THESIS

THE EFFECTS OF A SACCHAROMYCES CEREVISIAE FERMENTATION PRODUCT ON LIVER ABSCESS PREVALENCE, MICROBIOMES, AND RESISTOMES OF CATTLE RAISED TO PRODUCE NATURAL BRANDED-BEEF

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ABSTRACT

THE EFFECTS OF A SACCHAROMYCES CEREVISIAE FERMENTATION PRODUCT ON LIVER ABSCESS PREVALENCE, MICROBIOMES, AND RESISTOMES OF CATTLE RAISED TO PRODUCE NATURAL-BRANDED BEEF

Use of antibiotics in livestock production has received increased scrutiny due to public health concerns over the development and dissemination antimicrobial resistance. As a result, there are efforts to replace the use of medically important antimicrobial drugs in food producing animals with novel non-antimicrobial alternatives to treat disease. One target for antimicrobial drug reduction and replacement within the beef cattle industry is the use of tylosin, a macrolide antibiotic, which is widely included within diets of feedlot cattle to reduce incidence of liver abscesses. Tylosin is considered medically important for human health, and therefore tylosin use in feedlot operations may be limited in the future. Liver abscesses are a leading cause of liver condemnation at slaughter, and result in significant financial losses to the beef cattle industry. Exposure of cattle to high concentrate diets is associated with rumen acidosis and rumenitis, leading to the formation of liver abscesses. Several non-antimicrobial strategies for the treatment of liver abscesses have been evaluated, including nutritional management, vaccines, other antimicrobial drugs, and feed additive products, although none have been shown to reduce liver abscesses as effectively as treatment with tylosin.

The studies in this thesis evaluated effects of a *Saccharomyces cerevisiae* fermentation product (SCFP) on cattle performance, health, and microbiology outcomes. Yeast products have been shown to benefit rumen fermentation and improve cattle performance; and therefore, SCFP

were hypothesized to reduce the occurrence of liver abscesses when included in diets of beef cattle raised without antibiotics. The first chapter of this thesis reviews current information about liver abscess pathogenesis, microbiology, and the use and efficacy of antimicrobial and non-antimicrobial interventions. There is a significant impact of diet, treatment, and other management factors on rumen microbial ecology, including shifts in microbiomes attached to the rumen epithelium, rumen acidosis, and microbiology of liver abscesses. The second chapter focuses on a randomized block clinical trial conducted to evaluate the effects of SCFP on animal health, growth and production, liver abscess prevalence, fecal microbiomes, and fecal resistomes in cattle raised without antibiotics using 16S rRNA gene sequencing and shotgun metagenomic sequencing. In a randomized block clinical trial, there were no statistically significant, detectable differences of SCFP supplementation on any of the tested outcomes. The third chapter characterized the liver abscess microbial community from liver abscess contents using 16S rRNA marker gene sequencing. The liver abscess microbiome was diverse and polymicrobial, and shifts in liver abscess microbiomes across cattle enrollment group demonstrates that there is a potential impact of cattle source, feedlot environment, and other factors on liver abscess microbiomes. Given the diversity of the liver abscess microbiome demonstrated in this study, more work is needed to understand the role of liver abscess microbiomes for disease severity as pre-harvest feeding strategies are investigated further in feedlot cattle.

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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Liver abscesses are a costly, subclinical disease of cattle that are frequently detected at slaughter. The occurrence of liver abscesses in feedlot cattle are commonly associated with feeding high concentrate rations which lead to subacute rumen acidosis (SARA), rumenitis and infection of the liver through the portal venous drainage (Nagaraja and Chengappa, 1998). Therefore, the disease pathogenesis is commonly referred to as "Acidosis Rumenitis Liver Abscess Complex" (ARLC). The economic impacts of liver abscesses can be significant, particularly with severe liver abscesses, as they are associated with decreased cattle production and growth, carcass quality, and loss of the liver during harvest via condemnation (Brown and Lawrence, 2010; Reinhardt and Hubbert, 2015). The beef cattle industry has identified various strategies to mitigate the occurrence of liver abscesses, but most commonly it is through inclusion of in-feed antimicrobials in finishing diets. Due to concerns over developing antimicrobial resistance in feedlot environments and the food supply, use of medically important drugs in food animal production has come under scrutiny. In response, there is a push to develop and utilize non-antimicrobial feed additives, vaccines, and nutritional management strategies to provide effective alternative interventions for liver abscesses. However, the industry has not identified effective solutions that can maintain the same rate of gain while also reducing morbidity from liver abscesses in the absence of antibiotic intervention. This is in part due to a lack of comprehensive understanding of the epidemiology and pathogenesis of ARLC in cattle. The objectives of this literature review are to provide a critical assessment of the research and

research methods regarding the pathogenesis of ARLC, and to interpret the literature regarding antimicrobial and non-antimicrobial interventions for liver abscesses in feedlot cattle.

PATHOGENESIS OF ACIDOSIS RUMENITIS LIVER ABSCESS COMPLEX

Bacterial pathogenesis in liver abscess formation

Liver abscess formation in feedlot cattle is widely accepted to be secondary to rumen epithelial inflammation (i.e., rumenitis) that occurs commonly secondary to feeding diets high in rapidly fermentable starches which promotes SARA. This understanding originated from studies that detected the a linear relationship between feeding high concentrate diets, rumen pathology, and liver abscesses in feedlot cattle (Jensen et al., 1954a, 1954b; Smith, 1944). Many studies investigating the infectious etiology have focused on the bacterial content of liver abscesses using culture protocols, and this previous work has concluded that *Fusobacterium necrophorum*, a Gram-negative anaerobic bacterium, was most likely the primary etiologic agent of liver abscess formation (Lechtenberg et al., 1988; Nagaraja et al., 1999a; Nagaraja and Chengappa, 1998; Newson, 1938; Scanlan and Hathcock, 1983; Tadepalli et al., 2009; Tan et al., 1994). When the rumen epithelial lining is damaged by inflammation or physical injury, *F. necrophorum* can gain entry to the venous drainage of the rumen, and are transported to the liver via the portal vein. Once bacteria gain entry to the liver, several virulence factors, including protease and leukotoxin, can lead to a pyogranulomatous immune reaction (Tan et al., 1992).

Fusobacterium necrophorum are the most prevalent organisms identified from liver abscesses using anaerobic and aerobic culture methodology, however, several other bacteria have been isolated from liver abscesses, highlighting the potentially polymicrobial environment of liver abscesses. The second most frequently isolated bacterial species from liver abscesses is *Trueperella pyogenes*, which are Gram-positive, facultative anaerobes. This bacterial species is

thought to associate with the lining of the rumen epithelium because of the availability of oxygen associated with blood flow to this tissue (Narayanan et al., 1998; Tan et al., 1996). There is evidence that *F. necrophorum* and *T. pyogenes* may act synergistically (Tadepalli et al., 2009). The organisms are commonly isolated together in other cattle diseases, including foot rot (Nagaraja et al., 2005), metritis in dairy cows (Bicalho et al., 2012), and have nutritional and pathogenic synergy. Additional bacteria that have been isolated from liver abscesses include *Bacteriodes* spp., *Clostridium* spp., *Escherichia coli, Klebsiella* spp*., Proprionbacterium* spp*., Enterobacter* spp., *Mobiluncus* spp., *Mitsuokella* spp., *Pasterella* spp., *Peptostreptococcus* spp., *Porphyromonas* spp., *Prevotella* spp., *Proprionibacterium* spp., *Staphyloccus*, *Streptococcus*, and other unidentified Gram-positive and Gram-negative bacteria (Amachawadi and Nagaraja, 2016; Nagaraja and Chengappa, 1998; Scanlan and Hathcock, 1983; Weinroth et al., 2017). In addition, a recent study reported isolation of *Salmonella enterica* from liver abscesses collected from dairy cattle liver abscesses using anaerobic methods (Amachawadi and Nagaraja, 2015).

Other bacteria isolated from liver abscesses in feedlot cattle are not credited with playing a significant role in pathogenesis of liver abscess formation, and our current understanding is limited with respect to unculturable species. Though other bacteria are isolated less commonly compared to *F. necrophorum and T. pyogenes*, they could be playing a role in the formation and severity of liver abscesses. Other bacterial constituents could represent a significant gap in our current understanding of the mechanism for ARLC and factors determining disease severity. For example, *Bacteroides* spp., which are frequently identified in polymicrobial liver abscess cultures, have been implicated in bacteremia and abscess formation when they escape the gut environment in humans and other cattle diseases, in part due to potent virulence factors, evasion of the host immune system, purinergic signaling, and inflammasome activation (Van Metre,

2017; Wexler, 2007).Therefore, more work is needed to define both the microbial communities and secondly, understand the role that they could play in disease severity.

Dietary influences on microbial ecology and rumen acidosis

Diet is an important driver of host gut microflora, as demonstrated by the coevolution between mammals and their gut microbiota community structure across animal clades adapted to different diets (Muegge et al., 2011). When cattle are fed high grain diets that are low in roughage, the microbial ecology can shift, resulting in a dysbiosis that can range in severity from clinical to subclinical rumen acidosis. There are many nutritional management strategies to manage this, but it is important to understand what constitutes a "normal" or healthy vs. unhealthy microbiome is in cattle rumens (Nagaraja and Titgemeyer, 2007).

Ruminants are unique due to the symbiotic relationship they maintain with their rumen microbes to digest plants, relying on microbial communities to digest complex starches and cellulose prior to entering the true stomach (foregut fermentation). Conversion of feed into available energy sources to the ruminant, such as volatile fatty acids (VFA), is primarily performed by anaerobes. In one study, ruminant foregut microbial community composition and variability was determined from 32 species from 35 countries, including cattle, bison, buffalo, sheep, goats, deer, alpacas, llamas, and guanacos by sequencing regions of 16S rRNA genes and 18S rRNA genes to identify archaea, bacteria, and ciliated protozoal microbial communities (Henderson et al., 2015). Composition and variability were primarily attributed to diet, and to a lesser extent, host species and geography. They also found evidence for a "core microbiome" across all samples, consisting of more than 5,000 unique species, including members of microbial species, *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales.* In domesticated cattle, several studies have

utilized culture-independent methods to characterize the effects of diet on rumen microbiomes, including associations between feed efficiency and the microbiome (Hernandez-Sanabria et al., 2012), impact of pasture-fed versus grain-fed management on dairy cattle microbiomes (de Menezes et al., 2011), and feedlot cattle microbiomes under different management strategies (Shanks et al., 2011).

Effects of feeding cattle high concentrate diets on development of SARA and the constituents of the rumen microbiome is an area of active research (Fernando et al., 2010; Khafipour et al., 2009; Mao et al., 2013; Plaizier et al., 2017; Tajima et al., 2000). The pH of the rumen (normally 5.8-6.5) is important for rumen stability because decreased pH can decrease diversity of microbial populations, alter microbial fermentation products, and impact rumen motility and absorption capacity (Nagaraja and Titgemeyer, 2007). Following rapid intake of high concentrate feeds, rumen microbial fermentation rapidly increases, resulting in a cascade of changes that result in increased VFA production, lactic acid accumulation, reduced pH, decreased rumen motility, rumenitis, and mucosal changes to the rumen wall (Nocek, 1997). Historically, culture-based studies established that concurrent to these changes, rumen microbe populations shift, with proliferation of lactic acid-producing bacteria (i.e., *Streptococcus bovis*) and decreases in lactic acid-utilizing bacteria (i.e., *Megasperha elsdenii* and *Selenomonas ruminantium*). These shifts result in further increase in lactic acid accumulation, pH decline, and reduced absorption capacity of VFA, resulting in a downward spiral effect that leads to worsening acidosis and rumen wall damage (Russell and Hino, 1985). However, these studies were limited in their ability to account for microbial ecology *in vivo*, and in their technical ability to monitor populations of bacteria that are uncultivable.

Culture-independent methods, including high-throughput sequencing of marker genes, can quantify shifts in microbial composition, richness, and diversity of the microbial populations within the rumen, allowing for a more comprehensive understanding of microbial dynamics during complex disease processes such as SARA. Metagenomics methods, i.e., shotgun metagenomics and metatranscriptomics, can be used to quantify abundance of functional genes including enzymatic activity and antimicrobial resistance genes. Using these methodologies, several studies in cattle fed highly fermentable carbohydrates have found that feeding these diets (i.e., dried distillers grains) can lead to reduction in microbial richness and diversity across multiple intestinal locations, to variable extent (Callaway et al., 2010; Hook et al., 2011; Khafipour et al., 2016; Li et al., 2016; Mao et al., 2013; Petri et al., 2013; Plaizier et al., 2017; Rice et al., 2012; Tajima et al., 2000), however there are some variations in which microbes and genes shift across studies. Inconsistencies and variation across study system could be attributable to several factors, including host adaptation and microbial evolution in domesticated animals under different management operations, variation in starch content of the diet tested, feeding management, and experimental design, such as DNA isolation method, primer choice, sample collection methodology, bioinformatics analysis, etc. There is a need for standardization in microbiome studies to address these sources of both experimental and computational variation (Sinha et al., 2015).

Despite variation of methodology and lack of standardization in rumen microbiome studies, most studies concur that members from the phyla, *Firmicutes* and *Bacteroidetes,* are most abundant in the rumen microbial ecosystem. Additionally, relative ratios of these taxa tend to shift as grain concentration in the ration is increased, with an increase in the former relative to the later. Interestingly, one study (Fernando et al., 2010), did not observe this pattern with

respect to high grain diets. When SARA was induced, decreased relative abundance in *Bacteroidetes* spp.*,* along with *Fibrobacteres* spp., which resulted a loss in function for cellulose degrading bacteria in the rumen (Khafipour et al., 2009). In addition to losses of functional due to shifts in certain taxonomic groups, higher concentrate feed are associated with increased bacteria that have opportunistic or pathogenic potential, such as *Escherichia coli* and *Clostridium perfringens*, along with *F. necrophorum,* which has previously been attributed to lactate accumulation in the rumen, as it utilizes lactate as a substrate (Tadepalli et al., 2009).

The rumen epithelium microbiome

Though several studies have focused on characterizing rumen microbial dysbiosis in response to challenges with SARA, less is known about the microbiome of the rumen epithelial lining. It is likely that the liver abscess microbiome is associated with bacteria that are adherent to the rumen epithelial lining, since bacteria are thought to access the liver through rumen wall lesions via the portal vein circulation (Nagaraja and Chengappa, 1998; Narayanan et al., 1998; Rezac et al., 2014a). In a study characterizing the bacteria associated with ovine rumens, Mead and Jones, (1981) first proposed use of the term "epimural" to describe the flora associated with this niche. It is possible that further insight of epimural microbiome population shifts during SARA will help develop a more nuanced understanding of the pathogenesis for liver abscess formation, and possibly decipher what leads to severe versus mild abscess formation during.

In one unique study, Narayanan et al., (1998) collected samples of liver abscesses, rumen walls, and rumen contents from 59 cattle at slaughter. The anaerobic organisms that were isolated were subjected to characterization using ribosomal DNA restriction fragment length polymorphism analysis, or ribotyping. Interestingly, *Actinomyces pyogenes*, (now *Trueperella pyogenes)* was frequently isolated from the rumen epithelial lining, and had similar biochemical

characteristics to the corresponding isolates from liver abscesses; the authors concluded that they originated from the rumen epithelium. *F. necrophorum* is infrequently reported to be associated with the rumen epithelial lining, but it has been frequently associated with rumen lesions, such as parakeratosis (Garcia et al., 1971; Okada et al., 1999). As a facultative anaerobe that is sensitive to pH <7, it has been hypothesized that *F. necrophorum* is ideally suited to the niche of the rumen wall during subclinical rumen acidosis episodes (Tadepalli et al., 2009).

Several studies have used metagenomic sequencing techniques to characterize the epimural microbiome in cattle under different management and feeding conditions (De Mulder et al., 2017; Malmuthuge et al., 2014; Mao et al., 2015; Petri et al., 2013). Commonly identified phyla within the epimural community from these studies include *Bacteroidetes, Firmicutes,* and *Proteobacteria*, along with smaller abundances of archaeal species. However, the extent of our knowledge about epimural communities is limited by 16S rRNA gene sequencing, as this method does not allow for functional characterization, and both inactive and dead bacterial cells can be amplified. To characterize the functional role of the rumen epimural microbiota, a crossover study performed metatranscriptomic sequencing on rumen biopsies sampled from Holstein dairy cattle, comparing cows fed a forage-based diet with cows with induced SARA (Mann et al., 2018). Interestingly, shifts in the microbiome under conditions of SARA were not associated with differences in gene expression of epimural communities, but they were associated with enrichment of the phyla, *Bacteroidetes,* along with a decreased diversity. Based on metatranscriptomic sequencing, the most common genera associated with the rumen epithelium lining included *Clostridium*, *Campylobacter*, *Neisseria, Prevotella, Bacteroides, Treponema, Eubacterium*, and *Butyrivibrio.* Epimural rumen microbes contained higher gene expression levels for oxidative stress proteins, which may be an adaptive mechanism for these microbial

communities to survive exposure to reactive oxygen levels induced by changing redox potential in the rumen (Friedman et al., 2017). Gene expression was higher for genes involved in central metabolism, galactose, starch, and sucrose metabolism, as well as cellulose degradation. Another study characterized beef cattle epimural microbiomes in cattle during transition from a forage to a high concentrate diet using pyrosequencing of the V3 hypervariable region of 16S rRNA gene from beef heifers (Petri et al., 2013). They detected decreased bacterial diversity in cattle fed high grain diets compared to forage-fed diets. In addition, the microbial profiles in cattle experiencing clinical vs. subclinical acidosis found had increased relative abundances of *Acetitomaculum*, *Lactobacillus*, *Prevotella*, and *Streptococcus*. It is interesting to note that none of these studies have characterized *F. necrophorum* or *T. pyogenes* as large constituents in the epimural microbial communities, with or without SARA. Future studies that attempt to characterize the liver abscess pathogenesis during SARA should aim to characterize host-microbial interactions, particularly the epimural microflora and their relationship to liver abscess severity in feedlot cattle.

ANTIMICROBIAL DRUGS AND LIVER ABCESSES

Antimicrobial drugs for treatment of liver abscesses in U.S. feedlot cattle

The industry controls liver abscesses through both use of antimicrobials added to the feed (or water) and nutritional management. The FDA has recently taken steps to limit the use of medically important antimicrobials for use in the feed or water of food producing animals. Effective January 1, 2017, the Veterinary Feed Directive (VFD) brought the therapeutic use of medically important antimicrobials under the supervision of veterinarians to ensure judicial use of those drugs (Veterinary Feed Directive, 2015). The antibiotics that are currently approved for reduction of liver abscesses in feedlot cattle include bacitracin methylene disalicylate,

chlortetracycline, neomycin sulfate in combination with oxytetracycline, oxytetracycline, tylosin phosphate, and virginiamycin (Amachawadi and Nagaraja, 2016; FDA, 2018). Of these, chlortetracycline, tylosin phosphate, neomycin sulfate and oxytetracycline, and oxytetracycline are under the oversight of the VFD. According to the most recent national survey of beef cattle, 31.0% of all feedlots, and 71.2% of all cattle in feedlots greater than 1000 head utilize tylosin in the feed for the metaphylaxis of liver abscesses (USDA–APHIS–VS–CEAH–NAHMS).

Tylosin is frequently fed in combination with monensin in US feedlots. Ionophores do not fall under the VFD nor are they labelled for liver abscess prevention, but they are known to enhance feed efficiency and growth by influencing fermentation products in the rumen of cattle, and they are therefore commonly included in ration formulation (Cernicchiaro et al., 2016). From the NAHMS 2011 feedlot survey, over 90% of feedlots used ionophores in the feed and more than 90% of cattle in feedlots received ionophores, especially monensin. A total of 71.7% of feedlots used tylosin phosphate, but only 18.4% of cattle received chlortetracycline. Only 0.7% of feedlots and 0.2% of all cattle received virginiamycin. A total of 7.5% of feedlots used oxytetracycline in feed, with 0.5% of cattle receiving oxytetracycline.

Bacitracin is considered the least effective antimicrobial for reducing the incidence of liver abscesses, and tylosin is considered the most effective (Brown et al., 1973; Haskins et al., 1967; Rogers et al., 1995). Studies have shown that both chlortetracycline and tylosin (fed at 75 mg/head/day tylosin and 70 mg/head/day for chlortetracycline) were both effective for reduction of liver abscesses compared to control cattle, but tylosin was significantly more effective, comparatively (Brown et al., 1975). Tylosin is effective at reducing liver abscess prevalence, , by a range of 40% to 70%, when included in the ration at 8 to 10 g/ton or 90 mg/animal/day (Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007; Wileman et al., 2009). In

several studies that evaluated feed efficiency and liver abscess incidence in cattle fed tylosin, monensin, and a combination of the two, monensin was shown to have little no effect on liver abscess incidence but improves feed efficiency, while tylosin reduced liver abscess incidence significantly (Meyer et al., 2009; Potter et al., 1985). Wileman et al., (2009) performed a review of 6 studies, and found that feeding tylosin to feedlot cattle reduced the liver abscess risk from 30% to 8% in the studies examined, but did not evaluate liver abscess severity.

