DISSERTATION

THE EFFECT OF TYLOSIN EXPOSURE OR EXCLUSION ON LIVER ABSCESS PREVALENCE, FECAL PATHOGEN POPULATIONS, AND THE MICROFLORA OF FINISHED BEEF PRODUCTS FROM FEEDLOT CATTLE

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ABSTRACT

THE EFFECT OF TYLOSIN EXPOSURE OR EXCLUSION ON LIVER ABSCESS PREVALENCE, FECAL PATHOGEN POPULATIONS, AND THE MICROFLORA OF FINISHED BEEF PRODUCTS FROM FEEDLOT CATTLE

There is great pressure to reduce use of, and find alternatives to, antibiotics in animal production. More than 70% of feedlot cattle in 1000+ head lots are currently exposed to tylosin phosphate, a macrolide antibiotic used for the reduction and prevention of liver abscesses. As such, its potential removal from cattle feeding strategies could have a marked impact on both the economics of the beef industry and food safety. Additionally, little is known about the effect of tylosin exposure or exclusion on the microbiome of finished beef products. In light of these facts, a blinded, randomized, controlled field trial was conducted to evaluate the impact on the prevalence of liver abscesses and the characterization of the microbiome of feces, liver abscesses, carcasses, and finished beef products through 16S rRNA gene sequencing and shotgun sequencing from feedlot cattle with and without exposure to tylosin. Overall, liver abscess rates were lower among cattle exposed to tylosin. However, there were no differences among treatment groups for any of the sample types, suggesting that removal of tylosin from current feeding strategies will not upend the safety of the beef supply. The information gained in this study will provide valuable insight as the search for alternative feeding strategies to antibiotics continues.

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CHAPTER 1

Literature Review

Antibiotic Use in Animal Agriculture

In recent years, use of antibiotics in beef cattle production has come under intense scrutiny. Tylosin phosphate is an antibiotic commonly utilized prophylactically in cattle feeding for the reduction and prevention of liver abscesses. As a macrolide antibiotic, tylosin falls into a class of antibiotics that are considered important to human medicine, and with the implementation of the Veterinary Feed Directive (VFD), use of tylosin in beef feedlot systems now requires veterinary oversight (FDA, 2015). As public scrutiny of prophylactic antimicrobial use in animal production increases, it is imperative to investigate alternatives to using tylosin phosphate. However, as these alternatives are identified, it is equally important to consider any potential ramifications of alternatives on the safety and quality of beef products.

Background

For decades, use of antimicrobial drugs in food animal production has been the topic of great scrutiny and debate. As early as the 1940s, penicillin was used to treat bovine mastitis, marking some of the earliest usage of antibiotics in animal production (Gustafson and Bowen, 1997). In the ensuing years, several studies concluded that the addition of antibiotics to animal feed resulted in increased growth performance in poultry, swine, and cattle (Moore et al., 1946; Loosli and Wallace, 1950; Jukes and Williams, 1953; Perry et al., 1953). As use in animal production increased, antibiotics were commonly administered as a prophylactic measure across an entire herd or flock

upon appearance of one or two sick animals in order to maintain herd health (Gustafson and Bowen, 1997). As the frequency and reasons for use of antibiotics increased, so did debate concerning the relative risks and benefits of “sub-therapeutic” use, that is, the use of antibiotics for growth promotion and disease prophylaxis. In 1969, the United Kingdom formed a committee to look into such risks; the result of which was the recommendation that antibiotics used in human medicine be restricted to therapeutic use in animal production lest the increased use promote bacterial resistance to antibiotics (Swann, 1969). Following recommendations of the Swann Report, the European Community put forth a similar restriction in 1973. By 1986, Sweden banned use of growth-promoting antibiotics altogether with the entire European Union following suit by 2006 (EPC, 2005; Marshall and Levy, 2011).

Today, more than 100 antimicrobials are used in global food animal production for the prevention and treatment of diseases (Hao et al., 2014). These antimicrobials use a variety of mechanisms to affect their desired outcome. Antibiotics, specifically, fall into one of two categories depending on their mode of action: bacteriostatic or bactericidal (Pankey and Sabath, 2004). Bacteriostatic agents restrict cell growth and reproduction whereas bactericidal agents cause bacterial cell death. There are five basic modes of action by which antibiotics act to either inhibit or eliminate bacterial cells: 1) inhibition of cell wall synthesis; 2) alteration of cell membrane structure; 3) inhibition of protein synthesis; 4) inhibition of nucleic acid synthesis; and 5) disruption of other metabolic processes.

