.qza

#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-conv-AB-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-conv-AB-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-conv-AB-collapsed-table5.qza \
  --o-composition-table fecal-conv-AB-table-comp5.qza

#Run Family ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-conv-AB-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-conv-AB-ancom-fecal_timepoint5.qzv
```

```
#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-conv-AB-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-conv-AB-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-conv-AB-collapsed-table2.qza \
  --o-composition-table fecal-conv-AB-table-comp2.qza

#Run Phylum ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-conv-AB-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-conv-AB-ancom-fecal_timepoint2.qzv

#BC--------------------------------------------------------------------------------

#Filter for Timepoint BC
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('B', 'C') AND feed_program='conventional'" \
  --o-filtered-table fecal-table-ind-conv-BC-samples.qza

#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-conv-BC-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-conv-BC-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-conv-BC-collapsed-table5.qza \
  --o-composition-table fecal-conv-BC-table-comp5.qza

#Run Family ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-conv-BC-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-conv-BC-ancom-fecal_timepoint5.qzv
```

```
#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-conv-BC-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-conv-BC-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-conv-BC-collapsed-table2.qza \
  --o-composition-table fecal-conv-BC-table-comp2.qza

#Run Phylum ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-conv-BC-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-conv-BC-ancom-fecal_timepoint2.qzv

#CD--------------------------------------------------------------------------------

#Filter for Timepoint CD
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "fecal_timepoint IN ('C', 'D') AND feed_program='conventional'" \
  --o-filtered-table fecal-table-ind-conv-CD-samples.qza

#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-conv-CD-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-conv-CD-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-conv-CD-collapsed-table5.qza \
  --o-composition-table fecal-conv-CD-table-comp5.qza

#Run Family ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-conv-CD-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-conv-CD-ancom-fecal_timepoint5.qzv
```

```
#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-conv-CD-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-conv-CD-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-conv-CD-collapsed-table2.qza \
  --o-composition-table fecal-conv-CD-table-comp2.qza

#Run Phylum ANCOM: Time Point
qiime composition ancom \
  --i-table fecal-conv-CD-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-conv-CD-ancom-fecal_timepoint2.qzv


##############################################################################
#ANCOM: Within Program Across All Times

#!/bin/sh

#SBATCH --job-name=fecal-longitudinal-ANCOM
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Filter for Natural
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "feed_program IN ('natural')" \
  --o-filtered-table fecal-table-ind-nat-samples.qza
```

```
#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-nat-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-nat-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-nat-collapsed-table5.qza \
  --o-composition-table fecal-nat-table-comp5.qza

#Run Family ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-nat-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-nat-ancom-time5.qzv

#Phyum Level
qiime taxa collapse \
  --i-table fecal-table-ind-nat-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-nat-collapsed-table2.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-nat-collapsed-table2.qza \
  --o-composition-table fecal-nat-table-comp2.qza

#Run Family ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-nat-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-nat-ancom-time2.qzv

#------------------------------------------------------------------------------

#Filter for Conventional
qiime feature-table filter-samples \
  --i-table ind_rarefied_table.qza \
  --m-metadata-file metadata.txt \
  --p-where "feed_program IN ('conventional')" \
  --o-filtered-table fecal-table-ind-trad-samples.qza
```

```
#Family Level
qiime taxa collapse \
  --i-table fecal-table-ind-trad-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-trad-collapsed-table5.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-trad-collapsed-table5.qza \
  --o-composition-table fecal-trad-table-comp5.qza

#Run Family ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-trad-table-comp5.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-trad-ancom-time5.qzv

#Phylum Level
qiime taxa collapse \
  --i-table fecal-table-ind-trad-samples.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-trad-collapsed-table2.qza

#Add Family Pseudocount
qiime composition add-pseudocount \
  --i-table fecal-trad-collapsed-table2.qza \
  --o-composition-table fecal-trad-table-comp2.qza

#Run Family ANCOM: Management Program
qiime composition ancom \
  --i-table fecal-trad-table-comp2.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization fecal-trad-ancom-time2.qzv
```

```
################################################################################
##PAIRWISE DIFFERENCE COMPARISONS

#!/bin/sh

#SBATCH --job-name=fecal-14-pairwise-difference
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=00:10:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Beginning to End Shannon Differences
qiime longitudinal pairwise-differences \
  --m-metadata-file metadata.txt \
  --m-metadata-file core-metrics-fecal-comp/shannon_vector.qza \
  --p-metric shannon \
  --p-group-column feed_program \
  --p-state-column fecal_timepoint_numeric \
  --p-state-1 1 \
  --p-state-2 4 \
  --p-individual-id-column reassigned_lot \
  --p-replicate-handling random \
  --o-visualization shannon-1-4-pairwise-differences.qzv

#Beginning to End Richness Differences
qiime longitudinal pairwise-differences \
  --m-metadata-file metadata.txt \
  --m-metadata-file core-metrics-fecal-comp/observed_otus_vector.qza \
  --p-metric observed_otus \
  --p-group-column feed_program \
  --p-state-column fecal_timepoint_numeric \
  --p-state-1 1 \
  --p-state-2 4 \
  --p-individual-id-column reassigned_lot \
  --p-replicate-handling random \
  --o-visualization richness-1-4-pairwise-differences.qzv
```

```
#############################################################################
##NMIT

#!/bin/sh

#SBATCH --job-name=fecal-NMIT
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Family Level
qiime taxa collapse \
  --i-table comp_rarefied_table.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-comp-table5.qza

qiime feature-table relative-frequency \
--i-table fecal-comp-table5.qza \
--o-relative-frequency-table fecal-NMIT-table.qza

#Build Correlation Matrix
qiime longitudinal nmit \
  --i-table fecal-NMIT-table.qza \
  --m-metadata-file metadata.txt \
  --p-individual-id-column reassigned_lot \
  --o-distance-matrix nmit-dm-k.qza

#Test Management Program Differences
qiime diversity beta-group-significance \
  --i-distance-matrix nmit-dm-k.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization program-nmit-k.qzv

#Test Time Point Differences
qiime diversity beta-group-significance \
  --i-distance-matrix nmit-dm-k.qza \
  --m-metadata-file metadata.txt \
  --m-metadata-column fecal_timepoint \
  --o-visualization time-nmit-k.qzv
```