Mechanism of action for tylosin to reduce liver abscesses in feedlot cattle

Tylosin, is a Gram-positive bacteriostatic antimicrobial that inhibits bacterial growth (primarily Gram-positive) through inhibition of protein synthesis. It performs this action by binding to the 50S ribosomal subunit, resulting in dissociation of peptidyl tRNA during RNA translation (Tenson et al., 2003). Tylosin is thought to reduce liver abscesses through inhibition of *F. necrophorum* and *Trueperella pyogenes* in the rumen, liver or both (Nagaraja et al., 1999a). Though *F. necrophorum* is a Gram-negative anaerobe, it is sensitive to tylosin in the rumen contents of steers (Nagaraja et al., 1999b). However, as most studies have shown, tylosin does not completely prevent formation of liver abscesses, which has been hypothesized to be caused by treatment failure due to antibiotic resistance of *F. necrophorum*. To investigate this, a study compared the bacterial flora isolated from liver abscesses in cattle fed tylosin to untreated cattle. They tested the antimicrobial susceptibility of *F. necrophorum* to a panel of approved antimicrobials approved for treatment of liver abscesses in cattle. Although the mean minimum inhibition concentration of tylosin for *F. necrophorum* did not differ between groups, interestingly, *T. pyogenes* were in higher abundance in treated cattle. The latter finding was surprising given that *T. pyogenes* is a Gram-positive bacterium, and therefore, theoretically more susceptible to tylosin (Nagaraja et al., 1999a). This latter finding was attributed to synergistic

activity between *T. pyogenes* and *F. necrophorum,* as well as its association with the rumen epithelial lining. Several other studies have confirmed that *F. necrophorum* is pan-susceptible to the classes of antimicrobials with FDA approval for treatment of liver abscesses (Berg and Scanlan, 1982, 1982; Lechtenberg et al., 1998; Mateos et al., 1997).

Interestingly, in humans, macrolides are well known and often prescribed for their non-antimicrobial effects, including immunomodulation and immunity (Kanoh and Rubin, 2010). One possible explanation for the partial, but not complete, efficacy of tylosin in reducing liver abscess formation in beef cattle is its role on the systemic immune system and local immune reactions. The role of the host immune system and how it interacts with rumen microbes in feedlot cattle during SARA induction has not been studied or explored with regards to ARLC. Another possible explanation might be that other bacteria besides *F. necrophorum* and *T. pyogenes,* could have an active role in liver abscess formation. There are several gaps in our understanding of this disease with respect to microbial-host interactions, and the lack of understanding for the mechanism of action for tylosin in ARLC suggests that it should be an area of future research.

Effects of tylosin on rumen flora and antibiotic resistance

Considerable attention has been given to effects of in-feed tylosin on rumen and fecal bacteria and antibiotic resistance. The primary mechanism for macrolide resistance is through target-site modification by methylation or mutation, which can lead to cross-resistance to lincosamides, and streptogramins B; however, other mechanisms include drug efflux and drug inactivation that are less common (Leclercq, 2002). There is concern that the use of tylosin in livestock production could lead to increased antibiotic resistant determinants in the food supply, environmental contamination with livestock effluents, or via direct contact.

The effects of in-feed tylosin administration to cattle and its withdrawal prior to slaughter have been investigated using Enterococci as indicator bacteria in two studies. One study found that tylosin use increased the proportion of bacterial resistance to macrolides (*ermB* gene and *msrC* gene), but the withdrawal of tylosin 28 days prior to slaughter reduced the proportion of resistant Enterococci (Beukers et al., 2015). A separate study investigated potential for co-selection of macrolide and copper resistance while feeding copper and tylosin in a 2x2 factorial study design, using *Enterococci faecium* as an indicator taxa. They concluded that feeding dietary copper and tylosin alone, or in combination, resulted in elevated copper (*tcrB* gene) and tylosin resistance (*ermB* and *tetM* gene) in fecal enterococci, suggesting potential for co-selection for macrolide resistance with in-feed supplementation of copper (Amachawadi et al., 2015). The limitation with this methodology, which selects for only a few indicator bacteria, is that it draws conclusions about antibiotic resistance risk without consideration for other fecal microbial populations or resistance genes. This approach, while common in antibiotic resistance studies of feedlot systems, does not capture the complete picture of antibiotic resistance patterns from rumen or fecal microbiomes under different selection pressures.

Characterization of the entire antimicrobial resistance potential, or the resistome, using metagenomics techniques allows for a snapshot of the structure and functional characteristics of the gastrointestinal tract microbiome and resistome. In one study, metagenomic characterization of rumen, cecum, and colon samples from tylosin- and monensin-fed cattle, compared to untreated animals, indicated that there was decreased diversity in the rumen, but not distal gut, of treated animals (Thomas et al., 2017). No changes at the phylum level were detected, but reduced relative abundance of the genera *Ruminococcus*, *Erysipelotrichacceae* and *Lachnospiraceae* were detected in all sample locations in treated cattle. Though they detected an

increase in genes involved in detoxification, but there was no relationship between the composition of antimicrobial resistance genes in the gut, by treatment. Metagenomics studies allow for a comprehensive analysis for the entire antibiotic resistance potential or resistome; however, there still are challenges when designing studies to determine causality between certain antibiotic use and resistance in livestock management systems, and ultimately a combination of culture and culture-independent methods could work in concert to advance our understanding of antibiotic resistance patterns in feedlot cattle.

NON-ANTIMICROBIAL ALTERNATIVES AND LIVER ABSCESSES

Nutritional management

Digestive disorders, including bloat and rumen acidosis, are the second largest cause of morbidity and mortality, second only to respiratory disease in feedlot cattle; therefore, reducing acidosis is a priority for feedlot nutritional management (Nagaraja et al., 1998). Rumen acidosis can vary in severity, ranging from acute acidosis to subacute acidosis, with various possible sequelae, including liver abscesses, bloat, polioencephalomalacia, laminitis, and sudden death (Nagaraja and Titgemeyer, 2007). Rumen acidosis is related to feeding readily fermentable concentrates, causing a microbial dysbiosis or imbalance between microbial proliferation, utilization, and absorption of VFA. Several experimental model systems have evaluated feedlot nutritional management strategies, including the amount, source, and composition of starches, inclusion of dietary roughages, step-up, or gradually transitioning rations, dietary fibrous byproducts, feed additives, and bunk management, which is thoroughly reviewed elsewhere (Nagaraja and Titgemeyer, 2007). General recommendations to reduce SARA (leading to liver abscess formation) include increasing roughage level, improving ration mixing, providing adequate bunk space and water access, feeding at regular intervals to spread out intake and

reduce slug feeding and avoiding under- or over-feeding (Bartle and Preston, 1991; Elam, 1976; Jensen et al., 1954a; Reinhardt and Hubbert, 2015). There is a large focus for research in dairy cattle to reduce SARA through nutritional management as well, with a focus on transitional rations, monitoring particle size distribution, reducing feed sorting, and altering meal size and meal frequency (Humer et al., 2018). Although dairy and beef cattle have different nutritional requirements and environments, knowledge gained from the dairy cattle industry could be helpful and relavent for feedlot cattle in relation to bunk management. Furthermore, fed dairy cattle make up a significant amount of the beef cattle feedlot population, and dairy breeds are susceptible to the SARA and formation of liver abscesses. Therefore, it is important to carry over sound management strategies to the feedlot for Holstein cattle to reduce disease burden due to ARLC in these populations (Amachawadi and Nagaraja, 2016).

Vaccine approaches

Two commercial vaccines were developed to control liver abscesses caused by *F. necrophorum*, both of which take advantage of the bacteria's virulence factors. Fusogard (Elanco Animal Health) is a bacterin vaccine approved for the control of liver abscesses and footrot. Centurion, made by Merck Animal Health, was a combination of a leukotoxoid of *F. necrophorum* and a *T. pyogenes* bacterin, but it is no longer available. In a randomized field trial of cattle that were fed either high- or low- grain diets and vaccinated or unvaccinated with Fusogard, the vaccination reduced the prevalence of severe liver abscesses with a lower prevalence of liver abscesses, but was not effective for cattle fed the grain-diet that had high prevalence of liver abscesses (Checkley et al., 2005). In a separate clinical trial, 'natural' cattle were fed the same diet, and were administered placebo, Fusogard or Centurion, with no detectable difference in liver abscess severity or incidence between the treatment groups (Fox et

al., 2009). According to the most recent USDA NAHMS beef cattle study, the Fusogard vaccine is not currently utilized in surveyed beef cattle management protocols (USDA–APHIS–VS–CEAH–NAHMS). A possible target antigen of *F. necrophorum* is the outer membrane protein, which is necessary for bacterial adhesion (Kumar et al., 2013). This has been suggested as a future vaccine target, although this is not an area of active research.

Oil and oilseed feed additives for reduction of liver abscesses

There is substantial interest in both dairy and beef cattle industries to develop non-antimicrobial, feed additive alternatives to reduce SARA, improve production parameters, and reduce liver abscesses. Promising products undergoing evaluation include oilseeds, essential oils, direct-fed microbials, probiotics, and yeast products (including live, dead or yeast culture). Oilseed or oils have been investigated for their potential to increase beneficial fatty acids in the meat that improve flavor, as well as improvements in animal production. Gibb et al., (2004) performed two feedlot trials evaluating sunflower seeds as a dietary additive and roughage source, and they found that sunflower seeds improved carcass traits, meat flavor profiles, and fatty acid profiles in the tissues, although there were some inconsistencies noted between experiments in benefits to cattle performance. However, no adverse effects of sunflower seed supplementation on liver abscess prevalence and severity were reported. In a separate study, effects of dietary sunflower seeds on production factors, carcass characteristics, liver abscess incidence, and fatty acid composition in the muscle were determined for individually-penned steers fed a barley-based diet in a 2 x 2 factorial experiment to evaluate dietary sunflower seed and tylosin phosphate. Sunflower seeds or sunflower seed plus tylosin combination diet reduced liver abscesses compared to the control diet (Mir et al., 2008).The authors discussed potential mechanisms of action, including improvement in immune function in the liver, based on

evidence that conjugated linoleic acid can be sequestered in the liver of sheep (Ivan et al., 2001). Essential oils also have been proposed to act through disruption of the cytoplasmic membrane of bacteria cells, but this mechanism of action is not well understood (Nazzaro et al., 2013). Feedlot experiments comparing a commercial essential oil containing limonene and thymol tended to reduce liver abscesses, although the reduction was not statistically significant (Meyer et al., 2009). Other *in vitro* studies have shown that limonene and thymol can inhibit the growth of *F. necrophorum* in vitro*,* but other types of oils had no effect (Elwakeel et al., 2013).

Yeast products and yeast culture products for the reduction of liver abscesses

There is considerable interest in the use of yeast (dead, live, or yeast culture products) to improve animal production performance in both dairy and beef cattle, including the reduction of liver abscesses. As a result, several meta-analyses are available in the literature. The mechanism of action for these products is understood to not be a direct effect on rumen pH, but rather through regulation of fermentation by the rumen microbiome, particularly through stimulation of lactic acid-utilizing bacteria and increased populations of cellulolytic bacteria and fungi (Calsamiglia et al., 2012). A meta-analysis of yeast supplementation in dairy and beef cattle across 110 papers, 157 experiments, and 376 treatments found that live or dead yeast supplementation increased rumen pH (+0.03 on average) and rumen VFA concentration (+2.17 mM on average), and decreased rumen lactic acid concentration (-0.9 mM on average), suggesting an improvement in rumen fermentation (Desnoyers et al., 2009; Ishaq et al., 2017)Other studies have evaluated the effects of active dry yeast using 18S rRNA and fungal ITS gene sequencing, and found that supplementation mitigated SARA-mediated shifts in protozoa reduction, resulting in increased pH stabilization (Ishaq et al., 2017).

Saccharomyces cerevisiae fermentation products (SCFP) are available commercially, and they are produced by Diamond V (Cedar Rapids, IA). Studies *in vitro* have demonstrated beneficial effects of yeast culture, showing they provide soluble growth factors (i.e., organic acids, B vitamins, and amino acids) to stimulate growth of rumen bacteria that use lactate and digest cellulose (Callaway and Martin, 1997; Yang et al., 2015; Yoon and Stern, 1996). Therefore, supplementation with yeast culture products may stabilize rumen conditions for cattle that fed high concentrate diets. A meta-analysis evaluated effects of yeast culture product on milk production and other production measures in lactating dairy cows, and concluded that there are significant improvements in milk production outcomes including milk yield, milk fat, and milk protein yield (Poppy et al., 2012). A separate meta-analysis evaluated effects of SCFP supplementation on feedlot performance and carcass traits, and included 18 experiments: 9 of the studies were internal company reports, and 9 studies were published in peer reviewed journals (Wagner et al., 2016a). The analysis demonstrated that cattle fed SCFP had significant increases in final body weight (2.9 kg), average daily gain (6.5%), dry matter intake (1%), gain to feed ratio (2.6%), and carcass traits compared to controls. Only one study evaluated the effects of SCFP on liver abscess prevalence, performance, and carcass characteristics in comparison to cattle fed monensin, tylosin and direct-fed microbial feed additives. This study found no difference in final body weight, gain to feed ratio, carcass characteristics, morbidity and mortality, or in liver abscess prevalence (Scott et al., 2017). More research into effects of SCFP on ARLC is needed to determine effects on the rumen and liver abscess microbiome.

CONCLUSIONS

The interactions between the ruminant host and their commensal rumen bacteria play a key role in tipping the balance between mutually beneficial relationships and pathogenesis, and

this frontier is only starting to be explored as advanced molecular approaches become more affordable and widespread (Taschuk and Griebel, 2012). Going forward, it will be essential to determine factors that lead to differences in infection susceptibility in populations of cattle entering the feedlot environment (Malmuthuge and Guan, 2017). Developing an understanding for the relationship between host local and systemic immunity, wall-adherent, epimural microbiomes, and virulence factors of these microbial communities should be part of the next steps in elucidating pathogenesis for ARLC. Furthermore, as scrutiny over antibiotic use in the feedlot industry increases and regulatory changes are put into effect, it will be essential to monitor food safety implications and efficacy of novel alternative products, the impact use or removal of antibiotics, and alternative therapies, including feed additives. With application of sound epidemiologic study designs and advanced molecular sequencing technology in combination with traditional culture techniques, we can develop new perspectives and understanding about ARLC that can lead to better solutions for this complex disease. Advancement of our progress and understanding of novel therapeutic approaches is essential to reduce disease burden and economic losses associated with this disease of feedlot cattle.

CHAPTER 2: EVALUATING THE EFFECTS OF SACCHAROMYCES CEREVISIAE FERMENTATION PRODUCT SUPPLEMENTATION ON THE LIVER ABSCESS PREVALENCE, FECAL MICROBIOME, AND ANTIBIOTIC RESISTOME OF NATURAL BEEF CATTLE[1]

SUMMARY

Liver abscesses are a common disease of feedlot cattle associated with exposure to high concentrate diets, and represent a significant economic and animal welfare burden on the beef cattle industry. In most conventional feedlot operations, nutritional management combined with antimicrobial metaphylaxis are used to reduce the incidence of liver abscesses. Increased scrutiny for the use of antimicrobials in food producing animals has increased consideration for judicious antimicrobial use, increasing the need to evaluate alternative strategies for the treatment of liver abscesses in beef cattle. A block randomized clinical trial was conducted to evaluate the effects of a *Saccharomyces cerevisiae* fermentation product (SCFP) on liver abscess prevalence, cattle health, and production in steers raised without antibiotics in a commercial Colorado feedlot. To characterize the effects of SCFP on microbial ecology and antibiotic resistance genes, the fecal microbiome and resistome were characterized using 16S rRNA gene and shotgun metagenomic sequencing. Crossbred steers (n = 4,689) were randomly allocated into two groups: the control group of cattle received a basal ration only, and treatment group of cattle received a basal diet supplemented with SCFP at 17.8 g/head/day. At arrival, cattle were sorted

[1] Katherine L. Huebner, Jennifer N. Martin, Carla J. Weissend, Katlyn L. Holzer, Jennifer K. Parker, Steven M. Lakin, Enrique Doster, Margaret D. Weinroth, Zaid Abdo, Jessica L. Metcalf, Ifigenia Geornaras, Paul S. Morley, Keith E. Belk

by weight and vendor source into pens within a block (N = 28 pens). Prior to harvesting the cattle, fecal samples were collected from the pen floor and composited from multiple individuals to characterize the composition and diversity of microbial communities and antimicrobial, metal, and biocide resistance genes. At slaughter, liver abscess severity was scored on individual cattle using a standardized scoring system. There were no statistical differences detected in liver abscess prevalence, feedlot production variables, animal health variables, composited fecal microbiome or resistome by treatment group; however, block, determined based on cattle weight and source at trial enrollment—had a large influence on both. Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes, and Tenericutes were the most abundant phyla detected in fecal composites. The most abundant drug class of antimicrobial resistance included tetracycline ribosomal protection proteins and macrolide resistance efflux pumps. Supplementation with SCFP in the diet of feedlot cattle raised without antibiotics did not have statistically detectable differences on any measured outcomes; however, the differences between the blocks, or enrollment groups of cattle, raise interesting questions about the influence of cohabitation, diet, environmental and seasonal factors on cattle fecal microbial ecology.

Importance

Societal concerns regarding antimicrobial resistance and treatment failure in humans have driven the beef cattle industry to evaluate alternative approaches to treat cattle diseases in feedlot operations, including liver abscesses. However, removal of antimicrobials and exposure to new feed additives during the finishing period has unknown implications on populations of pathogenic and non-pathogenic microbes and their associated resistance genes, or resistomes. This randomized clinical trial characterized the effects of SCFP on animal production, animal health, liver abscess prevalence while also quantifying effects on the fecal composite microbial

ecology and resistome. There were no significantly detectable differences on any of the tested outcomes in this study population, which is informative for pre-harvest feeding strategies in cattle raised to produce natural beef. However, it may be possible that detectable SCFP treatment effects were obscured by the significant variation in microbiomes and resistomes for cattle within blocks, which could be due influence of cattle source, feedlot location, season of harvest, or other undocumented environmental factors. Further work is needed to understand factors that increase susceptibility to liver abscesses in feedlot cattle, including our understanding of the disease pathogenesis.

INTRODUCTION

Liver abscesses in cattle have a detrimental impact on animal growth and variety meat value, resulting in significant economic loss to the beef cattle industry (Brown and Lawrence, 2010; Nagaraja and Lechtenberg, 2007; Reinhardt and Hubbert, 2015; Rezac et al., 2014b). The recent National Beef Quality Audit-2016 reported that incidence of liver abscesses increased in feedlot cattle from the last audit (Eastwood et al., 2017). Liver abscesses are associated with exposure of feedlot cattle to diets that are high in fermentable carbohydrates (i.e., starches), leading to clinical and subclinical ruminal acidosis and rumenitis that lead to liver abscess formation (Amachawadi and Nagaraja, 2016; Nagaraja and Chengappa, 1998). Common management practices used to prevent and reduce the incidence of liver abscesses include nutritional management and inclusion of antibiotics in the feed or water. In the U.S., six antimicrobials are approved for the reduction of liver abscesses, however, tylosin phosphate (a macrolide antibiotic) fed at low inclusion doses is the most commonly used drug for this purpose. Over 70% of cattle in large commercial beef feedlots in the U.S. are reported to receive metaphylactic treatment of tylosin during the feeding period (USDA–APHIS–VS–CEAH–

NAHMS). The impact of tylosin exposure on enteric microbial ecology and antimicrobial resistance (AMR) in cattle is not well understood, yet regulatory changes are already in effect regarding its use. For instance, the use of medically important drugs, such as macrolides, recently came under veterinary oversight with the enactment of the Veterinary Feed Directive (FDA, 2013). Regulation of tylosin use as a feed additive throughout the entire duration of the finishing period of beef cattle may be limited in the future. As a result, the beef cattle industry needs to identify safe, effective, non-antimicrobial strategies to prevent liver abscesses in natural and conventional beef operations. There is also increasing demand for non-antimicrobial therapies in feedlot operations raising cattle with high susceptibility to liver abscesses, including cattle raised without antibiotics and dairy steer breeds (Amachawadi and Nagaraja, 2016).