Inhibition of cell wall synthesis can occur at a number of points in the synthesizing process. β-lactams such as penicillins, carbapenems, and cephalosporins

inhibit synthesis of peptidoglycans, a vital structural component of the cell wall. Glycopeptides, like vancomycin, allow peptidoglycan synthesis, but prevent formation of cross-linkages between peptidoglycans. Finally, antibiotics like topical bacitracin work to disrupt peptidoglycan precursors. In addition to antibiotics which utilize inhibition of bacterial cell wall biosynthesis, other classes such as lipopeptides, alter several cell membrane functions, leading to cell death.

Classes of antibiotics that perform protein synthesis inhibition to eliminate or stall the growth of bacteria include aminoglycosides, tetracyclines, macrolides, chloramphenicol, lincosamides, and streptogramins. The antibiotics prevent the production of enzymes and cellular structures required by the bacterial cells. Aminoglycosides, such as streptomycin and tetracyclines, act on the 30S ribosomal subunit. Aminoglycosides bind to the ribosomal proteins causing cell death while tetracyclines block the binding site for tRNA to the ribosome, thereby inhibiting bacterial growth. Macrolides, chloramphenicol, lincosamides, and streptogramins all act on the 50S ribosomal subunit and prevent peptide elongation.

Inhibition of nucleic acid synthesis may affect DNA or RNA synthesis. Quinolones, such as ciprofloxacin, bind to the alpha subunit of DNA gyrases which are required for the supercoiling of DNA, and metronidazole produces cytotoxins which disrupt DNA structure. Antimycobacterials like rifampicin bind to DNA-dependent RNA polymerase, thereby inhibiting RNA synthesis. Some classes of antibiotics utilize different methods of cell inhibition. Sulfonamides, for example, attack the folic acid pathway, preventing the production of folic acid, a necessary precursor for DNA synthesis. Each of these five modes of action could lead to either bactericidal or

bacteriostatic action depending on the antibiotic. For example, as referenced above, macrolides and streptogramins both inhibit protein synthesis; however, macrolides are bacteriostatic while streptogramins are bactericidal (Kapoor et al., 2017).

Antimicrobial Resistance

The Centers for Disease Control and Prevention (CDC) defines antimicrobial resistance (AMR) as the phenomenon that occurs when microbes develop the ability to defeat or resist the drugs that are designed to destroy them (CDC, 2018a). The AMR can be naturally-occurring or may be acquired through selective pressure or horizontal gene transfer. The mechanisms of resistance are as varied as the antibiotics against which they work. Naturally occurring mechanisms of resistance include a lack of target or receptor sites for the antimicrobial to attach, whereas acquired resistance mechanisms include efflux pumps; hydrolysis, phosphorylation, acetylation, or nucleotidylation of the target bacteria; and alteration of the target site (Davies and Davies, 2010).

Although the conversation regarding antimicrobial resistance has grown in recent years, AMR has affected humankind for centuries. Resistance genes have been identified in the mummified remains of an 11th century Andean female (Santiago-Rodriguez et al., 2015) and the Tyrolean mummy (Lugli et al., 2017). Additionally, naturally-occurring resistance genes were found in the microbiome of a New Mexican cave which dates back approximately 4 million years (Bhullar et al., 2012).

Though AMR can occur naturally in any environment, the increased use of antibiotics over the course of the last century has further contributed to an increased emergence of resistance genes (WHO, 2018). Shortly after the discovery of penicillin in

1928, a penicillinase was discovered in 1940 which allows for cleavage of penicillin, thus rendering it ineffective. With the wide-spread use of penicillin as a therapeutic agent, resistant bacterial strains were selected and allowed to proliferate (Abraham and Chain, 1940). The discovery of genetically-transferable resistance, called horizontal gene transfer (HGT), has further impacted the discussion around AMR (Davies, 1995). Although HGT has existed between bacterial species for millions of years, the rate at which AMR genes are being spread via HGT and the number of resultant resistant strains has increased over the past few decades, largely influenced by increased selective pressure (Barlow and Hall, 2002; Davies and Davies, 2010). Horizontal gene transfer occurs through transformation, transduction, and conjugation. In the process of transformation, bacteria incorporate free fragments of DNA, usually in the form of plasmids, into their genetic makeup whereas transduction involves introduction of new genetic material via bacteriophage (Rogers, 2011). Plasmid-associated conjugation is the most common mechanism of gene transfer (Norman et al., 2009). Doucet-Populaire et al. (1992) found that *Enterococcus* bacteria transferred resistance genes through HGT to *E. coli* in the digestive tract of mice.