```
#Build PCoA
qiime diversity pcoa \
  --i-distance-matrix nmit-dm.qza \
  --o-pcoa nmit-pc.qza

#Visualize Differences
qiime emperor plot \
  --i-pcoa nmit-pc.qza \
  --m-metadata-file metadata.txt \
  --o-visualization nmit-emperor.qzv

################################################################################
##FILES TO EXPORT FOR R ANALYSIS AND VISUALIZATION

sinteractive –time=01:00:00

#Phylum Relative Frequency
qiime feature-table relative-frequency \
--i-table fecal-collapsed-table2.qza \
--o-relative-frequency-table fecal-rf-table2.qza

qiime tools export \
--input-path fecal-rf-table2.qza \
--output-path fecal-phylum-rf-table

biom convert -i fecal-phylum-rf-table/feature-table.biom -o fecal-phylum-rf-table.tsv --to-tsv

#Family Relative Frequency
qiime feature-table relative-frequency \
--i-table fecal-collapsed-table5.qza \
--o-relative-frequency-table fecal-rf-table5.qza

qiime tools export \
--input-path fecal-rf-table5.qza \
--output-path fecal-family-rf-table

biom convert -i fecal-family-rf-table/feature-table.biom -o fecal-family-rf-table.tsv --to-tsv
```

```
#Collapsed Tables

#Family
qiime taxa collapse \
  --i-table ind_rarefied_table.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 5 \
  --o-collapsed-table fecal-collapsed-table5.qza

qiime feature-table summarize \
  --i-table fecal-collapsed-table5.qza \
  --o-visualization fecal-collapsed-table5.qzv \
  --m-sample-metadata-file metadata.txt

#Order
qiime taxa collapse \
  --i-table ind_rarefied_table.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 4 \
  --o-collapsed-table fecal-collapsed-table4.qza

qiime feature-table summarize \
  --i-table fecal-collapsed-table4.qza \
  --o-visualization fecal-collapsed-table4.qzv \
  --m-sample-metadata-file metadata.txt

#Class
qiime taxa collapse \
  --i-table ind_rarefied_table.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 3 \
  --o-collapsed-table fecal-collapsed-table3.qza

qiime feature-table summarize \
  --i-table fecal-collapsed-table3.qza \
  --o-visualization fecal-collapsed-table3.qzv \
  --m-sample-metadata-file metadata.txt

#Phylum
qiime taxa collapse \
  --i-table ind_rarefied_table.qza \
  --i-taxonomy fecal-taxonomy-silva-7-without-spaces.qza \
  --p-level 2 \
  --o-collapsed-table fecal-collapsed-table2.qza
```

```
qiime feature-table summarize \
  --i-table fecal-collapsed-table2.qza \
  --o-visualization fecal-collapsed-table2.qzv \
  --m-sample-metadata-file metadata.txt
```

FECAL R ANALYSIS AND VISUALIZATION CODE

```
#R Software Version 3.4.1
#R Studio Version 1.2.1335
#Load relevant packages
library(lme4)
library(forcats)
library(dplyr)
library(emmeans)
library(ggplot2)
library(reshape2)
library(cowplot)



################################################################################
#Import liver abscess data, rename columns, and set categorical variables as factors
LA_data <-
read.csv("C:\\Users\\fuern\\Dropbox\\School\\Projects\\Feedlot_Microbiome_Project\\R_Analysis\\Cattle_data.csv")
names(LA_data)[1]<-"Pen"
LA_data$Pen <- as.factor(LA_data$Pen)

#-------------------------------------------------------------------------
#Model total liver abscess occurrence
#Use logistic regression on raw count data with a binomial distribution
#Include management program as a fixed effect
#Include pen as a random effect to account for nesting of observational units within pen
LA_prev <- glmer(cbind(LA_yes,LA_no) ~ Program + (1|Pen) , data = LA_data,
                 family = "binomial")
LA_prev

#Display probability of abscess by management program by back-transformation from log odds scale
#Formally test the odds ratio for abscess occurrence by management program
emmeans(LA_prev, pairwise ~ Program, type = "response")

#-------------------------------------------------------------------------
#Model A+ liver abscess occurrence
LA_severity <- glmer(cbind(Severe_LA_yes,Severe_LA_no) ~ Program + (1|Pen) , data =
                       LA_data, family = "binomial")
LA_severity
```

```
#Display probability of A+ abscess by management program by back-transformation from log
odds scale
#Formally test the odds ratio for A+ abscess occurrence by management program
emmeans(LA_severity, pairwise ~ Program, type = "response")

#------------------------------------------------------------------------
#Model A liver abscess occurrence
LA_A <- glmer(cbind(A_LA_yes,A_LA_no) ~ Program + (1|Pen) , data = LA_data, family =
            "binomial")
LA_A

#Display probability of A abscess by management program by back-transformation from log
odds scale
#Formally test the odds ratio for A abscess occurrence by management program
emmeans(LA_A, pairwise ~ Program, type = "response")

#############################################################################
#Alpha Diversity

#Import alpha diversity, rename columns, and set categorical variables as factors
Alpha <-
read.csv("C:\\Users\\fuern\\Dropbox\\School\\Projects\\Feedlot_Microbiome_Project\\R_Analysi
s\\Fecal_Alpha_Diversity.csv")
names(Alpha)[1]<-"Sample"
Alpha$reassigned_lot <- as.factor(Alpha$reassigned_lot)

#------------------------------------------------------------------------
#Richness

OTU <- ggplot(Alpha,aes(x=fecal_timepoint, y=observed_otus, fill=feed_program)) +
  geom_boxplot(position=position_dodge(width = .95), width=.85) +
  labs(title="",x="Time Point", y = "Observed OTUs")+
  scale_y_continuous(limits=c(100, 700), expand = c(0, 0)) +
  theme_light() +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank()) +
  theme(axis.title.y = element_text(margin = margin(t = 0, r = 10, b = 0, l = 0)),
        axis.title.x = element_text(margin = margin(t = 10, r = 0, b = 0, l = 0))) +
  scale_fill_manual(name = "Management Program", values = c("#52be80", "#5dade2"))
OTU
```