Several non-antimicrobial strategies have been evaluated for their ability to control the incidence of liver abscesses, including the use of sunflower seeds (Gibb et al., 2004; Mir et al., 2008), essential oils (Elwakeel et al., 2013; Meyer et al., 2009), and vaccines (Checkley et al., 2005; Fox et al., 2009). *Saccharomyces cerevisiae* fermentation products (SCFP) are feed additives proposed to stimulate rumen microbial fermentation in cattle exposed to high-concentrate diets by providing amino acids, peptides, organic acids, and oligosaccharides that benefit rumen health and starch breakdown (Callaway and Martin, 1997; Mullins et al., 2013; Yang et al., 2015). Inclusion of SCFP in the diet of cattle has been demonstrated to improve performance characteristics, including increased milk production (Poppy et al., 2012), increased volatile fatty acid concentration, decreased rumen pH (Yoon and Stern, 1996), improved feed efficiency and carcass traits (Wagner et al., 2016b). Other studies have reported reductions in food safety pathogens and AMR in cattle supplemented with SCFP, including lymph node carriage, virulence and resistance of *Salmonella* spp. (Feye et al., 2016). A clinical trial

conducted within a conventional feedlot demonstrated a slight reduction of liver abscess prevalence in heifers fed SCFP alone compared to a heifers administered tylosin and monensin in the finishing ration, but this effect was not shown to be significant (Scott et al., 2017).

Given potential advantages of dietary inclusion of SCFP on cattle performance and liver abscess prevalence, a block randomized clinical trial was performed to evaluate effects of SCFP supplementation to reduce beef cattle liver abscess prevalence in a 'natural' beef production system. The objectives of this study were to evaluate beef feedlot cattle regarding effects of SCFP supplementation on 1) prevalence and severity of liver abscesses, 2) animal health, and feedlot performance variables, 3) fecal microbiome composition and diversity, and 4) fecal resistome.

METHODS

Study methods overview

A block-randomized clinical trial was designed to evaluate the efficacy of SCFP supplementation in comparison to a control ration in the finishing rations of beef cattle raised in a 'natural' beef production system without the use of antimicrobials or growth promotors. Upon enrollment to a Northern Colorado feedlot, steers were individually weighed. Cattle were randomly assigned to receive either an SCFP treatment or control ration within a block. The blocks consisted of cattle that had equal weight and cattle vendor source distributions. The cattle were fed within their pen until they reached a market weight for slaughter. Animal health and feedlot performance data were collected and analyzed at the pen level. Composited fecal samples (which we will also refer to as fecal samples in this paper) were collected from the pen floor prior to harvest, and the microbiome and resistome were characterized using 16S rRNA gene sequencing and shotgun metagenomic sequencing. Liver abscess scores, classified using a

standardized score system by abscess severity level, were measured on individual cattle and compiled at the pen level for analysis.

Clinical trial design

Steers (n = 4,689) were enrolled into the trial using a randomized, block-controlled design in a commercial feedlot in Northern Colorado. Upon arrival, cattle were individually weighed using a hydraulic chute, then sorted into a home pen. Cattle were then sorted into two adjacent pens based on equal weight distribution. Pens were of equal dimension and cattle stocking density to comply with standard management practices for this feedlot. Consistent with standard feedlot design for this region, animals were housed in open-air, dirt-floor pens with a central feed alley. Using a pre-assigned randomization spreadsheet, cattle placed in adjacent pens were randomly assigned to receive either the treatment diet supplemented with SCFP or the control ration. The treatment and control pen designated one block. Each block consisted of cattle with similar source location, breed, headcount, and weight distributions (N = 28 pens).

Study population and cattle handling

All procedures involving animals were approved by the Colorado State University Research Integrity and Compliance Review Office, and were determined to be exempt from IACUC oversight. Cattle eligible for enrollment were crossbred, yearling steers purchased domestically during March through June 2016. Cattle were purchased directly from producers located across multiple western states, and cattle were reared to produce beef products raised without antimicrobials or growth promotors. Cattle were handled using standard production practices. At arrival, all cattle were administered injectable and oral anti-parasitic treatments (Noromectin, Norbrook Laboratories, Northern Ireland; Synanthic, Boehringer Ingelheim; Standguard, Elanco Animal Health, Greenfield, IN). All cattle were also administered injectable

bacterin-toxoid vaccines protecting against bovine respiratory disease complex agents (including Infectious Bovine Rhinotracheitis virus, Bovine Virus Diarrhea virus, Bovine Parainfluenza$_3$, Bovine Respiratory Syncytial virus, *Mannheimia haemolytica*, and *Pasteurella multocida*) and clostridial diseases (*Clostridium chauvoei*, *Clostridium novyi*, *Clostridum Sordelli*, *Clostridum perfringens* types C & D) (Titanium 5+PH-M, Elanco Animal Health, Greenfield, IN; Vision 7, Intervet/Merck Animal Health, Omaha, NE), Lastly lot ear tags were inserted to uniquely identify cattle grouped within each pen.

Treatment and control pens of cattle within block were handled identically for all cattle movements, feed delivery, bunk management, and daily health inspection. Basal diets in both treatment and control groups consisted of predominantly steam flaked corn. The treatment diet contained the addition of SCFP product (NaturSafe, Diamond V, Cedar Rapids, IA) fed at 17.8 g/d, using dried distillers grain as the carrier (**Table 2.1)**. Cattle were fed twice daily on a consistent schedule. The feed mill and feed truck equipment were sanitized prior to feeding the treatment diet to minimize contamination, both before and after the treatment pens.

Table 2.1: Dry matter ingredient and chemical composition for basal finishing diets averaged over the feeding period.

Ingredient	DM Inclusion, %
Steam-flaked corn	63.1
Corn silage	12.6
Grass hay	1.9
Corn stalks	0.2
WDG	5.1
DDG[1]	8.7
Whey delactose permeate	1.8
Supplement	4.0
Vegetable oil	2.6
Feed additive[2]	
Chemical Composition	
Crude Protein, %	14.7
NDF, %	16.2
Ca, %	0.5

P, %	0.4

[1]The *Saccharomyces cerevisiae* fermentation product (SCFP; NaturSafe, Diamond V, Cedar Rapids, IA) treatment diet contained a separate supplement at 3.2% (dry matter basis (DM)) comprised of Dried Distillers grains (DDG) as the carrier and SCFP; this supplement displaced DDG in the basal diet.

[2]SCFP was fed at 17.8 g/d (vs. target of 18.0 g/d; 1,603 g/ton DM basis) for the treatment group.

The cattle were inspected daily for overt signs of illness by trained animal health personnel under supervision of a licensed veterinarian. All observers involved in cattle husbandry were masked to treatment group assignment throughout the duration of the trial. Sick cattle requiring treatment with antibiotics per feedlot protocol were removed from the study pen permanently. Morbidity and mortality data were recorded with classifications for the cause of illness or death (respiratory diseases, digestive diseases, or other diseases). Cattle housed together in a pen were shipped for harvest based on standard procedures used by this feedlot to determine when they have reached market weight (1400-1500 lbs), including average daily intake, estimated weight, market price, slaughter plant needs, and visual assessment. Treatment and control pens of cattle within each block were shipped on the same day, corresponding to the same order that they were randomly enrolled into the trial.

Sample and feedlot data collection

One day before harvest, a composite sample of fresh feces was collected from the pen floor for each pen within a block (n = 28 fecal samples). Composite fecal samples were collected using previously described methods (Noyes et al., 2016b; Yang et al., 2016). Briefly, samples from fresh fecal pats (~30 g each) were collected using a clean, gloved hand from 12 areas along crossing diagonals of the pen and combined in a sterile bag (Whirl-Pak, Nasco, Fort Atkinson, WI).

Computerized feedlot animal health data and averaged pen weight records from pen allocation to slaughter were collected, collated, and verified in a spreadsheet program (Microsoft Excel) by the manager of research at the feedlot. Individual animal weights were aggregated through calculation of the arithmetic mean at the pen-level weight. Average daily gain per pen of cattle was calculated by taking the difference of pen-averaged slaughter weight and starting weight divided by the number of days on feed. Dry matter intake to gain was calculated by dividing daily dry matter intake of feed by average daily gain. Crude morbidity attack rate was calculated as the total number of animals that were examined because they were judged to be sick, whether they received treatment or not, divided by the initial number of animals in the pen. Crude mortality was calculated as the total number of animals that died, whether they received treatment or not, divided by the initial total number of animals in the pen. Cause-specific morbidity attack rates for disease categories (respiratory diseases, digestive diseases, or other causes of disease), were calculated as the total number animals treated for a specific disease classification, divided by the initial number of animals in the pen. Cause-specific mortality attack rates for disease categories (respiratory diseases, digestive diseases, or other causes of death), were calculated as the total of animals that died, whether they died in the pen or after treatment, divided by the initial number of animals in the pen.

At harvest, cattle were humanely euthanized using standard beef processing protocols at a commercial abattoir located in Colorado. Identity of the cattle treatment assignment within pen was maintained at slaughter. Individual cattle liver abscess incidence and severity were classified by a trained observer using a modified Elanco Liver Check System (Elanco Animal Health, Greenfield, IN) as previously described (Rezac et al., 2014b). Briefly, the external surfaces of livers were observed visually for abscesses. Livers without visible abscesses were scored as

normal. Livers with resolved abscess scars or that contained ≤ 2 abscesses with diameters ≤ 2-cm were scored as an A-minus liver. Livers with 2 to 4 abscesses with diameters 2 to 4-cm were scored as an A liver. Livers with ≥ 1 abscess that was > 4-cm in diameter, or ≥ 4 abscesses ≥ 2-cm in diameter, abscesses adhered to the diaphragm, abscesses adhered to other organs, abscesses adhered to the abdominal wall, or abscesses that were ruptured or open were scored as A-plus livers. Other pathological liver abnormalities were not scored or considered for this study.

Sample processing and DNA isolation

Fecal composite samples were transported to the Colorado State University's Center for Meat Safety and Quality microbiology laboratory in insulated containers within one hour of collection. Composite fecal samples were manually homogenized, weighed into aliquots, and stored at -80°C.

Composited fecal sample DNA was extracted using the PowerMax Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) with modifications as previously described (Yang et al., 2016). Briefly, before extraction, 10 g of sample were thawed at 4°C. A sedimentation step was used to process the feces prior to DNA extraction, allowing for sedimentation of heavier debris, removal of large particulates and inhibitors, and release of bacterial cells into the supernatant. The sample was mixed with 30 ml buffered peptone water (BPW; Difco, Beckton Dickinson and Company) and allowed to separate into two layers. The top layer was centrifuged (4300 x g, 10 min), and the pellet rinsed with molecular-grade sterile phosphate buffered saline (PBS) and centrifuged again to pellet cells. The supernatant was removed, the resulting pellet was resuspended in PowerBead solution, and the remainder of the standard DNA extraction kit protocol followed. As per the manufacturer's instructions, DNA was eluted in 3 ml of C6 elution buffer.

To maximize DNA yield, the eluate was passed through the membrane filter twice. Samples were concentrated using standard ethanol-sodium acetate precipitation techniques. To the DNA sample, 10% volume (300 µl) of 3 M sodium acetate (pH 5.0), along with 200% volume of 100% cold molecular grade ethanol (6.6 mL), were added. The sample was mixed by inversion, incubated at -20°C for ≥ 30 minutes and then centrifuged at 11,000 x g for 20 min at 4°C. Supernatant was carefully discarded and the DNA pellet air dried before suspension in 150 µl of C6 elution buffer.

Library preparation and sequencing

16S rRNA gene amplification and sequencing for composited fecal samples was performed by a commercial sequencing company (Novogene Corporation, Beijing, China). Following DNA extraction, 1500 ng, 30 µl aliquots for each sample of purified DNA were delivered to the sequencing facility. DNA from the V4 region of the 16S rRNA gene was amplified using the primer set 515F/806R (Caporaso et al., 2012), with reverse primers containing unique barcode sequences. Library sequencing (paired-end, 2x250 bp) was performed on an Illumina HiSeq 2500 instrument and loaded into the sequencer according to the manufacturer's instructions (Illumina, San Diego, CA).

Composited fecal samples were also sequenced using shotgun metagenomics techniques. Following DNA extraction, 3 µg, 50 µl aliquots for each sample of purified DNA were delivered to the University of Colorado Genomics and Microarray Core (Aurora, CO) for sequencing. Genomic libraries for all samples were prepared using commercial kits (TruSeq DNA PCR-Free Library Prep Kit, Illumina, San Diego, CA), following the manufacturer's protocol to obtain an average insert size of 350 bp. Library sequencing was completed on the Illumina HiSeq 4000 instrument with 17 samples loaded into each lane, Mode V4 chemistry and paired-end reads of

150 nucleotides in length. Sequencing data were transferred electronically to a computer server at Colorado State University.

16S rRNA gene bioinformatics and statistical analysis

The 16S rRNA gene forward and reverse reads were imported into Qiime2-2017.12. The DADA2 pipeline (Callahan et al., 2016) was used for detecting and correcting Illumina amplicon sequences, removal of primers and chimeric reads, and assembly into sequence variants (SV). The DADA2 pipeline has increased resolution compared to customary construction of Operational Taxonomic Units (OTUS), resolving sequences exactly to the level of single nucleotide differences over the sequenced gene region (Callahan et al., 2017). Implemented in the q2-dada2 plugin, the parameters that were used in quality filtering included primer trimming (--p-trim-left-f-19 and –p-trim-left-r-20), and --p-trunc-len 200, which truncates each sequence at position 200, to remove low quality sequence less than a Phred score of 30. Taxonomy was assigned using a Naïve Bayes classifier trained on the Greengenes 13_8_99% database, where the sequences have been trimmed to only include 250 bases from the V4 region of the 16S rRNA gene that was sequenced in this analysis. Sequences classified as chloroplasts or mitochondria were filtered, and sequences with a count less than 10 were filtered out of the dataset, and assumed to be spurious. Taxonomic composition of the microbiome was visualized using a bar plot at the phylum, class, order, and family level. A rooted phylogenetic tree was created through *de novo* multiple sequence alignment using the MAFFT program, version 7 (Katoh and Standley, 2013), highly variable positions were removed to decrease noise in the tree, and FastTree-2 was applied to generate a phylogenetic tree from the masked alignment (Price et al., 2010).

Alpha and beta diversity metrics, statistical testing, and interactive visualizations were generated using q2-core-metrics-phylogenetics metric plugin. In this step, the feature table was

rarefied (subsampled without replacement to even sampling depth) to the level of the lowest sequence depth, 65,000 sequences, allowing retention of all 28 samples. Beta diversity analysis using the distance matrix generated from the weighted UniFrac phylogenetic metric (Lozupone et al., 2006) was visualized in a principle coordinates analysis (PCoA), using the software program, Emperor for plot generation (Vázquez-Baeza et al., 2013a). Alpha diversity was estimated using the Shannon diversity metric and was visualized in box whisker plots. To visualize alpha diversity as a function of sequencing depth, rarefaction curves were generated for each sample and samples were rarefied from a minimum depth of 10 to a maximum of 80,000 reads, a step size of 100 reads, and 5 iterations for each subsampling using the q2-diversity alpha-rarefaction plugin.

Testing for significant effects of categorical metadata, including treatment group, on alpha and beta diversity metrics were performed on rarefied feature tables (as described above). Differences for alpha diversity were determined using non-parametric Kruskall-Wallis test. Beta diversity was assessed for statistical differences using the unweighted Unifrac phylogenetic distance metric with the permutation-based statistical test, PERMANOVA (Anderson, 2001). Differential abundance testing was performed using Analysis of Composition of Microbiomes (ANCOM) procedure (Mandal et al., 2015). To meet the assumption for ANCOM that few (less than ~25%) of SV change between groups, SV not present within 10% of samples were filtered out. Since ANCOM does not tolerate zero frequencies, an arbitrary pseudocount of 1 was added to the feature table across all SV.

Shotgun metagenomic bioinformatics and statistical analysis

The AmrPlusPlus pipeline, a previously described for AMR analysis (Lakin et al., 2017), was utilized to identify and characterize AMR at the class, mechanism, and group level of

classification from the shotgun metagenomics data (v.1.20.1). The pipeline was executed using Nextflow programming language (v.0.26.0) (Tommaso et al., 2017), for which installation and documentation are publicly available (http://megares.meglab.org/amrplusplus). The pipeline consists of several steps, including quality control, resistome characterization, and microbiome characterization. The shotgun metagenomic sequence data were filtered and quality controlled using Trimmomatic (v0.36) (Bolger et al., 2014). Host (bovine) genomic DNA contamination was removed by aligning the quality controlled reads to the *Bos taurus* genome (NCBI accession AC_000158.1) using the Burrows Wheeler Aligner (BWA-MEM with default settings) (Li, 2013), and then removing the *B. taurus* genome using SAMtools (Li et al., 2009). Remaining reads were aligned to the MEGARes AMR resistance database (v1.01) and select, hand-annotated genes from the BacMet database using BWA-MEM (Lakin et al., 2017; Pal et al., 2014). An 80% gene fraction threshold (i.e., 80% of the full length of each AMR gene accession within each sample) was applied to identify potential positive AMR gene accessions in the samples. A small number of the gene accessions within the MEGARes reference database encode for resistance determinants that modify antimicrobial drug targets, i.e., elongation factor proteins. In some circumstances, these genes are closely related to cellular housekeeping genes, differing by only one single nucleotide polymorphism (SNP). A conservative approach to evaluating these gene accessions was to visually confirm 100% coverage of the reads to the central 95% portion of the reference gene nucleotide sequence. This is a conservative approach to prevent false positive gene accession classifications in the analysis, and visualization of coverage was performed using Integrative Genomics Viewer (Robinson et al., 2011). Overall, 33 gene accessions required SNP confirmation, but only 3 of these fit the confirmation criteria, so they were excluded from analysis.

The population count data for AMR determinants was obtained from the bioinformatics pipeline, and were normalized using cumulative sum scaling and analyzed using zero-inflated Gaussian mixed-model regression as implemented in the "metagenomeSeq" R package (v1.20.1) (Paulson et al., 2013), with treatment as a fixed effect and block as a random effect. Measures for alpha diversity were calculated on data normalized to the lowest sample size using the "vegan" package (v.2.4-6) for the R programming language (Dixon, 2003). Ordination was performed using Non-Metric Multidimensional Scaling (NMDS) with Bray-Curtis distance as implemented in the "vegan" package. Log-fold changes and *P*-values for regression coefficients were calculated by calculating pair-wise contrasts between treatment levels. *P*-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (Benjamini and Hochberg, 1995). Similar to the analysis presented in (Noyes et al., 2016b), the resistome from composited fecal samples in this study was screened for selected high profile resistance genes and drugs of great concern to the scientific and medical community. The count table was evaluated for alignments to the following gene accession classifications: class A beta-lactamases (SME, IMI, GES, KPC, TEM, SHV, CTX, CMY), class B beta-lactamases (NDM, CPHA), class C beta-lactamases (CMY), macrolide-streptogramin-lincosamide drug resistance genes (VGA, VGAB, VGAD, VATA, VATB, VATC, CATD, VATE), and phenicol resistance genes (CFRA). Database files, analytic data files, and analytic code are available at the following repository: (to be included in final manuscript using a public GitHub repository).

Feedlot health, production and liver abscess statistical analysis

All data were analyzed blind to treatment allocation (SAS, release 9.4; SAS Institute, Cary, North Carolina, USA). Data were analyzed using pen as the experimental unit. Animal health variables included in the analysis were attack rates for crude and cause-specific morbidity

and mortality classifications, and liver abscess classifications. These variables were compared between treatment groups using mixed-effects Poisson regression, with pen specified a repeated measure within blocks. Feedlot production variables included in the analysis were dry matter conversion (feed to gain ratio), dry matter intake (lbs/head/day), average daily gain (lbs/head/day, and days on feed). For all production variables, the arithmetic mean at the pen level was calculated, and performance variables were compared between experimental groups in a generalized linear model adjusting for pen and block structures. Least squares means were considered significantly different if P was ≤ 0.05.

RESULTS

Animal health, production performance, and liver abscess rates

In total, 4,689 steers were enrolled in the study; 2,345 steers within 14 pens were administered the control diet, and 2,344 cattle within 14 pens were administered the SCFP diet. The average pen size in this trial contained an average of 167 cattle (range of 104 to 225 head per pen). Overall, 41 steers enrolled in the trial died, including both steers removed from the pen for treatment that died in addition to cattle found dead within the pen. Overall, 362 steers required antimicrobial treatments for disease in compliance to the feedlot treatment protocols, and those animals were removed from the trial. Controlling for population structure, there were no differences detected in production or animal health variables detected between pens of cattle receiving the SCFP treatment and control rations (**Table 2.2, Table 2.3;** $P > 0.05$). Across all trial pens, the duration of days on feed varied, but days on feed did not change between SCFP treatment and control groups (average, 205 d; range 171 to 262 d). There were too few morbidity events to allow for comparisons between morbidity attack rate in the digestive diseases or between mortality attack rates for respiratory disease or other disease classifications.