The Veterinary Feed Directive

As the discussion regarding AMR evolves, the role of human antibiotic use in the promotion of resistance is undeniable. However, there is growing concern that the use of antimicrobials in food animals has a direct impact on humans and the antibiotics used in human medicine. Early studies by Levy et al. (1976) and Linton et al. (1977) first discussed spreading of resistant *E. coli* throughout poultry flocks, and from poultry to humans; the possible link between antibiotic use in animal agriculture and increased

AMR in humans has been a topic of great controversy since (Gustafson and Bowen, 1997; Witte, 2000; Davies and Davies, 2010; Landers et al., 2012).

In the United States, antibiotic usage is under the purview of the Food and Drug Administration (FDA). Prior to 1996, the FDA had two distribution categories for animal drugs: over-the-counter and prescription. At the time, antibiotics included in animal feeds were generally approved for over-the-counter use—meaning they could be purchased without a prescription or without veterinary oversight. The introduction of the Animal Drug Availability Act (ADAA) in 1996 created a new class of animal drugs called veterinary feed directive (VFD) drugs. As defined by the ADAA, VFD drugs are new animal drugs intended to be used in animal feeds and must be used only under the supervision of a veterinarian. In the ensuing years, however, very few VFD drugs were approved, as the process for use was considered extremely burdensome, and concern regarding the impact of antibiotic use in animal production on antimicrobial resistance grew. In April 2012, FDA released a statement of guidance, GFI #209, which detailed concerns about the injudicious use of medically-important antimicrobials in food animal production. This document suggested the limitation of medically-important drug use in food animals to therapeutic use under the care of a veterinarian. A second guiding document, GFI #213 outlined a potential timeline for implementation of GFI #209 (FDA, 2015). The VFD Final Rule was released in 2015, and full implementation of GFI #209 and #213 was complete by December of 2016, limiting the use of medically-important antibiotics for prevention, control, and treatment of specific diseases identified by a veterinarian (FDA, 2017).

Tylosin Phosphate

One of the antibiotics affected by the VFD rule is tylosin phosphate. Belonging to the macrolide class of antibiotics, tylosin falls into the World Health Organization's category of critically-important antimicrobials (WHO, 2016). As of 2011, tylosin was used in 71.2% of 1000+ head feedlots for the reduction and prevention of liver abscesses (USDA, 2013). Still used in the majority of feedlots today, the search for tylosin alternatives is imperative in light of the increased concern regarding antimicrobial resistance.

Liver abscesses are currently thought to be caused by the bacterium *Fusobacterium necrophorum*, which is native to the gastrointestinal system of beef cattle (citation). As a macrolide antibiotic, tylosin binds to the 50S ribosomal subunit and causes dissociation of peptidyl-tRNA from the ribosome, resulting in the interruption of peptide elongation during RNA translation (Tenson et al., 2003). The mode of action of tylosin with regard to liver abscesses is believed to be the inhibitory effect it exerts on *F. necrophorum*. Macrolides are primarily effective on gram-positive organisms. *F. necrophorum* is a gram-negative organism, however, it has shown susceptibility to tylosin phosphate (Lechtenberg et al., 1998). Potter et al. (1985) studied tylosin and the ionophore monensin alone and in conjunction with one another, and found that while monensin reduced feed intake and improved feed efficiency, it did not affect liver abscesses. Conversely, the inclusion of tylosin reduced the occurrence of liver abscesses by 18% resulting in improved average daily gain (ADG).

Alternative methods of controlling liver abscesses include addition of roughage in finishing diets, use of direct-fed microbials, inclusion of essential oils in the diet, and

vaccination. Because liver abscesses are thought to be caused by bacteria that escape the rumen during acidosis, increased roughage levels in the finishing diet have been suggested as potential method of mitigation. Several studies have shown that increased levels of roughage in the finishing diet are correlated with lower prevalence and severity of liver abscesses (Harvey et al., 1968; Gill et al., 1979; Zinn and Plascencia, 1996). However, studies conducted by Kreikemeier et al. (1990) and Stock et al. (1990) both indicated no difference in prevalence of liver abscess with increased intake of roughage.