```
#-----------------------------------------------------------------------------
#Shannon Diversity

Shan <- ggplot(Alpha,aes(x=fecal_timepoint, y=shannon, fill=feed_program)) +
  geom_boxplot(position=position_dodge(width = .95), width=.85) +
  labs(title="",x="Time Point", y = "Shannon") +
  scale_y_continuous(limits=c(03, 09), expand = c(0, 0)) +
  theme_light() +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank()) +
  theme(axis.title.y = element_text(margin = margin(t = 0, r = 10, b = 0, l = 0)),
        axis.title.x = element_text(margin = margin(t = 10, r = 0, b = 0, l = 0))) +
  scale_fill_manual(name = "Management Program", values = c("#52be80", "#5dade2"))
Shan

#-----------------------------------------------------------------------------
#Combine Plots

Blank <- ggplot() + theme_void()

AlphD <- plot_grid(OTU, Blank, Shan, labels=c('A', '', 'B'), ncol = 1,
              align="hv", axis="r", rel_heights = c(8,.5,8))
AlphD

#############################################################################
#Import phylum relatiove abundance, rename columns, and set categorical variables as factors
Phylum <-
read.csv("C:\\Users\\fuern\\Dropbox\\School\\Projects\\Feedlot_Microbiome_Project\\R_Analysis\\Fecal_Phylum_Abundance.csv")
names(Phylum)[1]<-"Program"


PRA <- melt(Phylum, id=c("Program", "Timepoint"), variable.name = "Phyla")
```

```
P_Plot <- PRA %>%
  mutate(percent = value*100) %>%
  ggplot(aes(x = Program, y = percent, fill = Phyla)) +
  geom_bar(stat = "identity", position = "fill", width = .85) +
  scale_y_continuous(labels = scales::percent) +
  labs(x="Management Program", y = "") +
  theme_light() +
  theme(panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        strip.background = element_blank(),
        strip.text = element_text(colour = 'black')) +
  facet_wrap(~Timepoint, nrow = 1) +
  theme(axis.title.x = element_text(margin = margin(t = 10, r = 0, b = 0, l = 0))) +
  scale_fill_manual(name = "Phyla",
                    labels = c("Firmicutes",
                               "Bacteroidetes",
                               "Spirochaetes",
                               "Actinobacteria",
                               "Proteobacteria",
                              "Tenericutes",
                               "Rare Taxa"),
                    values = c("#2e86c1", "#aed6f1",
                               "#27ae60", "#7dcea0",
                               "#c0392b", "#d98880",
                               "#95A5A6")) +
  theme(
    legend.title = element_text(size = 10),
    legend.text = element_text(size = 10))

P_Plot

#---------------------------------------------------------------------------
#Import family relative abundance, rename columns, and set categorical variables as factors
Fam <-
read.csv("C:\\Users\\fuern\\Dropbox\\School\\Projects\\Feedlot_Microbiome_Project\\R_Analysis\\Fecal_Family_Abundance_Simple.csv")
names(Fam)[1]<-"Program"

FRA <- melt(Fam, id=c("Program", "Timepoint"), variable.name = "Families")

cp4 <- c("#2e86c1", "#5dade2", "#aed6f1",
         "#27ae60", "#52be80", "#7dcea0",
         "#c0392b", "#cd6155", "#d98880",
         "#d68910", "#f5b041", "#fad7a0",
         "#6c3483", "#8e44ad", "#bb8fce",
         "#AAB7B8")
```

```
F_Plot <- FRA %>%
  mutate(percent = value*100) %>%
  ggplot(aes(x = Program, y = percent, fill = Families)) +
  geom_bar(stat = "identity", position = "fill", width = .85) +
  scale_y_continuous(labels = scales::percent) +
  labs(x="Management Program", y = "") +
  theme_light() +
  theme(panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        strip.background = element_blank(),
        strip.text = element_text(colour = 'black')) +
  facet_wrap(~Timepoint, nrow = 1) +
  scale_fill_manual(values = cp4,
                    labels = c("Ruminococcaceae",
                               "Prevotellaceae",
                               "Rikenellaceae",
                               "Lachnospiraceae",
                               "Bacteroidaceae",
                               "Spirochaetaceae",
                               "Muribaculaceae",
                               "Peptostreptococcaceae",
                               "Bifidobacteriaceae",
                               "Clostridiaceae 1",
                               "Erysipelotrichaceae",
                               "Christensenellaceae",
                               "Acidaminococcaceae",
                               "Bacteroidales RF16",
                               "Succinivibrionaceae",
                               "Rare Taxa")) +
  theme(axis.title.x = element_text(margin = margin(t = 10, r = 0, b = 0, l = 0))) +
  theme(legend.title = element_text(size = 10), legend.text = element_text(size = 10))
F_Plot

#To add italic labels: expression(italic("Label Name")) into labels = c()

#------------------------------------------------------------------------------
#Combine Plots

Blank <- ggplot() + theme_void()

RA <- plot_grid(P_Plot, Blank, F_Plot, labels=c('A', '', 'B'), ncol = 1,
                align="hv", axis="r", rel_heights = c(8,.5,8))
RA
```

```
################################################################################
#Import alpha diversity for composite samples with liver abscess prevalence and severity

LA_Cor <-
read.csv("C:\\Users\\fuern\\Dropbox\\School\\Projects\\Feedlot_Microbiome_Project\\R_Analysis\\Comp_Div_LA.csv")
names(LA_Cor)[1]<-"Pen"

cor(LA_Cor$abscess_prevalence,LA_Cor$period_shan_change, method = "spearman")
cor(LA_Cor$abscess_prevalence,LA_Cor$period_otu_change, method = "spearman")

cor(LA_Cor$abscess_prevalence,LA_Cor$D_shan, method = "spearman")
cor(LA_Cor$abscess_prevalence,LA_Cor$D_otu, method = "spearman")

cor(LA_Cor$abscess_prevalence,LA_Cor$C_shan, method = "spearman")
cor(LA_Cor$abscess_prevalence,LA_Cor$C_otu, method = "spearman")

cor(LA_Cor$severe_abscess_prevalence,LA_Cor$period_shan_change, method = "spearman")
cor(LA_Cor$severe_abscess_prevalence,LA_Cor$period_otu_change, method = "spearman")

cor(LA_Cor$severe_abscess_prevalence,LA_Cor$D_shan, method = "spearman")
cor(LA_Cor$severe_abscess_prevalence,LA_Cor$D_otu, method = "spearman")

cor(LA_Cor$severe_abscess_prevalence,LA_Cor$C_shan, method = "spearman")
cor(LA_Cor$severe_abscess_prevalence,LA_Cor$C_otu, method = "spearman")
```

APPENDIX B: LIVER ABSCESS ANALYSIS CODE

LIVER QIIME2 ANALYSIS CODE