Table 2.2: Baseline and production performance data summary, averaged at the pen-level.

	Experimental Group[1]			
Variable[2]	Control (n = 28)	Treatment (n = 28)	Standard Error[3]	P value
Days on feed	203.90	205.60	6.99	0.81
Daily dry matter intake (lbs/animal/day)	22.21	22.21	0.32	0.99
Average daily gain (lbs/animal/day)	2.90	2.87	0.06	0.74
Dry matter intake:gain	7.69	7.75	0.13	0.65

[1]See text for description of the treatment protocols.
[2]See text for pen level production variable calculations.
[3]Calcuated using standard generalized linear model of pen averages for experimental group effects and correcting for intra-pen clustering within block.

Table 2.3: Animal health data summary[1].

Experimental Group[2]	Control (n = 2345)			Treatment (n = 2344)			
Animal Health Variable	Adjusted risk (%)	95% CI[3]	n	Adjusted risk (%)	95% CI	n	P value
Crude Morbidity	7.2	5.4 - 9.6	168.0	8.3	6.0 - 11.3	194.0	0.51
Respiratory	4.6	3.4 - 6.4	109.0	5.7	3.8 - 8.5	134.0	0.44
Digestive	0.0	N/A	0.0	0.0	N/A	0.0	N/A
Other Causes	0.9	0.4 - 1.9	21.0	0.6	0.2 - 1.5	14.0	0.51
Crude Mortality	0.7	0.5 - 1.2	17.0	1.0	0.8 - 1.3	24.0	0.17
Respiratory	N/A[4]	N/A[4]	0.0	N/A[4]	N/A[4]	4.0	N/A[4]
Digestive	0.3	0.1 - 0.5	7.0	0.5	0.3 - 0.7	11.0	0.14
Other Causes	N/A[4]	N/A[4]	10.0	N/A[4]	N/A[4]	9.0	N/A[4]

[1]See text for animal health variable calculations and protocols for handling of sick or treated animals.
[2]See text for description of the treatment protocols.
[3]Calcuated for each adjusted rate using Poisson regression in a log linear model for experimental group effects and correcting for intra-pen clustering within block with generalized estimating equations.
[4]For death from respiratory or other causes, the model would not converge due to the small number of events.

In total, 4,324 individual cattle were scored for liver abscess presence and severity. The total adjusted risk of cattle having liver abscesses, at any severity level, was 38.9% (95% CI = 38.0 to44.5) and 38.1% (95% CI = 31.4 to 46.2) for cattle fed the control ration and cattle fed the SCFP ration, respectively (**Table 2.4**). Controlling for population structure, cattle receiving the control ration did not have an increased risk of having a liver abscess compared to the SCFP ration ($P = 0.79$). The relative risk of cattle having a severe (A-plus), moderate (A) or minor (A-minus) liver abscess did not change between treatment and control groups ($P > 0.05$).

Table 2.4: Liver abscess prevalence by grade and treatment group.

Experimental Group[1]	**Control (n = 2171)**			**SCFP Treatment (n = 2153)**			
Liver Abscess Score[2]	**Adjusted risk (%)**[3]	**95% CI**	**n**	**Adjusted risk (%)**	**95% CI**	**n**	***P* value**
A-minus	17.6	15.0 - 20.6	382.0	17.4	15.0 - 20.2	375.0	0.93
A	5.7	3.9 - 8.5	122.0	5.1	3.3 - 7.8	106.0	0.26
A-plus	15.2	14.1 - 16.5	341.0	15.3	14.1 - 16.5	337.0	0.98
Total abscesses	38.9	34.0 - 44.5	845.0	38.1	31.4 - 46.2	818.0	0.79

[1]See text for description of the treatment protocols.
[2]Gross liver pathology at slaughter: normal livers; A-minus livers had resolved abscess scars or $\leq$ 2 abscesses, $\leq$ 2-cm in diameter; Livers with 2 to 4 abscesses, 2 to 4-cm in diameter were scored as A. Livers with $\geq$1 abscess, $\geq$ 4-cm in diameter, or > 4 abscesses, > 2-cm in diameter, abscesses adhered to the diaphragm, adhered to other organs or the abdominal wall, abscesses that were ruptured were scored as A-plus abscesses.
[3]Calcuated for each adjusted rate using Poisson regression in a log linear model for treatment group effects and correcting for pen clustering within block with generalized estimating equations.

Microbiota composition and diversity

High quality sequences totaling 13,749,784 reads and representing 28 composited fecal samples were imported into Qiime2-2017.12. Following quality control in the DADA2 pipeline, there were 11,572,084 sequences remaining that made up 11,197 SV. Overall, there were 8 SV that classified to chloroplasts of plant origin and mitochondrial sequences. Following filtering and removal of these sequences, there were 11,571,241 total SV remaining across 28 samples, which were utilized for downstream analysis (Read count summaries, by sample, at each processing step are shown in **Appendix A; S.Table 2.1**). To visualize species richness as a function of sequencing depth, an alpha rarefaction curve was generated; the plateau of the curve began at approximately 200,000 reads for most samples, indicating that samples were sequenced to adequate depth to allow evaluation of microbial diversity (**Figure 2.1**).

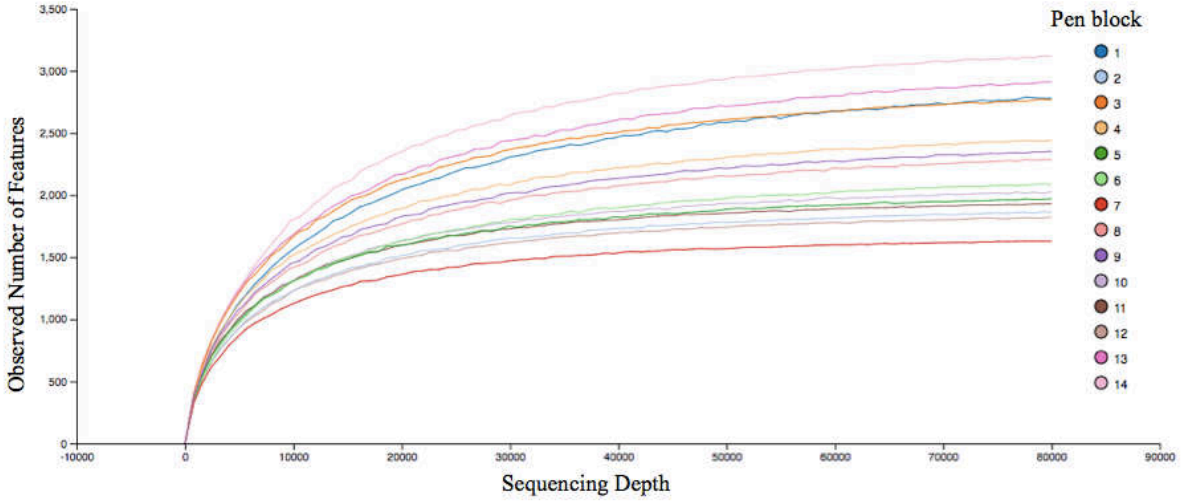

Figure 2.1: Alpha rarefaction curve for the observed species in composited fecal microbial communities. Each curve represents an experimental block. The rarefaction curve was generated using the numbers of exact SVs richness estimation. Samples were rarefied at an even depth of 80,000 sequences per sample.

The fecal microbiome of samples collected from both treatment groups across 28 samples was dominated by the phyla Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes, and Tenericutes (**Figure 2.2**). At the family level, taxonomic composition of fecal microbiomes in both groups was dominated by Ruminococcaceae, Lachnospiraceae, Bacteroidaceae, and Paraprevotellaceae. Rare phyla that accounted for < 1 % of all read counts across 28 samples included Actinobacteria, Acidobacter, Fibrobacteres, Verrucomicrobia, Cyanobacteria, Nitrospirae, Chloroflexi, Gemmatimonadetes, Planctomycetes, Euryarchaeota (kingdom Archaea), WS3, Elusimicrobia, Fusobacteria, TM7, WPS-2, Armatimonadetes, Chlamydiae, Chlorobi, AD3, OP3, TM6, Lentisphaerae, Synergistes, FCPU426, GN04, WWE1, Deferribacteres, BRC1, NC10, FBP, Caldithrix, Thermi, OD1, Crenarchaeota (kingdom Archaea), GOUTA4, NKB19, GN02, SC4, OP8, BHI80-139, SBR1093, LCP-89 and OC31.

Differential abundance testing (ANCOM) revealed that SCFP-fed pens of cattle did not have distinct microbial composition compared to the control fed cattle, at any taxonomic level. However, differential taxa were detected among blocks, with blocks 4, 7, and 9 having variable abundance of the genus, *Lactococcus,* compared to the other blocks. Microbial diversity, estimated using the Shannon diversity index, did not differ between the SCFP treatment and control cattle (**Figure 2.3.A**; $P = 0.43$). Though not significant, Shannon alpha diversity varied among blocks, with lower diversity in block 14 (**Figure 2.3.B**; $P = 0.19$). Degree of differentiation in the microbial communities, or beta diversity, was not significantly different between treatment groups (**Figure 2.4.A**; $P = 0.99$); however, block accounted for the differences in fecal microbiomes, demonstrated by the clustering effect on the PcOA plot (**Figure 2.4.B**; $P = 0.001$). The first axis explained 38.8% of the variation, which was largely driven by microbial differences in block 14.

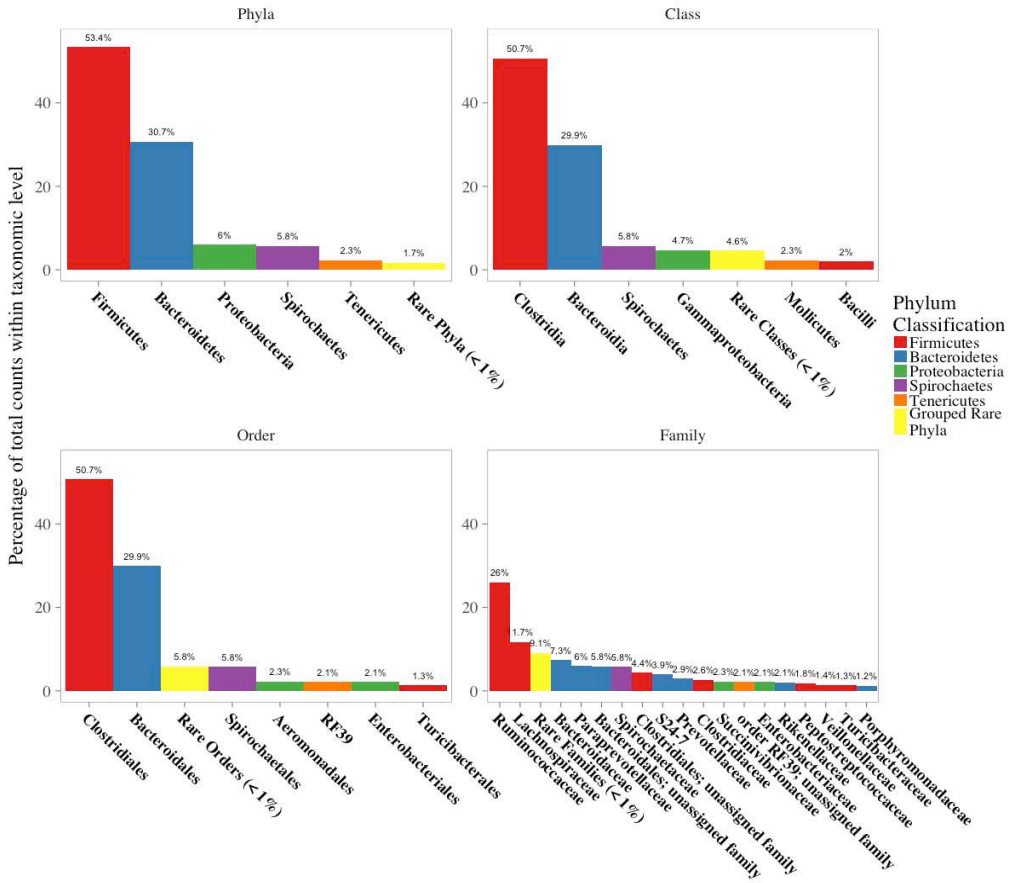

Figure 2.2. Relative proportions (count within taxonomic group / total count) of composited fecal microbiome of trial cattle at Phylum, Class, Order, and Family level across 28 composited fecal samples of trial cattle. Rare taxa (defined as having relative proportions <1%) were summed together and put into a distinct category at each level.

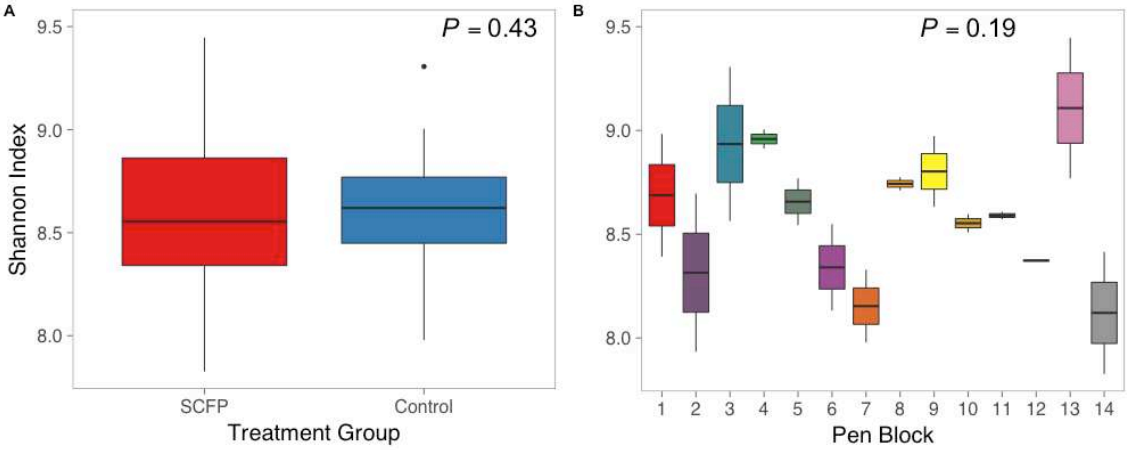

Figure 2.3: Shannon alpha diversity by treatment group (**A**) and block (**B**). Alpha diversity, or within-sample microbial diversity, did not differ by treatment group or block. Statistical differences were determined using non-parametric Kruskall-Wallis tests.

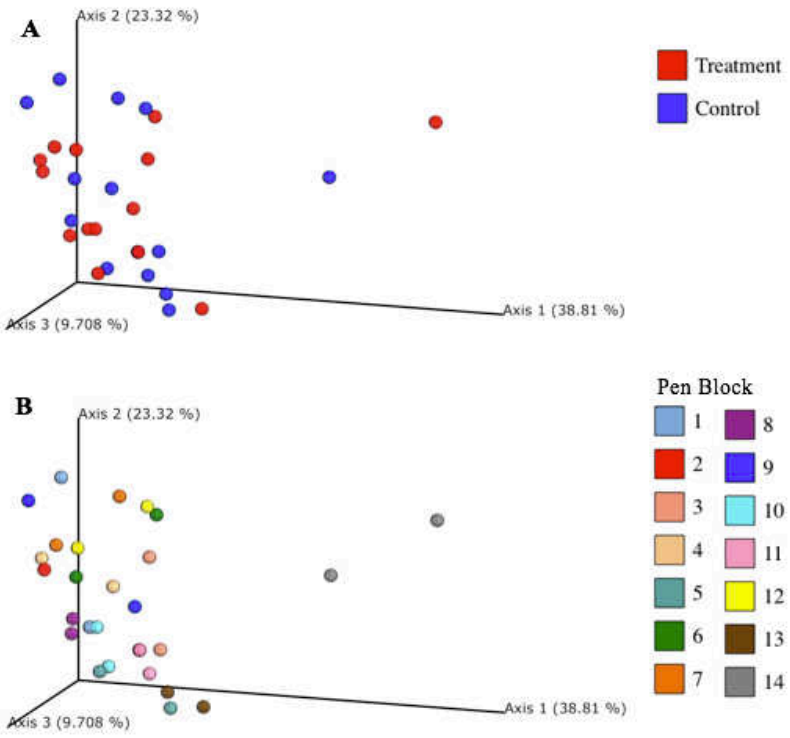

Figure 2.4: PcoA plot based on 16S rRNA amplicon weighted UniFrac distance by treatment group (**A**) and block (**B**). The microbial community composition of the composited fecal microbiome did not differ by treatment group, but did by block.

Resistome composition and diversity

Across the 28 composited fecal samples, shotgun sequencing generated 994,459,164 pairs of forward and reverse reads. The average quality score across reads by sample was 38 (range 36 to 38), indicating an error rate of 1.6 out of 10,000 base calls from sequencing. Following quality filtering and host (*Bos taurus* genome) removal, 895,733,367 read pairs remained. Of these, 1,610,843 read pairs mapped to the MEGARes and BacMet databases (**Appendix; S.Table 2.2**). There were no differences in the number of raw or trimmed reads or quality scores between the treatment and control groups, suggesting that there was no systematic bias in the sequencing effort between sample groups.

Overall, 151 unique gene accessions were identified (**Appendix; S.Table 2.3**). At the mechanism level, 0.27% of reads mapped to AMR determinants for metals and biocides and 99.73% of reads mapped to AMR determinants for antimicrobial drugs. At the class level, most reads mapped to AMR determinants for tetracycline resistance ribosomal protection proteins (62.3%) and macrolide resistance efflux pumps (25.6%). Of reads that mapped to AMR determinants for biocides, biocide resistance protein (*glpF*) and biocide resistance regulators (*rpoS, gadA* and *sugE*) were the most abundant. Of reads that mapped to AMR determinants for metal resistant mechanisms, most of them conferred resistance to multiple metals (**Appendix; S.Table 2.4**).

The composition and taxonomic richness of antimicrobial, metal and biocide resistance determinants did not differ by treatment group at expression levels high enough to infer biological significance at any taxonomic level (**Figure 2.5.A-B**). NMDS ordination did not show significant separation between the two treatment groups, at any taxonomic level (**Figure 2.6**). However, the resistome composition and richness differed when evaluating the resistome change between block (**Figure 2.7.A-B**), and this shift was observed at the Mechanism, Class, Group and gene accession levels.

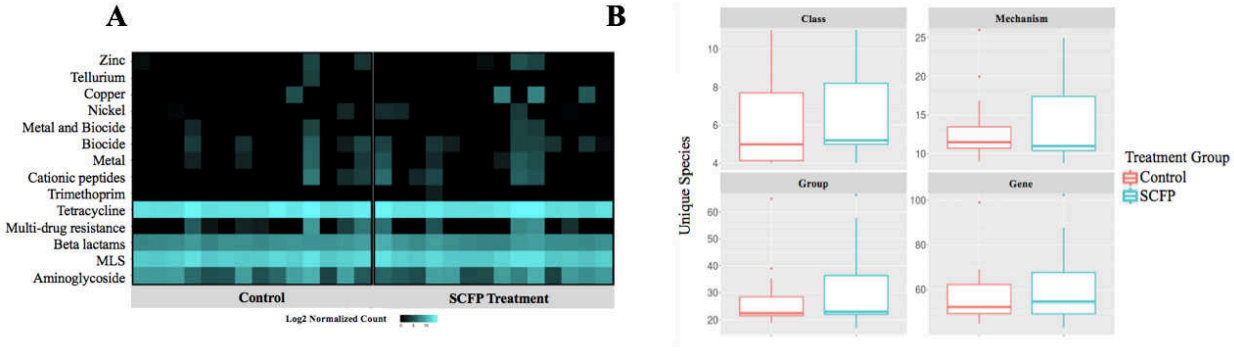

Figure 2.5: Heatmap of treatment comparison of composited fecal resistome of normalized counts of antimicrobial, metal and biocide resistance determinants, by drug class (**A**). Boxplot species richness comparison for composited fecal resistome at each taxonomic level, measured as unique species by inverse Simpson's index, by treatment group (**B**).