As interest in finding alternatives to using antimicrobials in feedlot diets has increased, studies to evaluate efficacy of direct-fed microbials (DFM), vaccines, and essential oils on rumen conditions, pathogen control, feedlot performance, and carcass characteristics were conducted. Studies of *Lactobacillus*-based DFM demonstrated a positive effect on *E. coli* shedding in feedlot cattle but had little effect on feedlot performance (Brashears et al., 2003; Younts-Dahl et al., 2005). DiLorenzo et al. (2006) compared use of polyclonal antibodies with tylosin to reduce rumen populations of *F. necrophorum* in beef steers. While populations of rumen of *F. necrophorum* were reduced compared to steers receiving no antibody supplementation, tylosin continued to produce a greater population reduction. There has also been great interest in developing a vaccine which would target the pathogenicity and virulence of *F. necrophorum*. Two vaccines have reached the commercial market; however, neither has proven to be consistently efficacious for mitigation of liver abscesses or reduction in abscess severity (Checkley et al., 2005; Fox et al., 2009). As a proposed alternative to antibiotics, it is thought that essential oils disrupt the cytoplasmic membrane of bacterial cells and thereby destroy the cell; however the exact mechanism is not well known

(Nazzaro et al., 2013). Limonene, a naturally-occurring compound found in lemons, oranges, and grapefruits (Castillejos et al., 2006), was found to reduce populations of rumen *F. necrophorum*, both *in vitro* and *in vivo,* and may have potential to mitigate incidence of liver abscesses (Elwakeel et al., 2013; Samii et al., 2016).

Of particular interest among those studying natural alternatives to antibiotics are yeast cultures and yeast fermentation products. Several studies have focused on the impact of feeding *Saccharomyces cerevisiae* live cultures to rumen microflora populations and rumen pH levels. These studies have indicated an efficacy of *S. cerevisiae* for increasing ruminal microbial populations, as well as stimulating lactate utilization, thereby reducing lactic acid concentrations and increasing rumen pH (Yoon and Stern, 1996; Callaway and Martin, 1997; Desnoyers et al., 2009; Calsamiglia et al., 2012). Although yeast cultures and fermentation products have shown success in reducing subacute acidosis, their effects are likely due to modification of the fermentation process in the rumen rather than a direct impact on pH levels themselves (Calsamiglia et al., 2012). Meta-analyses of *S. cerevisiae* fermentation products (SCFP) suggest that SCFP may provide benefits in both the dairy and beef cattle sectors. Inclusion of SCFP in the diets of dairy cattle resulted in increased dry matter intake (DMI) during early lactation and an overall increase in milk yield (Poppy et al., 2012). A review by Wagner et al. (2016) highlighted positive impacts of SCFP inclusion in feedlot diets, including increased ADG, DMI, and feed efficiency (G:F). The review also found nine studies that evaluated the impact of SCFP on carcass characteristics and noted an overall improvement in carcass quality grade. The favorable impact of SCFP on rumen

conditions and feedlot performance suggest that the effect of SCFP may extend beyond the rumen and provide aid in mitigation of liver abscesses.

Liver Abscesses

Prevalence

Bovine liver abnormalities have impacted the feedlot and beef industries for decades. First identified in 1940, studies have shown that liver abscesses negatively impact ADG, feed efficiency, and carcass characteristics (Smith, 1940; Brown et al., 1973; Rust et al., 1980; Brink et al., 1990). The leading cause of liver condemnations today, abscesses occur in both beef-type and dairy-type cattle (Eastwood et al., 2017). Liver abscesses commonly occur in grain-fed cattle, and prevalence can range from 1 to 100% in any particular group; however, no scientific literature suggests the cause of such a wide range in liver abscess prevalence (Nagaraja and Chengappa, 1998). The 2016 National Beef Quality Audit (NBQA) reported 17.8% of livers from commercial beef cattle in the U.S. were condemned due to the presence of an abscess-- an increase from 13.7% in the 2011 NBQA (McKeith et al., 2012; Eastwood et al., 2017). Liver abscesses are generally thought to be directly related to feedlot management, specifically the rapid transition to high-concentrate diets; however, a number of factors may influence their formation including diet, days on feed, cattle type, breed, gender, geographic location, and season (Reinhardt and Hubbert, 2015). Inclusion of roughage in feedlot diets has resulted in limited improvement in prevalence of liver abscesses, and the effects of grain type, dry hay vs. silage, and grain processing also have been studied as dietary factors which may contribute to the incidence of liver abscesses (Hale, 1985; Stock et al., 1987; Mader et al., 1991). Among all feedlot cattle, abscess

prevalence and severity have consistently been greater in steers than in heifers and in Holstein cattle compared with beef breeds. Higher abscess rates in steers and Holsteins are attributed to greater DMI and increased days on feed for steers and Holsteins than for heifers or beef breeds, respectively (Amachawadi and Nagaraja, 2016).