```
################################################################################
##Project: Feedlot Microbiota: Liver Abscess
##Version: QIIME2-2019.4
##Sequencing Date: 6/10/19
################################################################################

################################################################################
##IMPORT DEMUX

#!/bin/sh

#SBATCH --job-name=r1-demux-import
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=05:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

qiime tools import \
  --type 'SampleData[PairedEndSequencesWithQuality]' \
  --input-path /projects/fuerniss@colostate.edu/feedlot-microbiome/raw-data/run1/20190619/r1-
manifest.txt \
  --output-path r1-demux.qza \
  --input-format PairedEndFastqManifestPhred33V2

#Summarize
qiime demux summarize \
  --i-data r1-demux.qza \
  --o-visualization r1-demux.qzv
```

```
################################################################################
##DENOISE

#!/bin/sh

#SBATCH --job-name=r1-dada2
#SBATCH --nodes=1
#SBATCH --ntasks=2
#SBATCH --time=20:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#dada2
qiime dada2 denoise-paired \
  --i-demultiplexed-seqs r1-demux.qza \
  --p-trunc-len-f 250 \
  --p-trunc-len-r 250 \
  --p-n-threads 2 \
  --o-table r1-table.qza \
  --o-representative-sequences r1-rep-seqs.qza \
  --o-denoising-stats r1-denoising-stats.qza

#Visualize
qiime metadata tabulate \
  --m-input-file r1-denoising-stats.qza \
  --o-visualization r1-denoising-stats.qzv

qiime feature-table summarize \
  --i-table r1-table.qza \
  --o-visualization r1-table.qzv \
  --m-sample-metadata-file r1-metadata.txt

qiime feature-table tabulate-seqs \
  --i-data r1-rep-seqs.qza \
  --o-visualization r1-rep-seqs.qzv
```

```
#############################################################################
##FILTER FOR ONLY LIVER

sinteractive --time=00:30:00

#Activate Qiime2
source activate qiime2-2019.4

#Filter
qiime feature-table filter-samples \
  --i-table r1-table.qza \
  --m-metadata-file r1-metadata.txt \
  --p-where "liver_analysis_control='yes'" \
  --o-filtered-table complete-liver-table.qza

#Visualize
qiime feature-table summarize \
  --i-table complete-liver-table.qza \
  --o-visualization complete-liver-table.qzv \
  --m-sample-metadata-file r1-metadata.txt


#############################################################################
##TAXONOMY

#Run pretrained Greengenes
#Train and run SILVA
#Compare results

#############################################################################
##DOWNLOAD DATABASES

#download pretrained Greengenes database
wget https://data.qiime2.org/2019.4/common/gg-13-8-99-515-806-nb-classifier.qza

#download pretrained Silva database
wget https://data.qiime2.org/2019.4/common/silva-132-99-515-806-nb-classifier.qza

#download untrained SILVA
wget https://www.arb-silva.de/fileadmin/silva_databases/qiime/Silva_132_release.zip
```

```
################################################################################
##LIVER GG TAXONOMY

#!/bin/sh

#SBATCH --job-name=liver-taxonomy-gg
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Run GG Taxonomy
qiime feature-classifier classify-sklearn \
  --i-classifier gg-13-8-99-515-806-nb-classifier.qza \
  --i-reads r1-rep-seqs.qza \
  --o-classification liver-taxonomy-gg.qza

#Visualize GG Taxonomy
qiime metadata tabulate \
  --m-input-file liver-taxonomy-gg.qza \
  --o-visualization liver-taxonomy-gg.qzv

#Taxa GG Barplot
qiime taxa barplot \
  --i-table complete-liver-table.qza \
  --i-taxonomy liver-taxonomy-gg.qza \
  --m-metadata-file r1-metadata.txt \
  --o-visualization liver-taxa-bar-plots-gg.qzv

################################################################################
##TRAIN SILVA CLASSIFIER

sinteractive --time=00:30:00

#Activate Qiime2
source activate qiime2-2019.4

#Unzip Silva
unzip Silva_132_release.zip
```

```
#Import Sequences
qiime tools import \
  --type 'FeatureData[Sequence]' \
  --input-path SILVA_132_QIIME_release/rep_set/rep_set_16S_only/99/silva_132_99_16S.fna \
  --output-path silva_132_99_16S.qza

#Import Taxonomy
qiime tools import \
  --type 'FeatureData[Taxonomy]' \
  --input-format HeaderlessTSVTaxonomyFormat \
  --input-path SILVA_132_QIIME_release/taxonomy/16S_only/99/taxonomy_all_levels.txt \
  --output-path 16S-all-levels-99-ref-taxonomy.qza

#Train Classifier Using Script

#!/bin/sh

#SBATCH --job-name=silva-classifier
#SBATCH --nodes=1
#SBATCH --partition=smem
#SBATCH --ntasks=1
#SBATCH --time=12:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Extract Reference Reads
qiime feature-classifier extract-reads \
  --i-sequences silva_132_99_16S.qza \
  --p-f-primer GTGYCAGCMGCCGCGGTAA \
  --p-r-primer GGACTACNVGGGTWTCTAAT \
  --o-reads ref-seqs.qza

#Train Classifier
qiime feature-classifier fit-classifier-naive-bayes \
  --i-reference-reads ref-seqs.qza \
  --i-reference-taxonomy 16S-all-levels-99-ref-taxonomy.qza \
  --o-classifier silva-99-classifier.qza
```

```
################################################################################
#TEST CLASSIFIER
#tested on Moving Pictures Tutorial Rep Seqs