Ribosomal protection protein mechanisms comprised most of the alignments within the tetracycline resistance class, including *tetQ*, *tetO*, *tetW*, *tet32, tet44, tet40*, and *tetW*. Less abundant mechanisms within the tetracycline drug class included inactivation enzymes (*tetX*) and the major facilitator superfamily (MFS) efflux pumps (*tetA*, *tetB*). Macrolide efflux pumps (*MefA*) comprised most alignments within the macrolide, lincosamide, streptogramin resistance class, followed by 23S rRNA methyltransferase target modification genes (*ermq*), macrolide phosphotransferase genes (*mphb*), and the lincosamide nucleotidyltransferases (*lunc*). Within the aminoglycoside class, aminoglycoside O-nucleotidyltransferase mechanism (*ant9*) was most abundant. Within the beta-lactam class, the class A beta-lactamase (*cfx*) AMR mechanisms and groups were abundant. Of the critically important AMR resistance gene accessions screened, one sample representing a SCFP pen of cattle aligned to extended spectrum beta-lactamase (*ctx)*. However, the relative abundance was low, representing only 23 hits, or 0.038% of all hits in that sample.

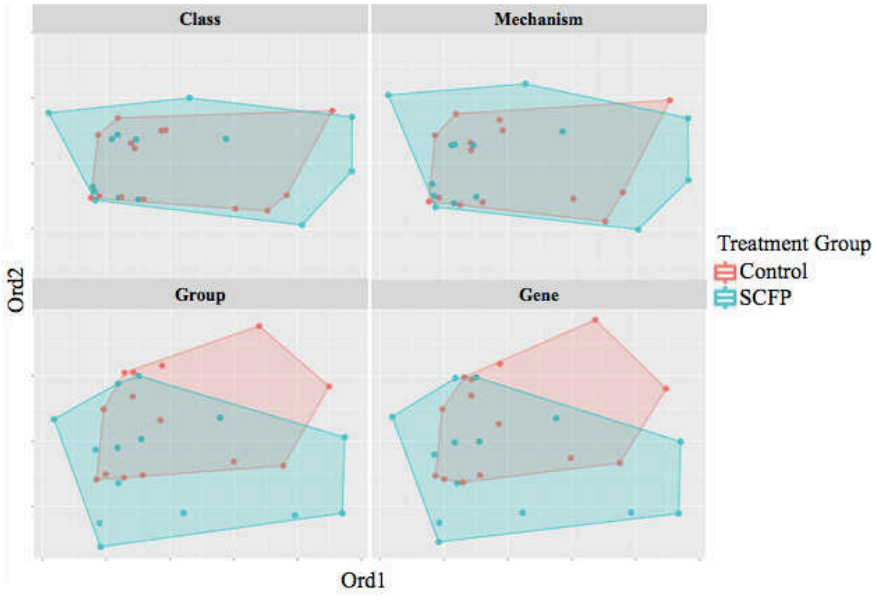

Figure 2.6: Non-metric multidimensional scaling (NMDS) ordination showing composited fecal resistomes at the class, mechanism, group, and gene accession level, by treatment group.

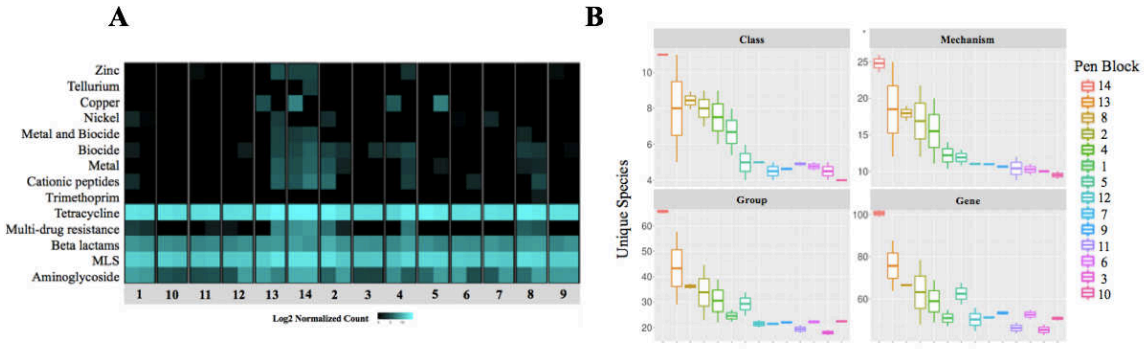

Figure 2.7: Heatmap of block comparison of normalized counts of antimicrobial, metal and biocide resistance genes, by drug class (**A**). Boxplot species richness comparison for fecal resistome at the class, mechanism, group, and gene level, measured as unique species by inverse Simpson's index, by block (**B**).

DISCUSSION

Using a block randomized clinical trial design, this study did not detect significant differences in any of the measured outcomes between the SCFP treatment and control diets. However, it was possible that the SCFP treatment effect may have been obscured by the significant variation in microbiomes and resistomes by block, driven by other factors including cattle source, feedlot location, and season of harvest. Overall, there was a high prevalence of liver abscesses in the study population (38%), which is approximately twice the national average for commercial beef cattle within the U.S (Eastwood et al., 2017). The increased occurrence of liver abscesses in this study population may be attributed to the lack of using prophylactic antibiotics, such as tylosin (Rezac et al., 2014b).

Supplementation of SCFP was hypothesized to reduce liver abscess prevalence and severity through modulation of the rumen microbiome to enhance fermentation during subacute acidosis in feedlot cattle exposed to high concentrate diets. However, no significant treatment differences were detected in the fecal microbiome, resistome or liver abscess prevalence. In dairy

cattle (Zhu et al., 2017), detected a SCFP dose response with SCFP supplementation on shifts of microbial populations for cellulolytic, lactate-utilizing and lactate-producing bacteria. However, the basal diet in that study was supplemented with up to 10 times higher levels of SCFP compared to the present study. Based on this, it is possible that the dose of SCFP in this clinical trial was below the minimal threshold for detection. Liver abscess severity in feedlot cattle may be related to population shifts in the bacteria adherent to the rumen epithelial lining, also known as "epimural" bacteria (Nagaraja and Chengappa, 1998; Narayanan et al., 1998; Rezac et al., 2014a). Given this mechanism for liver abscess formation during subclinical rumen acidosis, if SCFP altered the microbiome of solid or liquid rumen fractions, but not epimural microbial communities, the product may not have impacted the liver abscess prevalence or severity.

This clinical trial characterized the microbiome and resistome in pen composited feces, but other locations of the gastrointestinal tract were not sampled. The cattle gastrointestinal microbiome composition, diversity and abundance has previously been characterized as heterogeneous across segments of the gastrointestinal tract, with significant microbial shifts across samples collected from the rumen, small and large intestines, and feces (Mao et al., 2015; Plaizier et al., 2017; Thomas et al., 2017). In this study, composited fecal samples at the level of pen were evaluated, therefore the impact of SCFP treatment within other gastrointestinal compartments is not known. Future work directed towards evaluation alternative feed additive effects on the microbiome of cattle should be directed at evaluation of treatment effects within other gastrointestinal compartments, in addition to fecal samples.

The composition and richness of antibiotic, metal, and biocide AMR classes within the fecal resistome did not have detectable differences between treatment groups. However, there were several AMR classes detected within the resistome, despite no antimicrobial exposures to

this population of cattle raised for natural beef products. The predominant antimicrobial resistance classes detected within the feces of cattle were tetracycline resistance ribosomal protection proteins and macrolide resistant efflux pumps, beta-lactamases and aminoglycoside resistance. The high abundance of AMR determinants to these classes within the feces of feedlot cattle is consistent with other metagenomic analyses of feces collected from feedlot and dairy cattle operations (Noyes et al., 2016b, 2016a; Vikram et al., 2017). This study added to a growing body of evidence that there is baseline resistome in fecal contents of feedlot cattle. Interestingly, similar patterns have been described using metagenomics tools in human feces and waste (Feng Jie et al., 2017; Pal et al., 2016), in ancient arctic permafrost (D'Costa et al., 2011; Perron et al., 2015), and isolated cave environments (Bhullar et al., 2012). More baseline studies are needed to characterize the baseline resistome of feedlot cattle under different antimicrobial exposures to better understand the changes imposed by anthropogenic and agricultural impacts of antibiotic use on AMR in these systems.

One pen from this trial was positive for low numbers of alignments to extended spectrum beta lactamase gene accessions, *ctx*. The *ctx* gene confers resistance to third-generation cephalosporins (i.e., cefotaxime) and is classified as critically important in humans when expressed in disease-causing agents, such as *Escherichia coli* (Bonnet, 2004; Courpon-Claudinon et al., 2011; WHO, 2017). The cattle in this study were not treated with drugs within the cephalosporin class, so it is interesting that this mechanism for resistance was detected, despite a lack of direct antimicrobial exposure to this drug. This AMR gene was also detected in another study of feedlot cattle that characterized fecal resistome in populations raised without antibiotics, although the abundance was higher in the conventionally treated cattle comparison group (Vikram et al., 2017). This finding of *ctx* in this population of cattle not exposed to this drug

class further highlights the complexity of AMR dissemination and mechanisms in feedlot operations.

Although the primary objective of this trial was to evaluate the effect of SCFP treatment, the heterogeneity of the fecal microbiome and resistome differed significantly by block. At enrollment into the trial, cattle were randomly assigned to either a treatment or control pens within each block, such that blocks consisted of cattle groups that had equal distributions of weight, vendor source, location within the feedlot, and entry and harvest dates. Because these variables are confounded with block, it is not possible to determine which factor(s), if any, are driving the variation in microbiome and resistome by block. This result generates interesting questions about the influence of cattle cohabitation on the fecal microbiome and resistome in cattle housed together in pens. Numerous microbiome studies in both humans and animals have demonstrated that microbiomes are shaped by different factors including cohabitation, genetics, diet, age, and disease. One possibility for the 'block effect' observed this trial is that cohabitation of cattle originating from similar source populations could impact convergence of the microbiome over time in a pen. To evaluate this effectively, samples collected at arrival to the feedlot would be needed. In humans, genetically-related individuals, i.e., twins, share more of their gut microbes than unrelated individuals (Stewart et al., 2005). However, other studies have demonstrated that the shared environment can impact the microbiome of unrelated, cohabitating humans and animals, independent from genetic relatedness ((Song et al., 2013; Turnbaugh et al., 2009, Friswell et al., 2010). The effect of cohabitation on the cattle microbiome is not well characterized, however this effect could have major implications for cattle disease and feedlot management.

CONCLUSIONS

This study utilized a comprehensive approach for characterization of the microbiome and resistome in cattle treated with SCFP metagenomic tools in a rigorous randomized clinical trial design. However, metagenomics approaches have limitations, including the inability to estimate sample size to appropriately power study designs in feedlot clinical trials. Currently, there are no established methods for performing power calculations for metagenomics study designs, due to lack of baseline information about cattle microbiome or resistome effects under different management conditions and antimicrobial treatment exposures. As a result, it may be possible that the SCFP treatment effect may have been obscured by the significant variation in microbiomes across block, driven by other factors including cattle source, feedlot location, and season of harvest. Future study designs may be better powered for the ability to detect treatment differences in cattle exposed to diets containing different feed additives through use of more replicates at the pen or individual animal-level. Additionally, future work should be directed towards investigation of underlying microbiome factors influencing formation and severity of liver abscesses in cattle.

CHAPTER 3: CHARACTERIZATION OF MICROBIOME IN LIVER ABSCESSES FROM NATURAL COLORADO FEEDLOT CATTLE[2]

SUMMARY

Feedlot cattle exposed to high concentrate diets during the finishing period are prone to developing liver abscesses caused by the synergistic action of *Fusobacterium necrophorum* and *Truepurella pyogenes*. However, recent evidence from culture-independent techniques has suggested that microbial communities within liver abscesses contain diverse microbial communities, with unknown effects on cattle disease severity. The objective of this study was to characterize liver abscess microbial composition and diversity in beef cattle raised without antibiotics or growth hormones in a Northern Colorado feedlot. Liver abscess samples were collected from carcasses of feedlot cattle enrolled in a randomized-block clinical trial in Colorado that evaluated the effects of *Saccharomyces cerevisiae* fermentation products on liver abscess prevalence, cattle health and productivity, and fecal microbiomes and resistomes. Each clinical trial enrollment group, or block, consisted of one treatment and one control pen of cattle that shared equal distributions of cattle by weight, source location, feedlot placement location, and season of placement and harvest. The study did not demonstrate significant differences between treatment and control group on any of the measured outcomes, but there were differences between enrollment groups of cattle. The total liver abscess prevalence overall was 38% (95% CI 37.0 to 39.9). A convenience sample of encapsulated abscesses (5 abscesses per

[2] Katherine L. Huebner, Jennifer N. Martin, Carla J. Weissend, Katlyn L. Holzer, Jennifer K. Parker, Steven M. Lakin, Margaret D. Weinroth, Zaid Abdo, Jessica L. Metcalf, Tony C Bryant, Keith E. Belk, Paul S. Morley

trial pen, n = 133 liver abscesses) was collected from individual cattle livers from 14 enrollment groups (n = 28 pens). Liver abscesses were excised from the severely abscessed livers of cattle enrolled into the trial and purulent material was aseptically extracted. The liver abscess microbiome was characterized by 16S rRNA gene sequencing of the V4 region on an Illumina HiSeq platform. Data were analyzed using the Qiime202917.12 bioinformatics pipeline to characterize liver abscess microbial diversity and composition. The liver abscess microbiome was polymicrobial, with Gram-negative anaerobic bacteria predominating. Overall, classification to 66 phyla were represented by high relative abundance of Fusobacteria (66.9%), Bacteroidetes (23.4%), Proteobacteria (4.1%), Firmicutes (3.0%) and Actinobacteria (0.9%). Pens of cattle with higher liver abscess prevalence (45-60%) during the trial had significantly increased abundance of the phyla Acidobacteria, Chlorobi, and Chloroflexi in the liver abscess microbome compared to pens of cattle with less than 45% liver abscess prevalence. Given the diversity of the liver abscess microbiome demonstrated in this study, more work is needed to understand the role of liver abscess microbiomes in disease severity as pre-harvest feeding strategies are investigated.

INTRODUCTION

Liver abscesses are costly to the feedlot cattle industry, with severe abscesses contributing the largest impact on diminished carcass value (Brown and Lawrence, 2010; Nagaraja and Lechtenberg, 2007; Reinhardt and Hubbert, 2015). The National Beef Quality Audit- 2016 reported an increased proportion of liver condemnations due to liver abscesses since the last audit (Eastwood et al., 2017). During the finishing period, diets high in readily fermentable carbohydrates (i.e. starch) predisposes cattle to rumen acidosis, inflammation and infection of the rumen epithelium, and infection of the liver via the portal vein circulation

(Jensen et al., 1954a; Nagaraja and Lechtenberg, 2007). This disease complex is commonly referred to as rumen acidosis liver abscess complex (ARLC), since the primary foci of infection is the rumen epithelium, and liver abscesses form secondary to bacterial emboli from the rumen. To control and prevent ARLC within U.S. beef cattle housed in feedlots during the finishing period, the industry primarily utilizes nutritional management strategies, such as gradual transition to high concentrate feeds, coupled with antimicrobial treatment.

Several studies have used anaerobic and aerobic culture methods to characterize the bacterial content of liver abscesses. Most commonly, *Fusobacterium necrophorum* and *Trueperella pyogenes* are isolated, and the leading hypothesis is that these bacteria act synergistically to contribute to liver abscess formation and severity (Lechtenberg et al., 1988; Nagaraja et al., 1999a; Nagaraja and Chengappa, 1998; Newson, 1938; Scanlan and Hathcock, 1983; Tadepalli et al., 2009; Tan et al., 1994). Available liver abscess interventions for feedlot cattle, including macrolide antibiotics (i.e., tylosin) and bacterin vaccines, are labeled to target these bacteria, with incomplete efficacy (Checkley et al., 2005; Nagaraja and Chengappa, 1998; Wileman et al., 2009). Though less common, several other bacteria have been isolated from liver abscesses, highlighting the polymicrobial potential of liver abscesses. For example, *Salmonella enterica* isolates were recently recovered from liver abscess purulent material for the first time from culled dairy cattle (Amachawadi and Nagaraja, 2015), and application of 16S rRNA marker gene sequencing has recently revealed diverse microbiomes sequenced from liver abscesses collected from different feedlot operations (Weinroth et al., 2017). Little is known about the potential role of less commonly isolated bacteria, or unculturable bacteria, on disease pathogenesis or severity during ARLC.

Advances in next generation sequencing technologies and bioinformatics tools have opened a new frontier for understanding cattle host and rumen microbial relationships, with implications for methane emission (Carberry et al., 2014; Shi et al., 2014), feed efficiency (Hernandez-Sanabria et al., 2012; Myer et al., 2015; Zhou et al., 2009), and rumen acidosis (Mao et al., 2013; Petri et al., 2013; Plaizier et al., 2017). Application of advanced molecular technologies could help elucidate the underlying shifts in microbial ecology contributing to complex metabolic diseases, including ARLC pathogenesis. In the future, new knowledge about host-microbial interactions will allow us to seek new opportunities to enhance animal performance and mitigate disease through manipulation of rumen fermentation and function (Malmuthuge and Guan, 2017). This will become increasingly important, as scrutiny over antimicrobial use in feedlot cattle increases, and there is increased demand for the industry to evaluate non-antimicrobial intervention strategies to reduce liver abscesses.

There is considerable interest in the use of yeast (i.e., dead, live, or yeast culture products) to improve animal production and reduce disease in both dairy and beef cattle, including liver abscesses. Yeast cultures, such as *Saccharomyces cerevisiae* fermentation products (SCFP) are produced using a proprietary industrial process and supplemented in cattle diets as commercial feed additives. Studies *in vitro* have shown that SCFPs provide soluble growth factors (i.e., organic acids, B vitamins, and amino acids) to stabilize rumen microbial populations in cattle exposed to transitioning or high concentrate diets (Callaway and Martin, 1997; Yang et al., 2015), with beneficial effects for dairy and beef cattle population health (Poppy et al., 2012; Wagner et al., 2016a). In a recent randomized clinical trial reported in Chapter 2, the effects of SCFP supplementation on liver abscess prevalence, animal health characteristics and the fecal microbiome and resistome composition in cattle raised without

antimicrobials was evaluated, concluding no detectable differences in any measured outcomes. Using liver abscess samples collected from the pens of cattle enrolled in that trial, the goals of this study were to characterize the microbiome composition and diversity of liver abscesses in cattle raised without antibiotics in a Colorado feedlot to contribute to our understanding of ARLC pathogenesis in beef cattle.

METHODS

Study population and study design

Liver abscess samples in this study were collected from carcasses of cattle that were placed on a clinical trial to evaluate a SCFP dietary feed additive on the animal production, health, liver abscess prevalence, fecal microbiomes and resistome composition in feedlot cattle, which is previously described in chapter 2. All procedures involving animals were approved by the Colorado State University Research Integrity and Compliance Review Office, and were exempt from IACUC oversight. Briefly, a randomized-block clinical trial was performed in a commercial feedlot located in Northern Colorado. Crossbred steers, sourced from multiple producers in the western U.S., were raised to produce 'natural' beef products without the use of antibiotics or growth hormones. As enrollment groups of cattle arrived, they were sorted into two adjacent home pens. Pens of cattle in each enrollment group were then randomly assigned to receive the SCFP treatment or control diet (N = 28 pens). Treatment and control pens of cattle within the same enrollment group (i.e. block), were handled identically during the finishing period, and consisted of cattle from similar vendor source with equivalent weight distributions (n = 14 enrollment groups). Treatment pens of cattle were fed a basal diet supplemented with SCFP (NaturSafe, Diamond V), and the control pens of cattle were fed a basal diet only. Diet formulation and cattle feedlot management details were outlined in Chapter 2. Across all pens of

cattle, the duration of days on feed varied, but were the same for within enrollment group (average 205 d; range 171 to 262). Enrollment groups were shipped for harvest together on the same day, and shipment date was determined based on standard procedures used by this feedlot to determine market weight (1400-1500 lbs per animal). At slaughter, cattle were humanely rendered insensible per standard slaughter plant protocol, and pen group identity was maintained through the slaughter process. All feedlot and research personnel were masked to treatment through to analysis.

Sample collection

Liver abscess number and severity data within each pen were scored and compiled by a commercial liver abscess scoring service using a modified Elanco Liver Check System (Elanco Animal Health, Greenfield, IN) as previously described (Rezac et al., 2014a). A trained observer evaluated livers from cattle visually at the offal table, and scored them as normal, A-minus, A, or A-plus to characterize abscess severity. Briefly, livers without a visible abscess were scored as normal. Livers with resolved abscess scars or < 2 abscesses < 2-cm in diameter were scored as A-minus. Livers with 2 to 4 abscesses 2 to 4-cm in diameter were scored as A. Livers with > 1 abscess > 4-cm in diameter, or > 4 abscesses > 2-cm in diameter, abscesses adhered to the diaphragm, abscesses adhered to other organs, abscesses adhered to the abdominal wall, or abscesses that were ruptured or open were scored as A-plus abscesses. Other pathological abnormalities were not scored or considered for this study. A convenience sample of encapsulated liver abscesses from each treatment group (corresponding to a trial pen) were excised using a sterile scalpel blade from the abattoir. The liver abscesses samples were placed into sterile bags and immediately transported on ice to Colorado State University (Fort Collins, CO) for processing.