Across all cattle breeds and genders, liver abscesses have significant economic ramifications for the beef industry, primarily impacting animal performance and carcass yield. If liver abscesses are scored at the time of harvest the Elanco Liver Check System is employed. The Elanco Liver Check System scores livers based on presence, number and size of abscesses. A healthy liver with no abscesses would receive a score of 0. Abscessed livers are categorized by A-, A, or A+ scores, with scores increasing with severity of the abscess. A- and A livers are considered mild to moderate and would contain one to four small abscesses under 2.54 cm in diameter, whereas a severely abscessed liver would be assigned a score of A+ and contain one or more large abscess and inflammation of the liver tissue. Severe abscesses are often accompanied by additional adhesions on the lung and diaphragm (Elanco, 2018).

Estimated annual losses due to liver abscess condemnations are $15.8 million across the United States beef industry (Hicks, 2011). Mild and moderate abscesses have little impact on live animal performance (Brown and Lawrence, 2010). However, severely abscessed livers have been shown to decrease ADG by as much as 11% and feed efficiency by as much as 9.7% (Brink et al., 1990). Additional research indicated that severely abscessed livers also negatively impact carcass value. The change in value can be attributed to decreased HCW, marbling score, yield grade, and dressing percentage (Brown and Lawrence, 2010). Davis et al. (2007) reported severe liver

abscesses associated with a 3.2 kg decrease in HCW, but noted that HCW dropped 13.2 kg on average when severe abscesses were detected in conjunction with carcass adhesions.

Etiology and Pathogenesis

Although bovine liver abscesses are thought to be associated with the high-concentrate diets of feedlot systems, there is some disagreement regarding the specific causative agent or agents. While liver abscesses are polymicrobial in nature, culture studies of liver abscesses have primarily indicated presence of *Fusobacterium necrophorum* and *Trueperella pyogenes* in addition to *Bacteroides* spp., *Staphylococcus* spp., and *Streptococcus* spp. among others (Scanlan and Hathcock, 1983). Due to its isolation in nearly all studies, *F. necrophorum* is considered to be the primary causative bacterium of bovine liver abscesses. Also considered to be the primary causative agent in calf diphtheria and foot rot, *F. necrophorum* is an anaerobic, gram-negative bacterium naturally occurring in the gastrointestinal system of both animals and humans. The ability of *F. necrophorum* to thrive within the liver environment is believed to be attributed to the virulence factors leukotoxin and endotoxic lipopolysaccharide (Tan et al., 1996).

In the rumen, *F. necrophorum* serves to ferment lactic acid primarily into acetate and butyrate, and is therefore in higher abundance in the rumen of cattle fed a ration high in grains such as corn. Nagaraja et al. (1999) reported that the number of *F. necrophorum* cells in a rumen exposed to a high-grain ration is 10-fold that of a rumen exposed to a forage-based diet. *F. necrophorum* also has been commonly isolated from the inflamed rumen lining associated with parakeratosis and ruminitis (Kanoe et al.,

1978). Investigators provided a high statistical correlation between increased rumen *F. necrophorum* populations in the presence of a high-grain diet and their subsequent isolation from inflamed rumen lining, along with levels of *F. necrophorum* isolated from bovine liver abscesses as evidence that *F. necrophorum* is the primary etiologic agent of liver abscesses. As the pH in the rumen declines and acidosis occurs with the introduction of a high-grain diet, *F. necrophorum* migrate to the wall of the rumen and attach via agglutination involving surface proteins (Nagaraja and Lechtenberg, 2007). Damage to the rumen epithelium, either due to acidosis resulting from excessively decreased pH levels or by physical damage from sharp feed particles results in lesions on the rumen wall, thus allowing *F. necrophorum* to escape into the blood stream and enter the liver via portal blood and the hepatic portal vein. Although anaerobic, *F. necrophorum* is thought to overcome the oxygen-rich environment of the liver. Colonization of *F. necrophorum* in the liver is the primary stage of liver abscess formation (Nagaraja and Chengappa, 1998).

Smith (1944) first observed the concurrent occurrence of ruminitis and liver abscesses in feedlot cattle, and the phrase “ruminitis-liver abscess complex” was later coined by Jensen et al. (1954). In contrast with the ruminitis-liver abscess complex, Wieser et al. (1966) failed to detect a correlating relationship between rumen inflammation or lesions with liver abscesses. A study conducted by Narayanan et al. (1997) provided further evidence of the link between ruminitis and liver abscesses through restriction fragment length polymorphism analysis of rRNA genes, confirming presence of the same strain of *F. necrophorum* in rumen contents, the rumen wall, and liver abscesses. In addition to *F. necrophorum*, *Salmonella enterica* was recently

anaerobically isolated from bovine liver abscesses. More virulent and invasive than aerobically grown *S. enterica*, the newly isolated bacterium called into question the role of *S. enterica* in abscess formation (Amachawadi and Nagaraja, 2015).