#!/bin/sh

#SBATCH --job-name=silva-test
#SBATCH --nodes=1
#SBATCH --partition=smem
#SBATCH --ntasks=1
#SBATCH --time=02:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

qiime feature-classifier classify-sklearn \
  --i-classifier silva-99-classifier.qza \
  --i-reads mp-rep-seqs.qza \
  --o-classification test-taxonomy.qza

qiime metadata tabulate \
  --m-input-file test-taxonomy.qza \
  --o-visualization test-taxonomy.qzv

################################################################################
##LIVER SILVA TAXONOMY

#!/bin/sh

#SBATCH --job-name=liver-taxonomy-silva
#SBATCH --nodes=1
#SBATCH --partition=smem
#SBATCH --ntasks=1
#SBATCH --time=05:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4
```

```
#Run Silva Taxonomy
qiime feature-classifier classify-sklearn \
  --i-classifier silva-99-classifier.qza
  --i-reads r1-rep-seqs.qza \
  --o-classification liver-taxonomy-silva.qza

#Visualize Silva Taxonomy
qiime metadata tabulate \
  --m-input-file liver-taxonomy-silva.qza \
  --o-visualization liver-taxonomy-silva.qzv

#Taxa Silva Barplot
qiime taxa barplot \
  --i-table complete-liver-table.qza \
  --i-taxonomy liver-taxonomy-silva.qza \
  --m-metadata-file r1-metadata.txt \
  --o-visualization liver-taxa-bar-plots-silva.qzv

#############################################################################
##LIVER PRETRAINED SILVA TAXONOMY

#!/bin/sh

#SBATCH --job-name=liver-taxonomy-silva7
#SBATCH --nodes=1
#SBATCH --partition=smem
#SBATCH --ntasks=1
#SBATCH --time=4:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Run Silva Taxonomy
qiime feature-classifier classify-sklearn \
  --i-classifier silva-132-99-515-806-nb-classifier.qza \
  --i-reads r1-rep-seqs.qza \
  --o-classification liver-taxonomy-silva7.qza

#Visualize Silva Taxonomy
qiime metadata tabulate \
  --m-input-file liver-taxonomy-silva7.qza \
  --o-visualization liver-taxonomy-silva7.qzv
```

```
#Taxa Silva Barplot
qiime taxa barplot \
  --i-table complete-liver-table.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --m-metadata-file r1-metadata.txt \
  --o-visualization liver-taxa-bar-plots-silva7.qzv

################################################################################
##SELECT BEST CLASSIFIER

#liver-taxonomy-silva.qzv --> QIIME2VIEW
#liver-taxa-bar-plots-silva.qzv --> QIIME2VIEW

#liver-taxonomy-gg.qzv --> QIIME2VIEW
#liver-taxa-bar-plots-gg.qzv --> QIIME2VIEW

#liver-taxonomy-silva7.qzv --> QIIME2VIEW
#liver-taxa-bar-plots-silva7.qzv --> QIIME2VIEW

#Compare proportion of unclassified taxa
#Pretrained Silva selected

################################################################################
##FILTER CHLOROPLASTS AND MITOCHONDRIA
##FILTER SAMPLES, CONTROLS, AND REPLICATES

#!/bin/sh

#SBATCH --job-name=liver-filtering
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=00:45:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Remove Chloroplasts and Mitochondria
qiime taxa filter-table \
  --i-table complete-liver-table.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --p-exclude mitochondria,chloroplast \
  --o-filtered-table liver-table-no-mito-chlo-silva7.qza
```

```
#Extract Only Samples
qiime feature-table filter-samples \
  --i-table liver-table-no-mito-chlo-silva7.qza \
  --m-metadata-file r1-metadata.txt \
  --p-where "liver_only IN ('yes')" \
  --o-filtered-table liver-table-samples.qza

#Visualize Samples
qiime feature-table summarize \
  --i-table liver-table-samples.qza \
  --o-visualization liver-table-samples.qzv \
  --m-sample-metadata-file r1-metadata.txt

#Samples Taxa Barplot
qiime taxa barplot \
  --i-table liver-table-samples.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --m-metadata-file r1-metadata.txt \
  --o-visualization liver-samples-taxa-bar-plots.qzv

#************************************************************

#Extract Only Negative Controls
qiime feature-table filter-samples \
  --i-table liver-table-no-mito-chlo-silva7.qza \
  --m-metadata-file r1-metadata.txt \
  --p-where "control_type IN ('negative')" \
  --o-filtered-table liver-table-neg-con.qza

#Visualize Negative Controls
qiime feature-table summarize \
  --i-table liver-table-neg-con.qza \
  --o-visualization liver-table-neg-con.qzv \
  --m-sample-metadata-file r1-metadata.txt

#Neg Con Taxa Barplot
qiime taxa barplot \
  --i-table liver-table-neg-con.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --m-metadata-file r1-metadata.txt \
  --o-visualization liver-neg-con-taxa-bar-plots.qzv
```

```
#************************************************************
#Extract Only Positive Controls
qiime feature-table filter-samples \
  --i-table liver-table-no-mito-chlo-silva7.qza \
  --m-metadata-file r1-metadata.txt \
  --p-where "control_type IN ('positive')" \
  --o-filtered-table liver-table-pos-con.qza

#Visualize Positive Controls
qiime feature-table summarize \
  --i-table liver-table-pos-con.qza \
  --o-visualization liver-table-pos-con.qzv \
  --m-sample-metadata-file r1-metadata.txt

#Pos Con Taxa Barplot
qiime taxa barplot \
  --i-table liver-table-pos-con.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --m-metadata-file r1-metadata.txt \
  --o-visualization liver-pos-con-taxa-bar-plots.qzv

#************************************************************

#Extract Only Technical Reps
qiime feature-table filter-samples \
  --i-table liver-table-no-mito-chlo-silva7.qza \
  --m-metadata-file r1-metadata.txt \
  --p-where "tech_rep_pairs IN ('pair', 'rep')" \
  --o-filtered-table liver-table-tech-reps.qza

#Visualize Technical Reps
qiime feature-table summarize \
  --i-table liver-table-tech-reps.qza \
  --o-visualization liver-table-tech-reps.qzv \
  --m-sample-metadata-file metadata.txt