Sample processing

The encapsulated liver abscess samples were stored at 4°C for up to 48 hours before processing. The external surface of the abscess capsule was sterilized by dipping it into 100% ethanol to coat the surface, followed by flaming the entire surface. Following surface sterilization, a sterile scalpel was used to open the abscess capsule, and sterile tongue depressors were used to extract the purulent material. Purulent liver abscess contents from samples were placed in a sterile conical vial and stored at -80°C until further processing for sequencing.

DNA isolation

After thawing at room temperature, the DNA was isolated from 0.1 g to 0.4 g of purulent abscess material using the PowerFecal DNA Isolation Kit (MoBio Laboratories) with the following modifications. The Mini-Beadbeater-16 (Biospec Products) was used for bead beating and samples were processed for 3 pulses of 30 s each. The DNA was eluted with 50 to 100 µl of the kit elution buffer. To maximize DNA yield, the eluate was passed through the membrane filter twice. Two blank samples (where the kit protocol was followed with reagents only) were extracted alongside liver abscess to serve as a negative control for the DNA isolation procedure.

16S rRNA gene sequencing and bioinformatics

16S rRNA gene amplification and sequencing for liver abscess samples was performed by a commercial sequencing company (Novogene Corporation). Control samples did not have sufficient quantities of DNA and were not sequenced (**Appendix; S.Figure 3.1)**. The V4 region of the 16S rRNA gene was amplified using the primer set 515F/806R (Caporaso et al., 2012), with reverse primers containing unique barcode sequences. Library sequencing (paired-end, 2x250 bp) was performed on an Illumina HiSeq 2500 platform (Illumina). The raw data were demultiplexed and quality filtered by Novogene prior to analysis.

Forward and reverse reads were imported into Qiime2-2017.12. The DADA2 pipeline was used for detecting and correcting Illumina amplicon sequence data and assembly into sequence variants (SVs) (Callahan et al., 2016, 2017). As implemented in the q2-dada2 plugin, the parameters that were used in quality filtering included primer trimming (--p-trim-left-f-19 and –p-trim-left-r-20), and --p-trunc-len n, which truncated each sequence at position 170, to remove low quality sequence less than a Phred score of 30. Taxonomy was assigned to sequences in the feature table using a Naïve Bayes classifier trained on the Greengenes 13_8_99% database, where the sequences have been trimmed to only include 250 bases from the V4 region of the 16S rRNA gene that was sequenced in this analysis. Sequences that were classified as chloroplasts and mitochondria were filtered out from the feature table. Raw sequence data are to be available for download from NCBI in final manuscript.

Taxonomic composition was visualized in a bar plot utilizing SV across all samples in a non-normalized feature table, at the phylum, class, order, and family level. In the table, rare taxa ($\leq$ 0.6%) were grouped together for visualization purposes to create a barplot using R (version 3.4.2). A rooted phylogenetic tree was created through *de novo* multiple sequence alignment using the MAFFT program, version 7 (Katoh and Standley, 2013), highly variable positions were removed to decrease noise in the tree, and FastTree-2 was applied to generate a phylogenetic tree from the masked alignment (Price et al., 2010).

Statistical analysis

Alpha and beta diversity metrics, statistical significance testing, and interactive visualizations were generated using q2-core-metrics-phylogenetics metric plugin. For distance based diversity analyses, the feature table was rarefied to the level of the lowest sequence depth, 65,000 sequences, allowing retention of all 133 samples. Beta diversity was visualized using

unweighted UniFrac distances (Lozupone et al., 2006) in a principle coordinates analysis (PCoA) plot using Emperor (Vázquez-Baeza et al., 2013b). Alpha diversity was estimated using the Shannon diversity metric, and were visualized in whisker boxplots.

Statistical testing for differences in categorical metadata were performed on the rarefied feature tables (as described above) for alpha and beta diversity. The categorical metadata comparisons of interest to this study included SCFP treatment, enrollment group, and liver abscess prevalence categories (15-30%, 30-45%, and 45-60%). Statistical differences in alpha diversity were determined using a non-parametric Kruskall-Wallis test, with a Benjamini and Hochberg correction applied for multiple comparisons (Benjamini and Hochberg, 1995). Statistical differences for beta diversity was tested using unweighted Unifrac distances with the permutation-based statistical test, PERMANOVA (Anderson, 2001). To visualize alpha diversity as a function of sequencing depth, rarefaction curves were generated for each sample as a function of the observed number of observed SV, where samples were rarefied multiple times from a minimum depth of 10 to a maximum of 80,000, a step size of 100 reads, and 5 iterations for each subsampling using the q2-diversity alpha-rarefaction plugin. Heatmaps were generated in q2-feature-table plugin. Comparisons were considered significantly different if P was ≤ 0.05.

Differential abundance testing for categorical metadata was performed using ANCOM at each taxonomic level (Mandal et al., 2015). This procedure has been shown to control for false discovery rate, and is very sensitive for drawing inferences about taxon abundance in the ecosystem (Weiss et al., 2017). The procedure compared relative abundance of a SV between two microbial communities by calculating Aitchison's log-ratio of abundance of each SV relative to the abundance of the others (Aitchison, 1982). Significance test significance was performed using Benjamini-Hochberg statistic, controlling for False Discovery rate at 0.05 (Benjamini and

Hochberg, 1995). For each SV, the number of tests that were rejected were counted, and the count random variable, *W,* represented the number of nulls among the tests that were rejected, and final significance of each SV were determined using the distribution of *W*. To meet the assumption that few (less than ~25%) of the SV change between groups, SV not present within 10% of samples were filtered out of the feature table. Since ANCOM does not tolerate zero frequencies, a pseudocount of 1 was added to the feature table across all SV. The filtered table was normalized by calculating the $\log_{10}$ of the table, then clustering was performed on SV using averaged normalized Euclidian distance.

RESULTS

Liver abscess prevalence and severity

The clinical trial results from which the study cattle originated are detailed in chapter 2. Briefly, there were no significantly detectable differences between cattle receiving SCFP treatment and control diet in any of the tested outcomes. Of the 4,324 steers that were scored for liver abscesses, 61.5% (95% CI = 60.1 to 63.0) of cattle had normal livers, and 38.4% (95% CI = 37.0 to 39.9) had an abscess overall. Of all the abscessed livers, 17.5% (95% CI = 16.4 to 18.6) had an A minus abscess severity score, 5.3% (95% CI = 4.6 to 5.9) had an A abscess severity score, and 15.7% (95% CI = 14.6 to 16.8) had a grossly detectable A plus abscess severity score (**Figure 3.1**). Liver abscess prevalence and severity varied by cattle enrollment group (**Figure 3.2**).

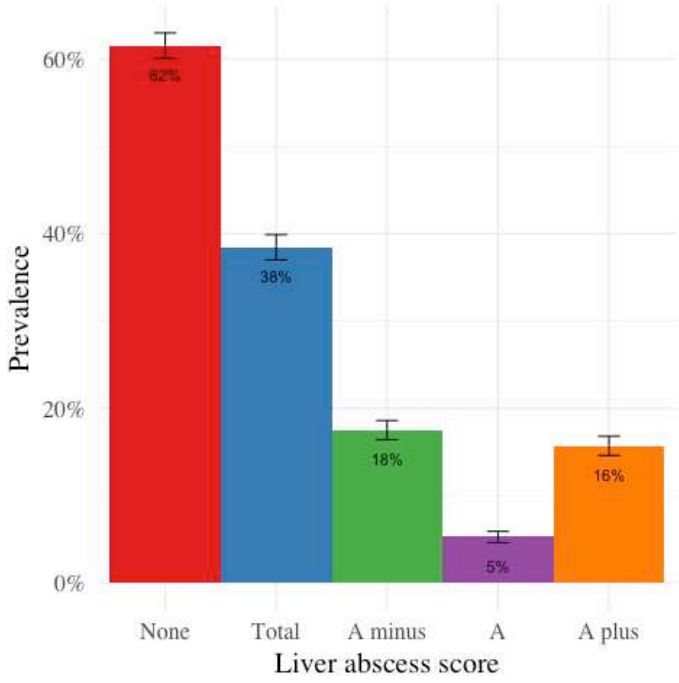

Figure 3.1: Liver abscess prevalence, by liver abscess severity in cattle enrolled within the *Saccharomyces cerevisiae* clinical trial.

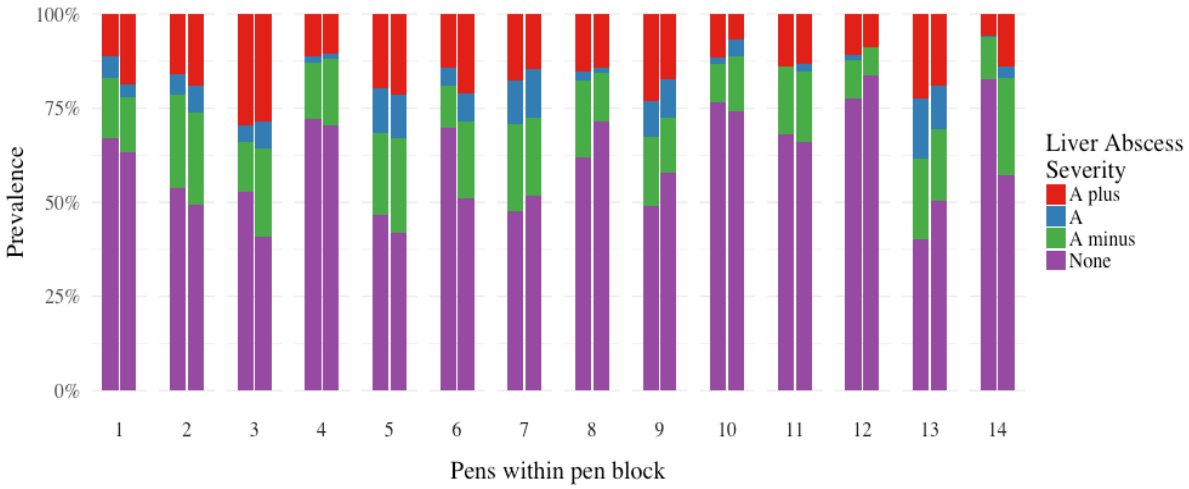

Figure 3.2: Liver abscess prevalence, by treatment or control pen within enrollment group, by liver abscess severity.

Liver abscess microbial community composition

Out of 28 pens of cattle, 27 pens were sampled for liver abscesses, resulting in 133 total liver abscess samples available for processing. A total of 28,596,801 sequencing reads were imported into Qiime2-2017.12 for analysis. Following quality control in the DADA2 pipeline, there were 25,686,444 sequences that were processed into 21,475 SV. Following removal of chloroplasts and mitochondrial SVs, there were 25,680,785 sequencing reads assigned to 21,475 SV remaining (**Appendix; S.Table 3.2**).

Sequence variant taxonomic classification represented 66 phyla, 196 classes, 391 orders, 656 families, and 1,234 genera (**Appendix; S.Table 3.3)**. While many of the SVs classified to genus level, some were classified at a higher level, which is a common finding with 16S rRNA sequencing data. Abundant SV included taxa classified to the phyla Fusobacteria (66.9%), Bacteroidetes (23.4%), Proteobacteria (4.1%), and Actinobacteria (0.9%). At the genus-level, *Fusobacterium* spp. (66.8%), *Bacteroides* spp. (16.8%)*, Porphyromonas* spp. (3.1%)*, Sporomusa* spp. (1.0%)*,* and *Campylobacter* spp. (0.8%) predominated (**Figure 3.3**). Taxa that classified to the genus, *Trueperella*, comprised 0.25% of all SV classifications. A small number of sequencing reads did not classify to a SV at any taxonomic level (0.01%), and three phyla were classified within the kingdom, Archaea, including the phyla Crenarchaeota (0.04%), Euryarchaeota (0.0004%), and Parvarchaeota (0.0001%).

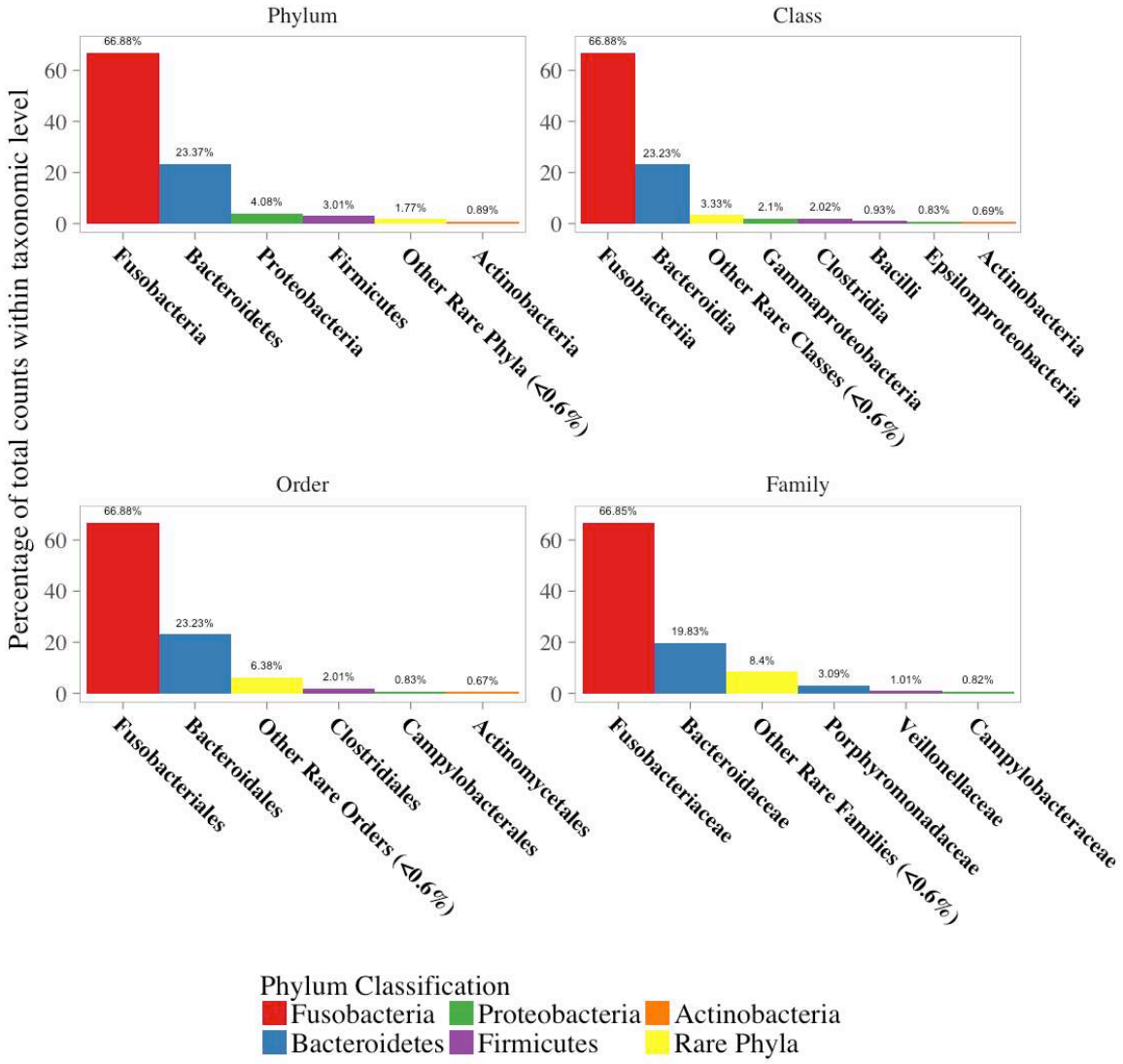

Figure 3.3: Relative proportions (count within taxonomic group / total count) of the liver abscess microbiome at the phylum, class, order, and family level across 133 liver abscess samples. Rare taxa (defined as having relative proportions <0.6%) were summed together and put into a distinct category at each level.

An Analysis of Composition of Microbiomes (ANCOM) revealed that the liver abscess microbiomes sampled from SCFP-fed cattle in the trial did not have distinct microbial composition compared to control-fed cattle, at any taxonomic level. However, the liver abscess microbial composition differed among the 14 enrollment groups of cattle. As described above, the enrollment groups consisted of cattle that had equal weight distribution, cattle vendor source,

within-feedlot location, and time of placement and harvest. Taxonomic shifts among cattle enrollment group microbial composition included members of the phyla Acidobacteria, Thermi, Chlorobi, Chloroflexi, Deferribacteres Gemmatimonadetes, Synergistetes, Tenericutes, Armatimonadetes, Planctomycetes, Actinobacteria, and OP1 (**Appendix; S.Table 3.4**), and these SV were in higher abundance in enrollment groups 2, 3, 4, 6, 7 and 9. A heatmap visualization also demonstrated the enrollment group differences in microbial community composition (**Figure 3.4**). The microbial composition also shifted between pens of cattle that had distinct category of total liver abscess prevalence, with pens having 15-30%, 30-45% liver abscess prevalence having lower abundances of the phyla Chlorobi, Acidobacteria, and Chloroflexi (**Appendix; S.Table 3.5; Figure 3.5**) compared to a liver abscess prevalence of 45-60%.

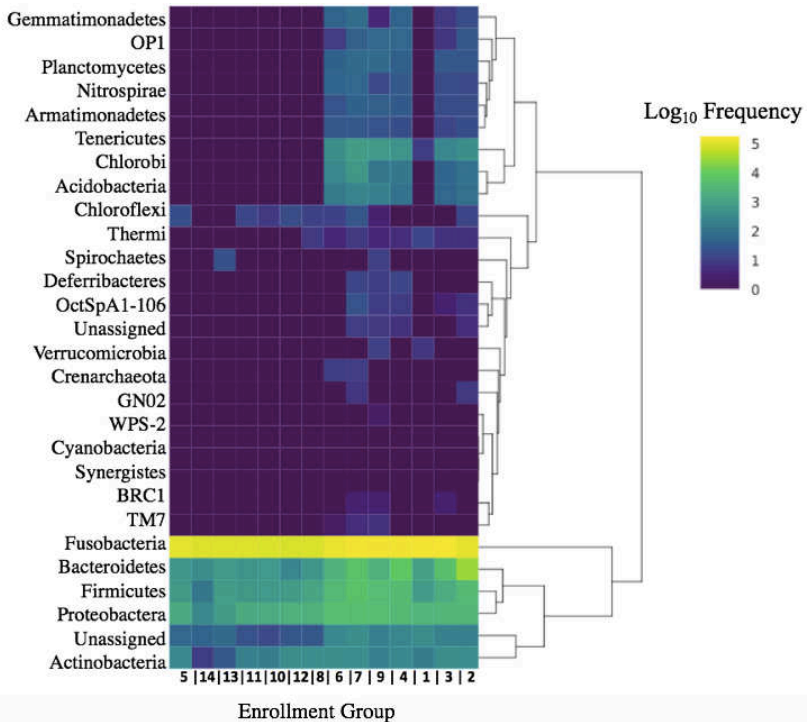

Figure 3.4: Heatmap of liver abscess phyla composition differences by trial enrollment group. The heatmap was constructed after the filtered feature table was normalized by adding a pseudocount of 1, then taking the $\log_{10}$ of the table. Clustering was performed on exact SV using averaged normalized Euclidian distance.

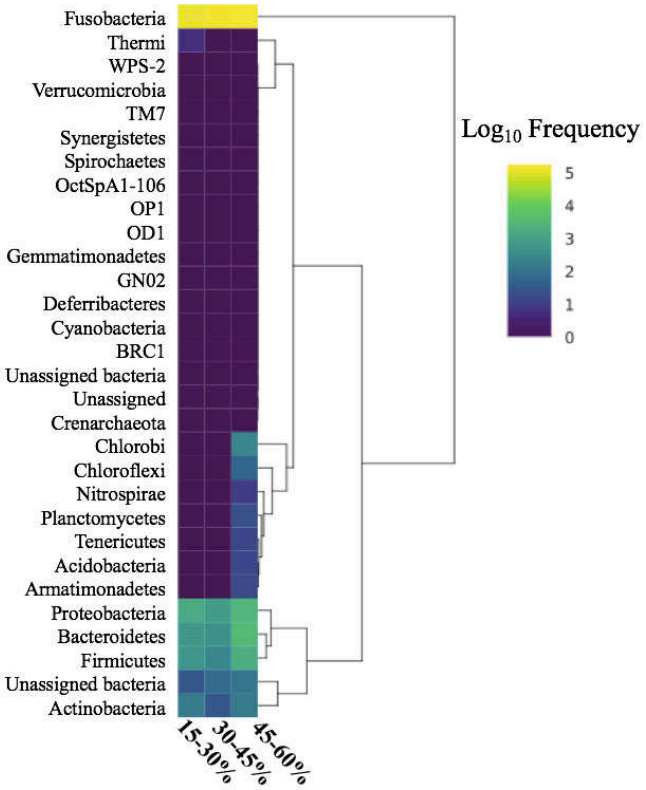

Figure 3.5: Heatmap of liver abscess phyla composition differences by liver abscess prevalence category. Each column represents a bin for range of liver abscess prevalence. The heatmap was constructed after the filtered feature table was normalized by adding a pseudocount of 1, then taking the log10 of the table. Clustering was performed on exact SV using averaged normalized Euclidian distance.