Although the exact etiological mechanism is not formally documented, the correlation between ruminitis and bovine liver abscesses is commonly accepted. However, from an epidemiological perspective, the concept of component causes can be applied to liver abscesses. Component causes, or multicausality refers to the possibility that many underlying factors that could contribute to occurrence of liver abscesses. It is possible that *F. necrophorum* may not be the primary causative agent of bovine liver abscesses, but rather an opportunistic bacterium which proliferates in the environment provided by the abscess (Rothman and Greenland, 2005). Similarly, in human colorectal cancer research, a theory termed the “driver-passenger” model has emerged and is gaining acceptance. Not unlike the driver-passenger theory of gene mutations, wherein “driver” mutations are found to enable rapid growth of cancerous cells while “passenger” mutations have little to no impact on cancerous cell growth, the driver-passenger model is now being applied to the entire bacterial flora associated with colorectal cancer (Tjalsma et al., 2012). This theory suggests that causative, or driver, bacteria, which aid in initiation of tumorgenesis, may be outcompeted by native commensal gut bacteria which thrive in the tumor microenvironment, ultimately overcoming and eliminating the driver bacteria from the environment. This model could be similarly applied to liver abscesses. As *F. necrophorum* is an anaerobic bacterium, it would not be expected to thrive in the oxygen rich environment of the liver and thereby initiate abscess formation. Instead, *F. necrophorum* may act as a “passenger” bacteria

proliferating and outcompeting the “driver” bacteria in the oxygen-deprived environment characteristic of liver abscesses.

Detection and Prevention

Diagnosis of liver abscesses is generally limited to the time of slaughter as cattle seldom experience any clinical symptoms. Rarely, an abscess will rupture causing extreme pain, widespread infection, and death (Rubarth, 1960). Liver enzyme tests have not proved to provide indication of liver abscess formation (el-Sabban et al., 1971). Ultrasonography, though useful for detection of liver abscesses is cost- and labor-prohibitive in addition to exposing feedlot animals to undue stress from additional, unwarranted handling. Ultrasonography can, however, be useful in research settings (Nagaraja and Lechtenberg, 2007).

The primary methods of liver abscess prevention have been discussed previously and include use of antimicrobial feed additives such as tylosin or natural alternatives such as DFM or essential oils, increased dietary roughage, and the creation of an effective vaccine. While the primary use of tylosin in feedlot systems is the prevention of liver abscesses, its inclusion or exclusion in the diets of feedlot cattle, may have unintended consequences on food safety.

Food Safety

Foodborne Illness in the United States

Foodborne illness is a national and global public health priority. The CDC estimated that foodborne pathogens cause more than 48 million cases of foodborne illness, 128,000 hospitalizations, and 3000 deaths annually in the U.S (CDC, 2017). The majority of these illnesses are caused by Norovirus, but *Salmonella enterica* is the

second leading culprit, with beef responsible for 99 *Salmonella* outbreaks since 1973 and 8 *E. coli* STEC outbreaks (O157:H7 and O26) since 2006 (Scallan et al., 2011; Laufer et al., 2015; CDC 2018d). First recognized as a pathogen in 1982, *E. coli* O157:H7 was declared an adulterant in 1994. Since that time, considerable effort has been made to reduce the occurrence of pathogens such as Shiga toxin-producing *E. coli* (STEC; includes *E. coli* O157:H7 and non-O157 *E. coli* shiga-toxin producing strains) and *Salmonella* in fresh beef products (Huffman, 2002; Koohmaraie et al., 2005; Wheeler et al., 2014). Cattle are natural harbors for STEC, and Elder et al. (2000) suggested that hide contamination is the most likely source of carcass contamination during processing. Outbreak data collected during the 2000s indicated that ground beef was a more common vessel for *Salmonella* infections (Laufer et al., 2015). Investigation into the transfer of antibiotic resistance genes from *Enterococcus* bacteria, a member of the Firmicutes phylum, to *Enterobacteriaceae* such as *E. coli* and *Salmonella* is ongoing. The National Antimicrobial Resistance Monitoring System (NARMS) included *Enterococcus* as one of the bacteria under surveillance in humans, retail meats, and food animals, making it an organism of interest in cattle feeding and beef safety (FSIS, 2018).

Pathogens and Indicator Organisms

E. coli is a rod-shaped, gram-negative bacterium belong to the phylum Proteobacteria. *E. coli* biotype I may be referred to as generic *E. coli* and is often used as an indicator organism with regard to the potential safety of fresh meats (Jay et al., 2005). However, a study of frozen beef patties found no evidence supporting a link between the presence of *E. coli* biotype I and *E. coli* O157:H7 (Pruett et al., 2002).