#Tech reps Taxa Barplot
qiime taxa barplot \
  --i-table liver-table-tech-reps.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --m-metadata-file r1-metadata.txt \
  --o-visualization liver-tech-reps-taxa-bar-plots.qzv
```

```
################################################################################
##PHYLOGENY

#!/bin/sh

#SBATCH --job-name=liver-sepp
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate Qiime2
source activate qiime2-2019.4

#Filter Rep Seqs for Only Samples
qiime feature-table filter-seqs \
  --i-data r1-rep-seqs.qza \
  --i-table liver-table-samples.qza \
  --o-filtered-data liver-samples-rep-seqs.qza

#Build Sepp Tree
qiime fragment-insertion sepp \
  --i-representative-sequences liver-samples-rep-seqs.qza \
  --o-tree liver-insertion-tree.qza \
  --o-placements liver-insertion-placements.qza

################################################################################
##ALPHA REREFACTION

sinteractive --time=00:15:00

#Activate Qiime2
source activate qiime2-2019.4

qiime diversity alpha-rarefaction \
  --i-table liver-table-samples.qza \
  --i-phylogeny liver-insertion-tree.qza \
  --p-max-depth 30000 \
  --m-metadata-file r1-metadata.txt \
  --o-visualization liver-alpha-rarefaction.qzv
```

```
################################################################################
##CORE METRICS

#!/bin/sh

#SBATCH --job-name=liver-core-metrics
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

qiime diversity core-metrics-phylogenetic \
  --i-phylogeny liver-insertion-tree.qza \
  --i-table liver-table-samples.qza \
  --p-sampling-depth 10049 \
  --m-metadata-file r1-metadata.txt \
  --output-dir core-metrics-liver


################################################################################
#ALPHA AND BETA DIVERSITY

#!/bin/sh

#SBATCH --job-name=liver-group-sig
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Unweighted
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-liver/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-liver/unweighted-unifrac-feed-program-significance.qzv \
  --p-pairwise
```

```
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-liver/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-liver/unweighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-liver/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-liver/unweighted-unifrac-pen-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-liver-prev-2/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file r1-metadata-prev.txt \
  --m-metadata-column Dominant_Genus \
  --o-visualization core-metrics-liver-prev-2/unweighted-unifrac-bact-significance.qzv \
  --p-pairwise

#Weighted
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-liver/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization core-metrics-liver/weighted-unifrac-feed-program-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-liver/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization core-metrics-liver/weighted-unifrac-source-significance.qzv \
  --p-pairwise

qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-liver/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization core-metrics-liver/weighted-unifrac-pen-significance.qzv \
  --p-pairwise
```

```
qiime diversity beta-group-significance \
  --i-distance-matrix core-metrics-liver-prev-2/weighted_unifrac_distance_matrix.qza \
  --m-metadata-file r1-metadata-prev.txt \
  --m-metadata-column Dominant_Genus \
  --o-visualization core-metrics-liver-prev-2/weighted-unifrac-bact-significance.qzv \
  --p-pairwise

#Shannon
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-liver/shannon_vector.qza \
  --m-metadata-file r1-metadata.txt \
  --o-visualization core-metrics-liver/shannon-group-significance.qzv

#Richness
qiime diversity alpha-group-significance \
  --i-alpha-diversity core-metrics-liver/observed_otus_vector.qza \
  --m-metadata-file r1-metadata.txt \
  --o-visualization core-metrics-liver/observed_otus_significance.qzv

################################################################################
##ANCOM

#!/bin/sh

#SBATCH --job-name=liver-ancom
#SBATCH --nodes=1
#SBATCH --ntasks=1
#SBATCH --time=01:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=fuerniss@rams.colostate.edu

#Activate qiime
source activate qiime2-2019.4

#Genus Level
qiime taxa collapse \
  --i-table liver-table-samples.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --p-level 6 \
  --o-collapsed-table liver-collapsed-table6.qza

#Add Genus Pseudocount
qiime composition add-pseudocount \
  --i-table liver-collapsed-table6.qza \
  --o-composition-table liver-table-comp6.qza
```

```
#Run Genus ANCOM: Management Program
qiime composition ancom \
  --i-table liver-table-comp6.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization ancom-feed_program6.qzv

#Run Genus ANCOM: Source
qiime composition ancom \
  --i-table liver-table-comp6.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization ancom-source6.qzv

#Run Genus ANCOM: Pen
qiime composition ancom \
  --i-table liver-table-comp6.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization ancom-pen6.qzv

#Phylum Level
qiime taxa collapse \
  --i-table liver-table-samples.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --p-level 2 \
  --o-collapsed-table liver-collapsed-table2.qza

#Add Phylum Pseudocount
qiime composition add-pseudocount \
  --i-table liver-collapsed-table2.qza \
  --o-composition-table liver-table-comp2.qza

#Run Phylum ANCOM: Management Program
qiime composition ancom \
  --i-table liver-table-comp2.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column feed_program \
  --o-visualization ancom-feed_program2.qzv

#Run Phylum ANCOM: Source
qiime composition ancom \
  --i-table liver-table-comp2.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column cattle_source \
  --o-visualization ancom-source2.qzv
```

```
#Run Phylum ANCOM: Pen
qiime composition ancom \
  --i-table liver-table-comp2.qza \
  --m-metadata-file r1-metadata.txt \
  --m-metadata-column reassigned_lot \
  --o-visualization ancom-pen2.qzv

##############################################################################
##FILES TO EXPORT FOR R ANALYSIS AND VISUALIZATION
#Box plots for alpha diversity

#Visualize Rarefied Table
qiime feature-table summarize \
  --i-table rarefied_table.qza \
  --o-visualization rarefied_table.qzv \
  --m-sample-metadata-file metadata.txt

#Visualize Phylum Rarefied Table
qiime taxa collapse \
  --i-table rarefied_table.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --p-level 2 \
  --o-collapsed-table liver-collapsed-rare-table2.qza

qiime feature-table summarize \
  --i-table liver-collapsed-rare-table2.qza \
  --o-visualization rarefied_table2.qzv \
  --m-sample-metadata-file metadata.txt