Microbial diversity

To assess species richness as a function of sampling depth, an alpha rarefaction curve was generated (**Figure 3.6**). The plateau of the curve began at approximately 20,000 sequences per sample, indicating that sequencing depth (reads/sample) was adequate to estimate sample microbial diversity. Group comparisons for species richness (alpha diversity) and degree of differentiation in microbial communities (beta diversity) and were evaluated for statistical differences between the trial SCFP treatment and control groups, but no statistical differences were detected **(***P* = 0.53; and *P* = 0.39, respectively; **Appendix; S.Figure 3.2)**.

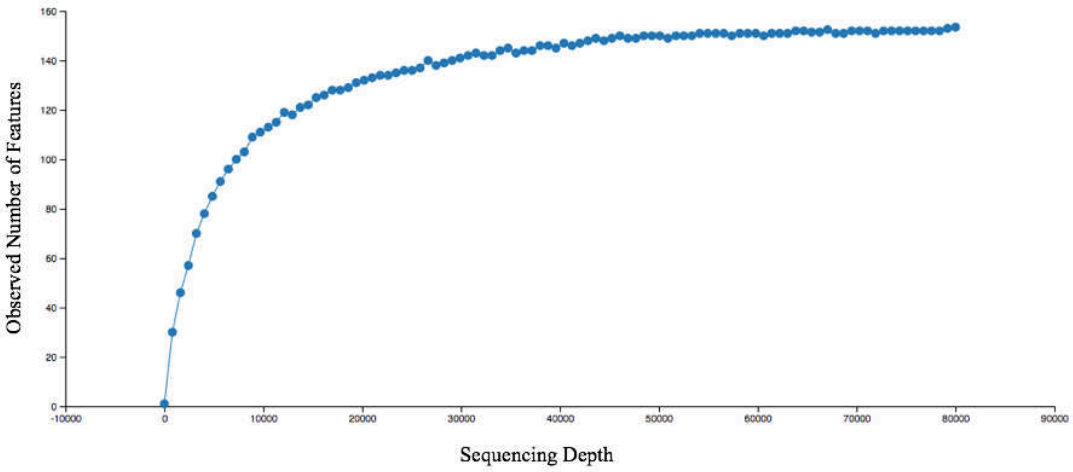

Figure 3.6: Alpha rarefaction curve for the observed species in liver abscess microbial communities across 28 liver abscess samples. The rarefaction curve was generated using the numbers of exact SVs richness estimation. Samples were rarefied at an even depth of 80,000 sequences per sample.

To assess differences in microbial diversity and community across the clinical trial enrollment groups and liver abscess prevalence categorizations, Shannon diversity index and unweighted Unifrac metrc were evaluated to compare alpha and beta diversity. There were statistically significant differences in Shannon alpha diversity between the enrollment groups of cattle ($P = 0.001$), as well as differences in microbial community composition, or beta diversity ($P = 0.001$; **Figure 3.7**). However, comparisons between liver abscess prevalence categories (15-30%, 30-45%, and 45-60%) did not show statistically significant differences in Shannon alpha diversity ($P = 0.42$); however, there were differences detected in beta diversity, which were largely driven by clustering of the 15-30% liver abscess prevalence category (P = 0.001; **Figure 3.8**)

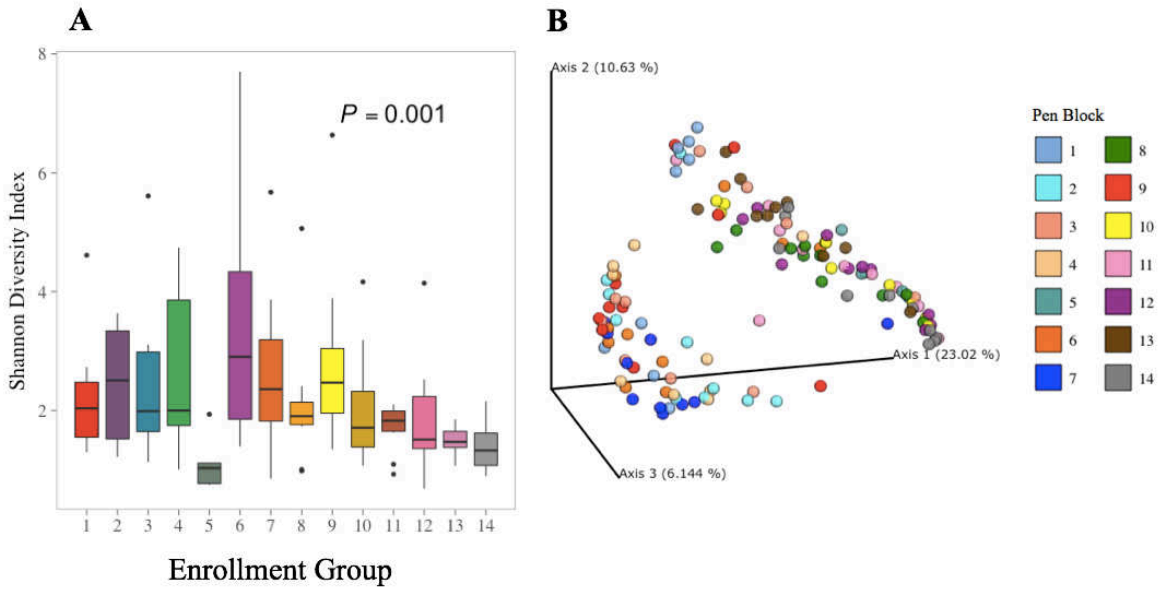

Figure 3.7. (**A**) Shannon alpha diversity by enrollment group of cattle from the clinical trial. Statistical differences were determined using non-parametric Kruskall-Wallis tests (test statistic, H = 33.9; *P* = 0.001). (**B**) PcoA plot based on 16S rRNA gene sequencing, showing unweighted UniFrac distance by enrollment group of cattle into the clinical trial. Statistical differences were determined using the PERMANOVA test (pseudo F statistic = 3.41; *P* = 0.001).

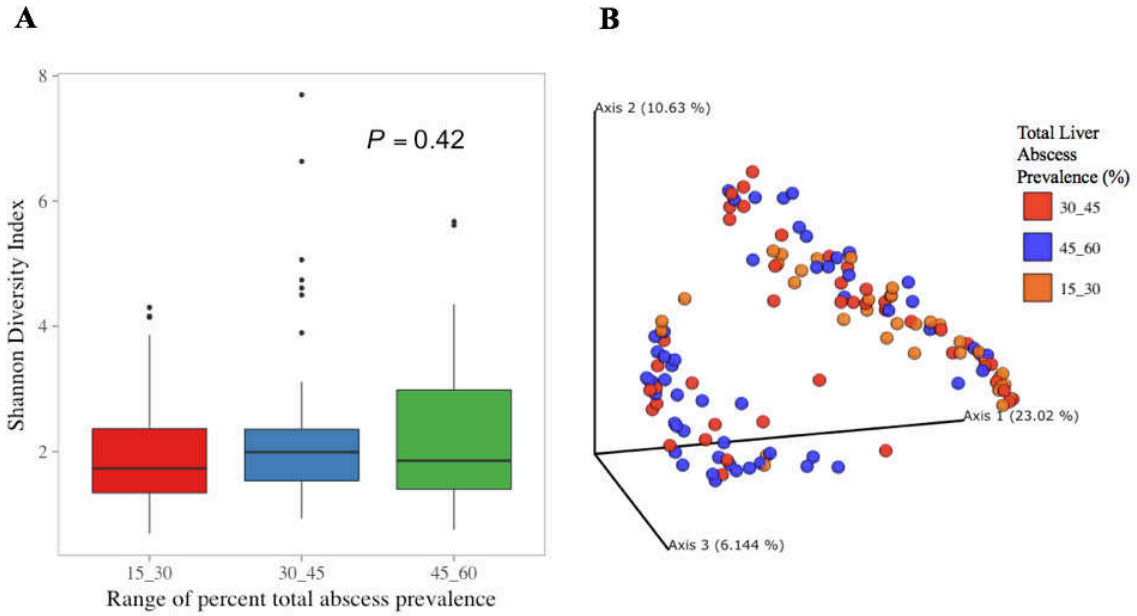

Figure 3.8. (**A**) Shannon alpha diversity by liver abscess prevalence category from cattle enrolled in the clinical trial. Statistical differences were determined using non-parametric Kruskall-Wallis tests (test statistic, H = 1.72; *P* = 0.42). (**B**) PcoA plot based on 16S rRNA amplicon, showing unweighted UniFrac distance grouped by liver abscess prevalence category. Statistical differences were determined using the PERMANOVA test (pseudo F statistic = 3.24; *P* = 0.001) (**B**).

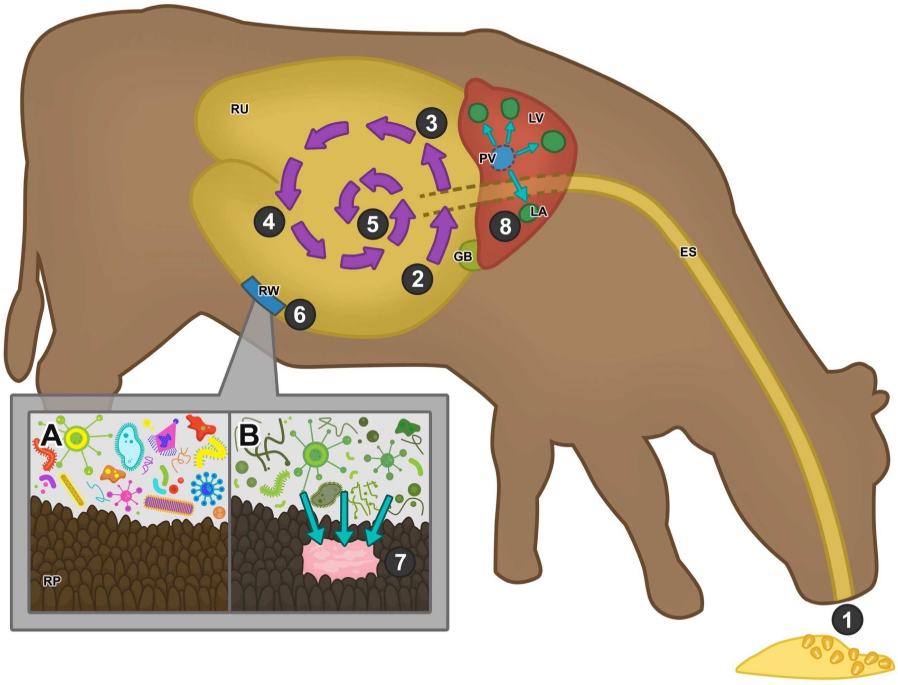

Figure 3.9. A revised paradigm for acidosis rumenitis liver abscess complex that considers microbial ecology in the mechanism for pathogenesis: 1) Cattle consumes or rapidly transitioned to highly fermentable carbohydrates; 2) microbial growth, populations shift to increased fermentation and VFA production; 3) rumen pH decreases (pH=5.5-6.6); 4) lactate-producing bacteria outgrow and outnumber lactate utilizing bacteria, lactate accumulates; 5) subacute rumen acidosis occurs (pH < 5.5); 6) focal or multifocal rumenitis lesions form (characterized by parakeratosis, blunted papillae or denuded areas of epithelium, and in more severe cases, ulceration and scar tissue formation); 7) Microflora associated with rumen epithelium gain entry to portal vein circulation, which is filtered by the liver; 8) liver abscess formation and severity is influenced by severity and duration of rumenitis, bacteremia, host immune status, and microbial virulence factors. ES = esophagus; RU = rumen; RW = rumen wall epithelium; RP = rumen papillae; LV = liver; LA = liver abscess; GB = gallbladder.

DISCUSSION

Using a marker gene sequencing approach, this study characterized the liver abscess microbiome collected from feedlot cattle as having diverse, polymicrobial communities which are predominated by Gram-negative anaerobes. Like other studies using selective culture techniques, *Fusobacterium* spp. dominated the liver abscess microbiome; however, there were increased abundance of less commonly identified bacteria, along with several unculturable

bacteria never detected previously from liver abscess samples. Applications of 16S rRNA sequencing coupled with bioinformatics tools provides a novel perspective for liver abscess bacteriology compared to selective culture techniques (Weinroth et al., 2017). This study contributed to the shifting paradigm that pathogenesis and severity of ARLC is a function of the dynamic interactions between the enteric rumen microbiome, cattle host factors, and feedlot environment (**Figure 3.9**). This exciting paradigm shift has already revolutionized our understanding and treatment approaches for several human gastrointestinal diseases, including inflammatory bowel disorders and malignant gastro-intestinal cancers (Coleman and Haller, 2017; Lam et al., 2017; Sittipo et al., 2018). This study detected significant compositional changes in the liver abscess microbiome among distinct cattle enrollment groups and among pens of cattle with distinct liver abscess prevalence, demonstrating that the liver abscess microbiome varies based on underlying factors related to cattle population health. This finding has implications for future studies that seek to evaluate novel feedlot management strategies, including antibiotic alternatives, for the treatment of liver abscesses in feedlot cattle.

Different enrollment groups in cattle in this study population varied with respect to vendor source, weight distributions, within feedlot location, and season of harvest, indicating that one or more of these factors can impact liver abscess microbial communities, and possibly ARLC disease severity. Few studies have documented the risk factors for pre-harvest factors on liver abscess formation with respect to variation in cattle source location, feedlot environment characteristics, or seasonal effects on liver abscess bacteriology or severity. Due to the design of the clinical trial from which the study population originated, it is not possible to determine which of these factors, if any, are responsible for driving the changes observed due to confounding. Interestingly, the phyla, Chloroflexi, Chlorobi, and Acidobacteria, were in higher relative

abundance in pens of cattle in higher liver abscess prevalence categories of 45-60%, therefore, future research directed at understanding the role of these bacteria during ARLC is warranted. Members of the acidophilic phylum, Acidobacteria, are frequently identified in marker gene surveys from a wide variety of environmental samples; however, they are unculturable, so little is known about its role in the rumen (Barns et al., 2007; Quaiser et al., 2003). There is limited information in the literature about the role of Chlorobi, or green sulfur bacteria, (Sakurai et al., 2010), in the rumen; however, one study correlated lipopolysaccharide intravenous injection and acidosis in dairy cattle with decreased abundance of Chlorobi (Jing et al., 2014). One limitation of the present study is that the sampling methods do not distinguish stage of abscessation at the time of sampling with respect to timing of abscess formation after rumen acidosis, which could impact associations between the liver abscess microbiome with liver abscess prevalence. There are no studies in the literature that accounted for microbial shifts in liver abscesses based on stage of progression. Understanding the microbial ecology of liver abscesses during different stages of progression warrants further investigation, as there is potential for significant shifts in microbial populations over time which could influence disease severity.

Using 16S rRNA sequencing, the most abundant bacteria classified within the liver abscess samples were *Fusobacterium* spp., which was consistent with the literature. This Gram-negative anaerobe proliferates in the rumen in cattle exposed to high concentrate diets due to the ability to utilize lactic acid (Nakagaki et al., 1991; Tan et al., 1996). Almost all studies have used anaerobic culture methodology to isolate *Fusobacterium necrophorum,* and it is widely accepted as the main etiologic agent in liver abscess formation (Amachawadi and Nagaraja, 2016; Nagaraja et al., 1999; Scanlan and Hathcock, 1983; Tadepalli et al., 2009). The second most commonly isolated species reportedly cultured from liver abscesses is *Trueperella pyogenes*,

however SV classifying to this genus only accounted for 0.25% of all classified SV in the liver abscess samples from this study.

Additional bacterial isolated from liver abscesses reported in the literature include *Bacteriodes* spp., *Clostridium* spp., *Escherichia coli, Klebsiella* spp*., Proprionbacterium* spp*., Enterobacter* spp., *Mobiluncus* spp., *Mitsuokella* spp., *Pasterella* spp., *Peptostreptococcus* spp., *Porphyromonas* spp., *Prevotella* spp., *Proprionibacterium* spp., *Staphyloccus*, *Streptococcus*, and other unidentified Gram-positive and Gram-negative bacteria (Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007). However, these bacteria are not credited with playing a large role, if any, in the pathogenesis of liver abscess formation. Our study detected several of these SV in high abundance within the liver abscess microbiome, particularly *Bacteroides* spp. and *Porphyromonas* spp., which are Gram-negative, obligate anaerobes. Virulence and contribution of these genera to the formation of liver abscesses is possibly underestimated. Both have been implicated in bacteremia and abscess formation when they escape the gut environment in humans and other cattle diseases, in part due to antibiotic resistance mechanisms, potent virulence factors, evasion of the host immune system, purinergic signaling, and inflammasome activation (Almeida-da-Silva et al., 2016; Olsen and Yilmaz, 2016; Van Metre, 2017; Wexler, 2007).

Recently, *Salmonella enterica* was isolated from a subset of dairy cattle liver abscesses, highlighting the potential for unknown microbial diversity in liver abscesses and possible food safety implications (Amachawadi and Nagaraja, 2015). In the present study, members of the family Enterobacteriaceae that were identified that included *Escherichia*, *Erwinia, Serratia, Buchnara, Providencia, Shigella, Citrobacter, Proteus, Morganella, Candidatus, Edwardsiella,* and *Dickeya*; however, there was no classification at the genus-level for *Salmonella* spp. One

limitation to marker gene studies is the poor sensitivity with regards to genus and species-level classifications. Therefore, is not known if *Salmonella* was present at the unclassified family-level in this study, or not. One interesting finding was that *Campylobacter* spp. were identified in relatively high abundance within liver abscess samples, which was also reported in a liver abscess by (Weinroth et al., 2017), but in lower abundance. During the slaughter process, liver abscesses are commonly adhered to other organs and/or rupture (Brown and Lawrence, 2010; Rezac et al., 2014a), causing carcass contamination, and potentially resulting in *Campylobacter* contamination of beef products which is a significant food safety concern (Abley et al., 2011; Wong et al., 2007). Though this classification was not specific enough to identify the species or strain level of this bacteria, it is concerning as many species of *Campylobacter* have been shown to infect humans and animals causing disease (Kaakoush et al., 2015).

One other study has utilized 16S rRNA gene sequencing approaches to describe liver abscess microbiomes, but it focused on evaluation of cattle under different management conditions and from different regions. In that report, the liver abscess microbiome also was characterized as diversely polymicrobial, identifying 5 phyla, 13 classes, and 17 orders of bacteria (Weinroth et al., 2017). Furthermore, Bacteroidetes, and not Fusobacterium was classified as the most abundant phyla, along with other changes in relative abundances of taxa. Another possible explanation for these differences among study results is the different sample size of liver abscesses, study populations, antimicrobial use, cattle source, and other unmeasured management and environmental conditions in the feedlots tested. Another possible explanation for the disparities may be due to differences in bioinformatics pipelines, as recent advances have made it possible to classify taxa using exact SVs rather than construction of molecular operational taxonomic units (OTUs), a method that clusters sequencing reads that differ by less

than a fixed dissimilarity threshold (Callahan et al., 2016, 2017). This highlights the importance to use care when interpreting marker gene data across distinct study designs and bioinformatics platforms, and also emphasizes the need for standardized analytical pipelines to increase reproducibility of microbiome projects (Sinha et al., 2015).