Responsible for the largest number of outbreaks attributed to beef, *E. coli* O157:H7 is part of a group of *E. coli* strains known as shiga-toxin producing *E. coli*, or STEC. There are six additional non-O157 STEC (O26, O45, O103, O111, O121, O145). In 2012, these six pathogens joined *E. coli* O157:H7 on the adulterant list. The CDC estimates that there are approximately 265,000 STEC infections each year (CDC, 2018b).

S. enterica is another rod-shaped, gram-negative organism of the family *Enterobacteriaceae*. *S. enterica* is categorized into subspecies or serotypes. *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis are the most common serotypes in the United States. *Salmonella* is responsible for an estimated 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths in the U.S. annually (CDC, 2018c). Though not considered an adulterant at this time, *Salmonella* is on the NARMS list of bacteria that are under surveillance (FSIS, 2018).

Another bacterium on the NARMS surveillance list is *Enterococcus* (FSIS, 2018). Identified as a gram-positive cocci belonging to the family Firmicutes and commonly found in the intestinal tract of humans and animals, *Enterococcus* is not a pathogen (Jay et al., 2005). However, it is identified as an indicator organism with regard to food safety due to its ability to readily transfer resistance genes to pathogenic bacteria, such as *E. coli* and *Salmonella* (Courvalin, 1994).

Food Safety Interventions

Implementation of pre- and post-harvest interventions are vital to the maintenance of food safety. The outbreak of *E. coli* in the Western U.S. during 1992-1993, which resulted in the death of four children, was the ignition point for a series of regulations and policies aimed at reducing risk of *E. coli* in the meat industry (CDC,

1993). The first of these policies was enforcement of zero tolerance for visible contamination, followed by mandated Hazard Analysis and Critical Control Point (HACCP) implementation a couple of years later. More recently, six non-O157 Shiga toxin-producing *E. coli* (STEC) were added to the adulterant list. With creation of industry groups such as the Beef Industry Food Safety Council (BIFSCo), food safety and interventions became a non-compete issue within the meat industry and relevant information was shared among companies (Wheeler et al., 2014).

As the hide has been shown to be the most common pathogen reservoir and, therefore, the primary source of carcass contamination, the majority of pre-harvest strategies for mitigation of foodborne pathogens are aimed at reducing pathogen loads on the animal and include DFM, bacteriophages, vaccines, and live animal washes. Direct-fed microbials, sometimes referred to as probiotics, have been studied as possible methods for pathogen reduction, but results have not been consistent (Arthur et al., 2010). A study conducted by Brashears et al. (2003) which utilized a *Lactobacillus*-based probiotic to reduce populations of *E. coli* O157:H7 in feedlot cattle has shown the most promise with regard to the use of DFM as a pre-harvest intervention. Though not currently widely used, as the push to remove antibiotics from animal production continues, probiotics may serve a larger role in feedlot systems.

Vaccines are the most difficult, and often costly, of the pre-harvest interventions to implement. Vaccines often require 2 to 3 doses to be considered effective, a process that requires additional man-power, time, and puts additional stress on the animals. Additionally, there is currently no incentive to vaccinate, and cattle can become re-contaminated upon arrival to the plant through contact with un-vaccinated cattle or

contact with feces from a previous load of un-vaccinated cattle (Wheeler et al., 2014; Ochieng' and Hobbs, 2016). The simplest of the pre-harvest strategies, live-animal washes at the site of the processing facility, often act as a final pre-harvest intervention to aid in removal of fecal matter and pathogens from the hide.

Post-harvest strategies are aimed at minimizing contact of the hide with the carcass surface and removing any potential contamination after removal of the hide. These strategies include physical, thermal, and antimicrobial interventions such as organic acid washes, steam vacuum, and physical removal of contamination via knife trimming. During beef harvest, any area where the hide is cut is a possible site of contamination. Steam vacuuming is a combination of physical and thermal interventions and has been shown to be effective, particularly in areas of the carcass that are known to be commonly contaminated. Carcass washes and sprays are utilized both before and after complete hide removal and may consist of a dilute organic acid such as lactic acid or peroxyaceteic acid (Wheeler et al., 2014).

During the harvest process, if contamination is noted, knife trimming and steam vacuuming is the most common methods by which the contaminated area is removed. Additional knife trimming is currently being utilized in most beef plants as of January 2018 to remove key peripheral lymph nodes from the beef carcass. As *Salmonella* has been isolated from peripheral lymph nodes, FSIS included in the 2017 guidelines a recommendation to remove the following lymph nodes from the beef carcass: 1) superficial cervical; 2) subiliac; 3) axillary; 4) popliteal; 5) coxalis; and 6) Iliofemoralis of the head (FSIS, 2017). (Koohmaraie et al., 2012; FSIS, 2017; Webb et al., 2017).