#Visualize Class Rarefied Table
qiime taxa collapse \
  --i-table rarefied_table.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --p-level 3 \
  --o-collapsed-table liver-collapsed-rare-table3.qza

qiime feature-table summarize \
  --i-table liver-collapsed-rare-table3.qza \
  --o-visualization rarefied_table3.qzv \
  --m-sample-metadata-file metadata.txt
```

```
#Visualize Order Rarefied Table
qiime taxa collapse \
  --i-table rarefied_table.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --p-level 4 \
  --o-collapsed-table liver-collapsed-rare-table4.qza

qiime feature-table summarize \
  --i-table liver-collapsed-rare-table4.qza \
  --o-visualization rarefied_table4.qzv \
  --m-sample-metadata-file metadata.txt

#Visualize Family Rarefied Table
qiime taxa collapse \
  --i-table rarefied_table.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --p-level 5 \
  --o-collapsed-table liver-collapsed-rare-table5.qza

qiime feature-table summarize \
  --i-table liver-collapsed-rare-table5.qza \
  --o-visualization rarefied_table5.qzv \
  --m-sample-metadata-file metadata.txt

#Visualize Genus Rarefied Table
qiime taxa collapse \
  --i-table rarefied_table.qza \
  --i-taxonomy liver-taxonomy-silva7.qza \
  --p-level 6 \
  --o-collapsed-table liver-collapsed-rare-table6.qza

qiime feature-table summarize \
  --i-table liver-collapsed-rare-table6.qza \
  --o-visualization rarefied_table6.qzv \
  --m-sample-metadata-file metadata.txt

qiime feature-table summarize \
  --i-table liver-collapsed-rare-table5.qza \
  --o-visualization rarefied_table5.qzv \
  --m-sample-metadata-file metadata.txt
```

```
#Phylum Relative Frequency
qiime feature-table relative-frequency \
--i-table liver-collapsed-table2.qza \
--o-relative-frequency-table liver-rf-table2.qza

qiime tools export \
--input-path liver-rf-table2.qza \
--output-path liver-phylum-rf-table

biom convert -i liver-phylum-rf-table/feature-table.biom -o liver-phylum-rf-table.tsv --to-tsv

#Genus Relative Frequency
qiime feature-table relative-frequency \
--i-table liver-collapsed-table6.qza \
--o-relative-frequency-table liver-rf-table6.qza

qiime tools export \
--input-path liver-rf-table6.qza \
--output-path liver-genus-rf-table

biom convert -i liver-genus-rf-table/feature-table.biom -o liver-genus-rf-table.tsv --to-tsv
```

LIVER R ANALYSIS CODE

```
#R Software Version 3.4.1
#R Studio Version 1.2.1335
#Load relevant packages
library(lme4)
library(forcats)
library(dplyr)
library(emmeans)
library(ggplot2)
library(reshape2)
library(cowplot)



################################################################################
#Import bacterial prevalence count data, fix column names, and set categorical variables as
factors
Bac_prev <- read.csv("File_Path")
names(Bac_prev)[1]<-"Pen"
Bac_prev$Pen <- as.factor(Bac_prev$Pen)

#-------------------------------------------------------------------------------------------
#Model Bacteroides occurrence within an individual abscess
Bactero <- glmer(cbind(Bacteroides_yes,Bacteroides_no) ~ Program + (1|Pen) , data =
                 Bac_prev, family = "binomial")
Bactero

#Display probability of Bacteroides by management program by back-transformation from log
odds scale
#Formally test the odds ratio for Bacteroides occurrence by management program
emmeans(Bactero, pairwise ~ Program, type = "response")

#-------------------------------------------------------------------------------------------
#Model Trueperella occurrence within an individual abscess
Trueper <- glmer(cbind(Trueperella_yes,Trueperella_no) ~ Program + (1|Pen) , data = Bac_prev,
                 family = "binomial")
Trueper

#Display probability of Trueperella by management program by back-transformation from log
odds scale
#Formally test the odds ratio for Trueperella occurrence by management program
emmeans(Trueper, pairwise ~ Program, type = "response")
```

```
#############################################################################
#Alpha Diversity

#Import alpha diversity data and fix column names
Alpha <- read.csv("File_Path")
names(Alpha)[1]<-"Sample"

#Summarize by Feed Program
aggregate(Alpha[, 5:6], list(Alpha$feed_program), mean)
aggregate(Alpha[, 5:6], list(Alpha$feed_program), sd)

#Summarize by Pen
aggregate(Alpha[, 5:6], list(Alpha$reassigned_lot), mean)
aggregate(Alpha[, 5:6], list(Alpha$reassigned_lot), sd)


#############################################################################
#Boxplot of Observed OTUs by Feed Program
OTU <- ggplot(Alpha, aes(x=feed_program, y=observed_otus)) +
  geom_boxplot(width=0.5) +
  labs(x="Management Program", y = "Observed OTUs") +
  scale_y_continuous(breaks=c(0,3,6,9,12), limits=c(0, 14), expand = c(0, 0)) +
  theme_light() + theme(panel.grid.major = element_blank(),
                        panel.grid.minor = element_blank()) +
  theme(axis.title.y = element_text(margin = margin(t = 0, r = 10, b = 0, l = 0)),
          axis.title.x = element_text(margin = margin(t = 10, r = 0, b = 0, l = 0))) +
          annotate("text", x = 1.5, y = 13, label = "italic(P) == .488", parse = TRUE)
OTU

#Boxplot of Shannon by Feed Program
Shan <- ggplot(Alpha, aes(x=feed_program, y=shannon)) +
  geom_boxplot(width=0.5) +
  labs(x="Management Program", y = "Shannon Diversity Index")+
  scale_y_continuous(breaks=c(0.0,0.5,1.0,1.5), limits=c(0, 2), expand = c(0, 0)) +
  theme_light() + theme(panel.grid.major = element_blank(),
                        panel.grid.minor = element_blank()) +
  theme(axis.title.y = element_text(margin = margin(t = 0, r = 10, b = 0, l = 0)),
          axis.title.x = element_text(margin = margin(t = 10, r = 0, b = 0, l = 0))) +
          annotate("text", x = 1.5, y = 1.85714286, label = "italic(P) == .356", parse = TRUE)
Shan

Blank <- ggplot()