CONCLUSIONS

Our understanding about the interactions between the ruminant host and their commensal rumen bacteria is relatively nascent, including mechanisms that underlie the balance between mutually beneficial relationships and pathogenesis (Taschuk and Griebel, 2012). Going forward, it will be essential to elucidate factors that lead to differences in infection susceptibility in order to detect novel ways to reduce disease burden (Malmuthuge and Guan, 2017). Developing an understanding of this relationship between rumen mucosal immunity, wall-adherent microbial communities, and local immune responses in the rumen wall and liver tissue should be part of the next steps in understanding acidosis rumenitis liver abscess complex. Furthermore, as scrutiny over antibiotic use in the feedlot industry increases and regulatory changes are implemented, it is important to focus liver abscess research efforts on high risk populations of cattle, including fed dairy and beef breeds raised without antibiotics. It will also be important to monitor food safety implications of novel alternative products, and the impact use or removal of antibiotics and alternative therapies will have foodborne pathogens in a microbial ecology context. With the proper application of advanced molecular sequencing technology to provide new perspectives, we can better understand acidosis rumenitis liver abscess complex to facilitate research and discovery of alternative treatments.

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APPENDIX

CHAPTER 2 SUPPLEMENTARY MATERIALS

Supplemental Table 2.1: 16S rRNA amplicon reads per sample frequencies and summary statistics at different stages of data processing, including importation of raw fastq sequences into Qiime2, following quality control in the DADA2 pipeline, and following filtering out mitochondrial and chloroplast sequences.

Sample ID	Imported Sequences	Following DADA2	Following Filtering
CF1	405942	348298	348298
CF2	436851	372814	372814
CF3	643540	520266	519923
CF4	465200	380085	380085
CF5	540561	467300	467300
CF6	1002314	852917	852917
CF7	327779	279321	279321
CF8	871911	744616	744613
CF9	600316	511071	511056
CF10	430094	359935	359677
CF11	366452	316713	316713
CF12	248945	218523	218518
CF13	277280	228743	228640
CF14	468365	401412	401406
CF15	696914	595602	595602
CF16	489723	410888	410888
CF17	282386	240601	240501
CF18	485072	415209	415209
CF19	352641	294369	294369
CF20	544153	466501	466501
CF21	504813	429127	429127
CF22	244950	207346	207346
CF23	451262	382182	382182
CF24	456146	388754	388754
CF25	482651	404829	404829
CF26	785434	650689	650684
CF27	505038	376912	376912
CF28	383051	307061	307056
	Summary Statistics		
Minimum frequency	244,950	207,346	207,346

1st quartile	378,901	314,300	314,299
Median frequency	491,064	385,468	385,468
3rd quartile	541,459	466,701	466,701
Maximum frequency	1,002,314	852,917	852,917
Mean frequency	13,749,784	413,289	413,259

Supplemental Table 2.2: Shotgun sequencing paired-end reads per sample frequencies and summary statistics at different stages of data processing, including raw fastq sequences, following quality control and host removal, and number of alignments to the BacMet and MEGARes databases using the AmrPlusPlus pipeline.

Sample ID	Paired-end reads/sample)	Quality Score	After quality control host removal	No. of alignments to database
CF1	43209371	37.7	39299378	67839
CF2	23216028	37.7	20907069	36996
CF3	24793292	37.7	22067343	33189
CF4	14853690	38.1	13419546	20511
CF5	23619704	37.9	21248030	43974
CF6	32284007	37.8	29014215	54721
CF7	19068839	36.1	17070809	28006
CF8	25819778	38.2	23502170	40019
CF9	56382659	37.7	51280927	93491
CF10	26320094	37.6	23648974	47166
CF11	23994450	37.8	21604334	38358
CF12	35921580	38.5	31916136	61311
CF13	24403110	37.8	22075567	40913
CF14	36985682	37.6	33248902	61155
CF15	50191940	36.5	44936754	76617
CF16	56687773	38.5	50853345	83789
CF17	27444715	37.7	24551659	35608
CF18	48122715	37.7	43544670	82627
CF19	40797472	36.4	37006659	57137
CF20	33062421	38.4	30127464	51521
CF21	26471970	37.9	23538950	38710
CF22	25310627	37.7	22951381	36447
CF23	47525379	37.8	43246811	95286
CF24	43772475	38.4	39254620	70683
CF25	56028423	38.0	50543553	95303
CF26	47536926	37.9	42552740	80368

CF27	50357870	37.9	45186233	89420
CF28	30276174	38.3	27135128	49678
		Summary Statistics		
Minimum	14853690.0	36.1	13419546.0	20511.0
1st Quartile	25181293.3	37.7	22732427.5	38622.0
Median	32673214.0	37.8	29570839.5	53121.0
Mean	35516398.7	37.8	31990477.4	57530.1
3rd Quartile	47528265.8	38.0	42726257.8	77554.8
Maximum	56687773.0	38.5	51280927.0	95303.0

Supplemental Table 2.3: Number and proportion of reads aligning to each category and mechanism of resistance.

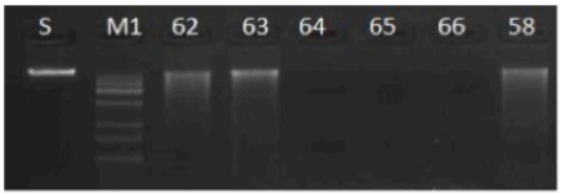
Resistance Mechanism	Total reads aligned	Proportion of reads	Resistance Mechanism	Total reads aligned	Proportion of reads
Antimicrobial Drug Resistance			**Biocide Resistance**		
Tetracycline resistance ribosomal protection proteins	226,940	62.264%	Biocide resistance protein	135	0.037%
Tetracycline inactivation enzymes	1,285	0.353%	Biocide resistance regulator	31	0.009%
Macrolide phosphotransferases	3,672	1.007%	**Metal Resistance**		
Tetracycline resistance major facilitator superfamily MFS efflux pumps	4,212	1.156%	Metal efflux pump	13	0.004%
Aminoglycoside O-phosphotransferases	195	0.054%	Metal resistance protein	87	0.024%
Aminoglycoside N-acetyltransferases	26	0.007%	Metal efflux regulator	57	0.016%
Penicillin binding protein	132	0.036%	Metal efflux protein	8	0.002%
Class C betalactamases	29	0.008%	Metal ABC efflux pump	13	0.004%
Lincosamide nucleotidyltransferases	9,820	2.694%	Nickel ABC efflux pump	18	0.005%
Aminoglycoside efflux pumps	299	0.082%	Nickel ABC efflux regulator	6	0.002%
Macrolide resistance efflux pumps	93,254	25.585%	Copper resistance protein	465	0.128%
Aminoglycoside efflux regulator	24	0.007%	Tellurium resistance protein	13	0.004%
Aminoglycoside O-nucleotidyltransferases	9,130	2.505%	Zinc ABC efflux pump	7	0.002%
Polymyxin B resistance regulator	90	0.025%	Zinc resistance regulator	64	0.018%
Multi-drug efflux pumps	1,031	0.283%	Metal resistance regulator	9	0.002%
23S rRNA methyltransferases	1,786	0.490%	**Cross-Category Resistance**		
Dihydrofolate reductase	2	0.001%	Metal and biocide resistance protein	46	0.013%
Class A betalactamases	10,855	2.978%	**Total**		
MDR regulator	455	0.125%	Total Reads	364,482	
Lipid A modification	273	0.075%			

CHAPTER 3 SUPPLEMENTARY MATERIALS

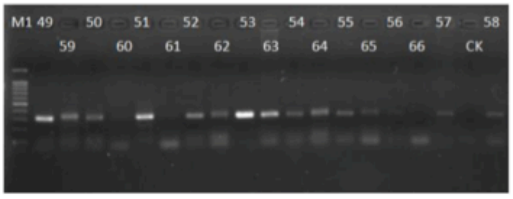
A

Sample Name	Qubit concentration (in MEG lab)	Genomic DNA Quantification using gel electrophoresis	Nanodrop concentration (after several rounds of amplification)
NC1	< 0.5 ng/uL	None	7.182 ng/ul, volume: 100 ul
NC2	< 0.5 ng/uL	None	8.441 ng/ul, volume: 128 ul
NC3	< 0.5 ng/uL	None	FAIL (0 ng/uL)

B



C



Supplemental Figure 3.1: DNA Quantification Results for negative control samples. Negative controls were extracted on the same date alongside liver abscess samples using only the PowerFecal DNA Isolation kit reagents (blank extractions) in the MEG (Microbial Ecology Group lab at Colorado State University). Sample concentrations following Qubit fluorometry are shown, along with DNA quantification results following PCR amplification (**A**). Agarose gel electrophoresis of the genomic DNA (**B**) and PCR products following amplification (**C**) agarose gel electrophoresis for NC1 (band 64), NC2 (band 65), and NC3 (band 66). PCR products were obtained after several rounds of amplification but were of insufficient quantity for sequencing.

Supplemental Table 3.2: 16S rRNA amplicon reads per sample frequencies and summary statistics at different stages of data processing, including importation of raw fastq sequences into Qiime2, after quality control in the DADA2 pipeline, and following removal of mitochondrial and chloroplast sequences.

Sample ID	Imported Sequences	Following DADA2 pipeline	Following Filtering
CL1a	250803	198706	198636
CL1b	204631	175946	175877
CL1c	232969	188461	188446
CL1d	601058	571496	571496
CL1e	248798	214219	214196
CL2a	237044	194827	194497
CL2b	584958	545793	545772
CL2c	283311	263449	263422
CL2d	500785	470334	470334
CL2e	743119	711057	710931
CL3a	183745	149636	149418
CL3b	560208	537292	537284
CL3c	324794	269477	269419
CL3d	307268	259403	259321
CL3e	114587	107017	107017
CL4a	751456	485384	485381
CL4b	178856	155476	155413
CL4c	106325	101121	101117
CL4d	484894	454372	454358
CL4e	249279	231550	231499
CL5a	188327	151470	151428
CL5b	170320	125572	125448
CL5c	219829	207838	207833
CL5e	325782	312401	312396
CL6a	289638	257340	257317
CL6b	455162	421891	421858
CL6c	106257	97860	97860
CL6d	208227	177212	177212
CL6e	215655	189664	189646
CL7a	441926	407171	407158
CL7b	212094	170112	170037
CL7c	211622	124010	123861
CL7d	210162	193702	193658

CL7e	564703	534499	534460
CL8a	447325	417367	417365
CL8b	262655	209816	209705
CL8c	278430	244105	244105
CL8d	685424	644770	644741
CL9a	99128	65826	65826
CL9b	211653	188029	187965
CL9c	318003	287474	287415
CL9d	303946	224402	224402
CL9e	592117	550219	549918
CL10a	319002	224621	224613
CL10b	167485	133908	133857
CL10c	179721	128997	128122
CL10d	148708	124754	124694
CL10e	267964	254764	254764
CL11a	203537	137554	137505
CL11b	469777	437714	437687
CL11c	218979	181761	181710
CL11d	812110	748968	748954
CL11e	275028	253511	253473
CL12a	491524	460744	460734
CL12b	275408	243846	243627
CL12c	367100	333977	333626
CL12d	225802	208357	208337
CL12e	100786	98501	98501
CL13a	369436	347263	347236
CL13b	144668	131453	131440
CL13c	426632	330321	330179
CL13d	235189	179377	179137
CL13e	119231	114279	114277
CL14a	119721	101685	101515
CL14b	361245	329807	329769
CL14c	282367	246498	246374
CL14d	237839	224130	224118
CL14e	108778	102796	102780
CL15a	106236	98965	98965
CL15b	107186	103458	103458
CL15c	102951	96712	96712
CL15d	106328	100549	100543
CL15e	101305	80212	80212

CL16a	119046	111879	111873
CL16b	101701	96232	96193
CL16c	104817	98974	98954
CL16d	116953	110969	110946
CL16e	107531	102877	102877
CL17a	192007	184948	184939
CL17b	111354	105308	105202
CL17c	111465	107244	107239
CL17d	103087	99838	99835
CL17e	110890	105878	105875
CL18a	112148	103856	103830
CL18b	100489	92739	92736
CL18c	100101	95891	95888
CL18d	109229	105408	105408
CL18e	116913	109189	109189
CL19a	101989	96659	96649
CL19b	182105	172364	172364
CL19c	116089	111505	111500
CL19d	110841	105940	105940
CL19e	119115	112480	112433
CL20a	103514	98247	98236
CL20b	110529	105554	105549
CL20c	109639	105876	105876
CL20d	116316	111078	111078
CL20e	111361	105350	105350
CL21a	104218	100053	99995
CL21b	102749	98664	98664
CL21c	100608	96705	96703
CL21d	104755	99844	99844
CL21e	191194	184455	184439
CL22a	106962	102429	102424
CL22b	113528	105827	105823
CL22c	110327	106552	106552
CL22d	184433	176624	176583
CL22e	117334	112028	112022
CL24a	112921	107367	107349
CL24b	205233	199239	199232
CL24c	115324	111940	111937
CL24d	110778	104982	104973
CL24e	112751	109912	109912

CL25a	138448	132316	132304
CL25b	101337	97537	97532
CL25c	100168	96396	96373
CL25d	114948	108884	108882
CL25e	102262	98917	98917
CL26a	110625	105806	105802
CL26b	114367	109846	109846
CL26c	119939	114431	114431
CL26d	100789	96498	96498
CL26e	102423	98425	98198
CL27a	102061	99400	99394
CL27b	115016	110505	110505
CL27c	110657	106575	106575
CL27d	110122	103573	103573
CL27e	105005	86734	86730
CL28a	119197	115209	115209
CL28b	100542	97062	97062
CL28c	88850	82338	82336
CL28d	116409	110190	110190
CL28e	111976	95650	95650
Summary Statistics			
Minimum frequency	88850	65826	65826
1st quartile	109639	103458	103458
Median frequency	119939	115209	115209
3rd quartile	262655	224402	224402
Maximum frequency	812110	748968	748954
Mean frequency	215014	193131	193089

Supplemental Table 2.3: Raw, non-normalized counts and relative proportion of phyla.

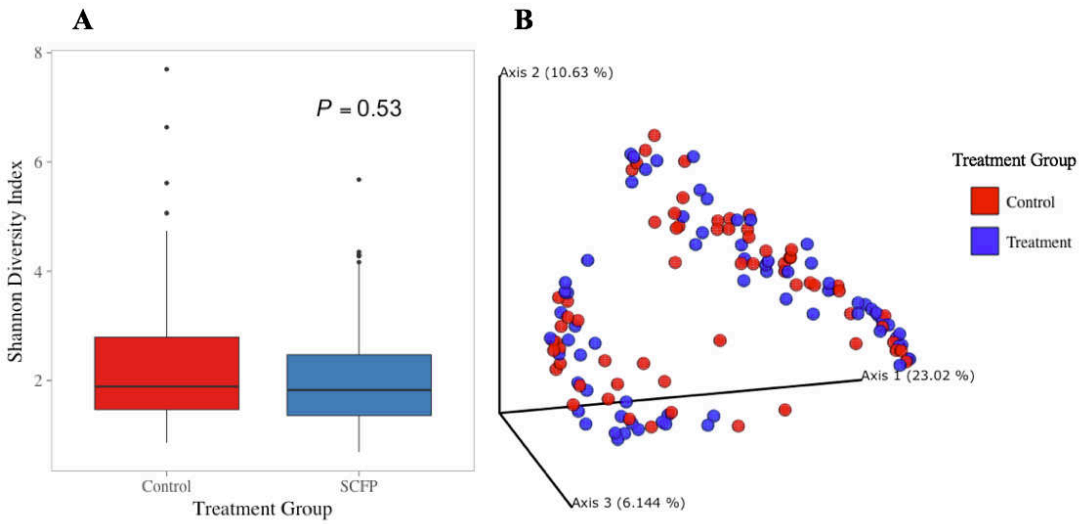
Phylum	Total count	Percent of total	Phylum	Total count	Percent of total
Fusobacteria	17146371	66.87903	GAL15	236	0.00092
Bacteroidetes	5992515	23.37367	NC10	217	0.00085
Proteobacteria	1046761	4.08287	Thermotogae	205	0.00080
Firmicutes	770470	3.00520	GN02	173	0.00067
Actinobacteria	228570	0.89153	Aquificae	172	0.00067
k__Bacteria;__	145802	0.56870	NKB19	171	0.00067
Acidobacteria	86231	0.33634	OP8	169	0.00066
Chlorobi	55601	0.21687	AC1	149	0.00058
Chloroflexi	39342	0.15345	OP9	147	0.00057
Gemmatimonadetes	20314	0.07923	AD3	135	0.00053
Nitrospirae	14829	0.05784	TM6	125	0.00049
Planctomycetes	12602	0.04915	Lentisphaerae	114	0.00044
Crenarchaeota[a]	11467	0.04473	Euryarchaeota	110	0.00043
Spirochaetes	9823	0.03831	SBR1093	82	0.00032
Verrucomicrobia	8105	0.03161	Hyd24-12	70	0.00027
OP1	6189	0.02414	TA06	67	0.00026
OctSpA1-106	5898	0.02301	WS1	65	0.00025
Armatimonadetes	5447	0.02125	WS2	63	0.00025
[Thermi]	5326	0.02077	OP11	58	0.00023
Tenericutes	4412	0.01721	Poribacteria	56	0.00022
Unassigned	4307	0.01680	Caldithrix	51	0.00020
Unassigned	3396	0.01325	GN04	45	0.00018
Cyanobacteria	2567	0.01001	BHI80-139	43	0.00017
TM7	2013	0.00785	WS5	39	0.00015
WS3	1531	0.00597	SR1	35	0.00014
OD1	1165	0.00454	Parvarchaeota[a]	30	0.00012
Deferribacteres	899	0.00351	FCPU426	30	0.00012
BRC1	754	0.00294	OP3	22	0.00009
FBP	586	0.00229	[Caldithrix]	21	0.00008
Elusimicrobia	444	0.00173	PAUC34f	18	0.00007
Synergistetes	380	0.00148	Caldiserica	12	0.00005
Chlamydiae	286	0.00112	WS4	12	0.00005
Fibrobacteres	277	0.00108	Dictyoglomi	11	0.00004
WPS-2	254	0.00099			

Supplemental Table 2.4: Analysis of Composition of Microbiomes among blocks for liver abscess 16S rRNA gene sequencing data.

Phylum	Reject null hypothesis	W
[Thermi]	TRUE	29
Acidobacteria	TRUE	29
Chlorobi	TRUE	29
Chloroflexi	TRUE	29
Deferribacteres	TRUE	29
Gemmatimonadetes	TRUE	26
Synergistetes	TRUE	25
Tenericutes	TRUE	25
Unnassigned Bacteria	TRUE	23
Armatimonadetes	TRUE	23
Planctomycetes	TRUE	23
Actinobacteria	TRUE	22
OP1	TRUE	22
Firmicutes	FALSE	21
GN02	FALSE	21
Nitrospirae	FALSE	21
OctSpA1-106	FALSE	21
Proteobacteria	FALSE	21
WPS-2	FALSE	20
Cyanobacteria	FALSE	19
Unassigned;__	FALSE	18
OD1	FALSE	17
TM7	FALSE	17
Verrucomicrobia	FALSE	17
k__Bacteria;__	FALSE	16
Fusobacteria	FALSE	16
BRC1	FALSE	14
Spirochaetes	FALSE	12
k__Archaea;p__Crenarchaeota	FALSE	11
Bacteroidetes	FALSE	7

Supplemental Table 2.5: Analysis of Composition of Microbiomes among pens with different levels of total liver abscess prevalence for liver abscess 16S rRNA gene sequencing data.

Taxa	Reject null hypothesis	W
Chlorobi	TRUE	23
Acidobacteria	TRUE	18
Chloroflexi	TRUE	18
Synergistetes	FALSE	13
[Thermi]	FALSE	12
Planctomycetes	FALSE	10
TM7	FALSE	10
WPS-2	FALSE	10
GN02	FALSE	9
OctSpA1-106	FALSE	9
Cyanobacteria	FALSE	8
Nitrospirae	FALSE	8
k__Bacteria;__	FALSE	7
Actinobacteria	FALSE	7
Armatimonadetes	FALSE	7
Gemmatimonadetes	FALSE	7
	FALSE	5
Tenericutes	FALSE	5
Fusobacteria	FALSE	4
OD1	FALSE	4
OP1	FALSE	4
k__Archaea;p__Crenarchaeota	FALSE	3
BRC1	FALSE	2
Firmicutes	FALSE	2
Proteobacteria	FALSE	2
Bacteroidetes	FALSE	0
Deferribacteres	FALSE	0
Spirochaetes	FALSE	0
Verrucomicrobia	FALSE	0
Unassigned;__	FALSE	0



Supplemental Figure 2.2. Shannon alpha diversity by treatment group from the clinical trial. Statistical differences were determined using non-parametric Kruskall-Wallis tests (test statistic, H = 0.39; *P* = 0.53) (**A**). PcoA plot based on 16S rRNA amplicon, unweighted UniFrac distance by treatment group. Statistical differences were determined using the non-parametric PERMANOVA tests (pseudo F statistic = 0.97; *P* = 0.39) (**B**). Microbial community composition and diversity did not differ by treatment group.