#Combine Boxplots
A <- plot_grid(OTU, Blank, Shan, labels=c('A', '', 'B'), nrow = 1, rel_widths = c(8,1,8))
A
```

```
#############################################################################
#Relative Abundance

#Phylum Abundance by Program
Phylum_S <-
read.csv("C:\\Users\\fuern\\Dropbox\\School\\Projects\\Feedlot_Microbiome_Project\\R_Analysis\\Phylum_Abundance_Simple.csv")
names(Phylum_S)[1]<-"Management"
names(Phylum_S)[4]<-"Rare Taxa"

PS <- melt(Phylum_S, id.vars = "Management", variable.name = "Phyla")
PA <- PS %>%
  mutate(Phyla = fct_relevel(Phyla, 'Fusobacteria', 'Bacteroidetes', 'Rare Taxa'),
         percent = value*100) %>%
  ggplot(aes(x = Management, y = percent, fill = Phyla)) +
  geom_bar(stat = "identity", position = "fill", width = .85) +
  scale_y_continuous(labels = scales::percent) +
  labs(y = "") +
  theme_light() +
  theme(panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        legend.position="bottom",
        axis.title.x=element_blank()) +
  scale_fill_manual(name = "",
                    labels = c("Fusobacteria",
                               "Bacteroidetes",
                               "Rare Taxa"),
                    values = c("#5dade2",
                               "#52be80",
                               "#AAB7B8"))+
  guides(color = guide_legend(override.aes = list(size = 0.35))) +
  theme(legend.title = element_text(size = 9),
        legend.text = element_text(size = 9))
PA

#Genus Abundance by Program
Genus_S <-
read.csv("C:\\Users\\fuern\\Dropbox\\School\\Projects\\Feedlot_Microbiome_Project\\R_Analysis\\Genus_Abundance_Simple.csv")
names(Genus_S)[1]<-"Management"
names(Genus_S)[4]<-"Rare Taxa"

GS <- melt(Genus_S, id.vars = "Management", variable.name = "Genera")
GA <- GS %>%
  mutate(Genera = fct_relevel(Genera, 'Fusobacterium', 'Bacteroides', 'Rare Taxa'),
         percent = value*100) %>%
```

```
  ggplot(aes(x = Management, y = percent, fill = Genera)) +
  geom_bar(stat = "identity", position = "fill", width = .85) +
  scale_y_continuous(labels = scales::percent) +
  labs(y = "") +
  theme_light() +
  theme(panel.grid.major = element_blank(),
      panel.grid.minor = element_blank(),
      legend.position="bottom",
      axis.title.x=element_blank()) +
  scale_fill_manual(name = "",
                    labels = c(expression(italic("Fusobacterium")) ,
                               expression(italic("Bacteroides")),
                               "Rare Taxa"),
                    values = c("#5dade2",
                               "#52be80",
                               "#AAB7B8")) +
  guides(color = guide_legend(override.aes = list(size = 0.35))) +
  theme(legend.title = element_text(size = 9),
        legend.text = element_text(size = 9))
GA


#Genus Abundance by Sample
Genus_ALL <-
read.csv("C:\\Users\\fuern\\Dropbox\\School\\Projects\\Feedlot_Microbiome_Project\\R_Analysi
s\\Genus_Abundance_Individual.csv")
names(Genus_ALL)[1]<-"Management"
names(Genus_ALL)[6]<-"Rare Taxa"

GRAA <- melt(Genus_ALL, id=c("Management", "ID"), variable.name = "Genera")
```

```
GAA <- GRAA %>%
  mutate(Genera = fct_relevel(Genera, 'Fusobacterium', 'Bacteroides', 'Porphyromonas',
'Caviibacter', 'Rare Taxa', ),
         percent = value*100) %>%
  ggplot(aes(x = ID, y = percent, fill = Genera)) +
  geom_bar(stat = "identity", position = "fill", width = .85) +
  scale_y_continuous(labels = scales::percent) +
  labs(x="Natural                                                    Conventional", y = "") +
  theme_light() +
  theme(panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        axis.text.x=element_blank(),
        axis.ticks.x=element_blank(),
        legend.position="bottom") +
  scale_fill_manual(name = "",
                    labels = c(expression(italic("Fusobacterium")) ,
                               expression(italic("Bacteroides")),
                               expression(italic("Porphyromonas")),
                               expression(italic("Caviibacter")),
                               "Rare Taxa"),
                    values = c("#5dade2",
                               "#52be80",
                               "#cd6155",
                               "#f5b041",
                               "#AAB7B8"))+
  guides(color = guide_legend(override.aes = list(size = 0.35))) +
  theme(legend.title = element_text(size = 9),
        legend.text = element_text(size = 9))

GAA

#Combine Plots
ggdraw() +
  draw_plot(PA, x = 0, y = .55, width = .45, height = .45) +
  draw_plot(GA, x = .55, y = .55, width = .45, height = .45) +
  draw_plot(GAA, x = 0, y = 0, width = 1.0, height = 0.5) +
  draw_plot_label(label = c("A", "B", "C"), size = 15,
                  x = c(0, 0.55, 0), y = c(1, 1, 0.5))
```

```
##############################################################################
#Correlation of Fusobacterium and Bacteroides
#Import proportion data, fix column names, and set both columns as number
Cor <- read.csv("File_Path")
names(Cor)[1]<-"Sample"

cor(Cor$Fusobacterium,Cor$Bacteroides, method = "spearman")

CP <- ggplot(Cor, aes(x=Fusobacterium, y=Bacteroides)) +
  geom_point()+
  labs(x=~italic("Fusobacterium")~ "Relative Abundance",
       y=~italic("Bacteroides")~ "Relative Abundance") +
  theme_light() + theme(panel.grid.major = element_blank(),
                        panel.grid.minor = element_blank()) +
  theme(axis.title.y = element_text(margin = margin(t = 0, r = 10, b = 0, l = 0)),
          axis.title.x = element_text(margin = margin(t = 10, r = 0, b = 0, l = 0))) +
  scale_x_continuous(expand = c(0, .04)) + scale_y_continuous(expand = c(0, .04)) +
  annotate("text", x = .75, y = .75, label = "Spearman Correlation: -0.886")
CP
```