Detection and Characterization of Foodborne Pathogens

Detection of foodborne pathogens and indicator organisms is an ever-moving field. Detection and diagnostics are critical to maintenance of a safe food supply. For years, the gold standard of pathogen detection has been traditional plate culture. However, this can be very costly, both in time and dollars spent. The ever-looming ideal of pathogen detection would combine speed with accuracy and cost-effectiveness. More recently, new methods have emerged, including enzyme-linked immunosorbent assays (ELISA) and molecular methods such as polymerase chain reaction (PCR) and use of PCR, specifically quantitative PCR (qPCR), has been investigated in the meat industry. However, no method is 100% specific and 100% sensitive; therefore, both methods are still widely utilized. Pathogen detection by PCR is largely the primary method of detection while culture techniques serve to confirm positive results received from PCR (Priyanka et al., 2016).

Though not ideal for rapid, day-to-day detection of pathogens, another new technology has emerged: next generation sequencing (NGS). Even with the development of selective media and refinement of culture techniques over more than 100 years, traditional culture methods have succeeded in isolating less than 1% of microorganisms (Slonczewski and Foster, 2014). A term coined by Staley and Konopka (1985), the Great Plate Count Anomaly, has been used to describe the difference in the number of cells that can be grown on culture media and those that are identified through microscopy. With the development of next generation sequencing, such as 16S rRNA gene amplification and sequencing and shotgun sequencing along with metagenomic analysis, the entire microbiome is open to evaluation. Though still new, many studies in

animal agriculture have already been completed and demonstrate the utility of NGS approaches to “old” challenges. Weinroth et al. (2017) used 16S rRNA gene sequencing to characterize liver abscesses of feedlot cattle while Thomas et al. (2017) and Mao et al. (2015) have utilized it to characterize the gut microbiota of feedlot and dairy cattle. Shotgun sequencing is also being used for detection of pathogens and resistance genes in beef production systems (Noyes et al., 2016; Yang et al., 2016). Across the agriculture and food industries, NGS will allow the exploration of many new frontiers as the technology continues to progress and change.

Impacts to Current and Future Research

With implementation of the Veterinary Feed Directive and the push to remove medically-important antibiotics, such as macrolides, from beef production, it is imperative to search for antibiotic alternatives that will reduce or prevent liver abscesses. Additionally, little is known regarding the impact of tylosin exposure or exclusion on the microbiome of feedlot cattle. Characterization of the microbiome of beef carcasses and trimmings is of utmost importance in order to maintain the safety and quality of beef in the Unites States. This study seeks to determine the impact of tylosin exposure and exclusion on liver abscess prevalence, fecal microbiome, and the microbiome of finished beef products.

CHAPTER 2

The Effect of Tylosin Exposure or Exclusion on Liver Abscess Prevalence, Fecal Pathogen Populations, and the Microflora of Finished Beef Products From Feedlot Cattle

Introduction

Liver abscesses are the most common liver defect in commercial cattle at the time of harvest (Brown and Lawrence, 2010). Utilization of tylosin phosphate, a macrolide, for reduction and prevention of liver abscesses in commercial feedlot cattle has long been a standard practice (Potter et al., 1985). Current, tylosin phosphate is widely used in feedlot cattle in the United States for the prevention of liver abscesses—in 2013, more than 70% of 1000+ head feedyards utilized this tool (USDA, 2013). Despite its efficacy in reducing liver abscesses, macrolides are considered an antibiotic important in human medicine. Consequently, in the past decade, significant pressure has been placed on the commercial cattle industry to identify alternatives to tylosin phosphate. Macrolides, which target gram-positive bacteria, are generally considered ineffective against gram-negative bacteria such as *Escherichia coli* or *Salmonella*; however, the full extent of the effect of tylosin on feedlot cattle feces, liver abscesses, carcasses, and beef trimmings has not been studied. .

Furthermore, as feeding practices are altered, it is imperative to examine the impact on populations of bacteria in beef production. Thus, the objective of this study was to determine the impact of tylosin exposure and exclusion on prevalence of liver abscesses in feedlot cattle and on the microbial populations of feces, liver abscesses, beef carcasses, and beef trimmings. This study employed not only traditional

assessments of microorganisms and their characteristics, but also 16S rRNA gene sequencing to gain a unique insight into the diverse microbial communities of a variety of samples derived from the beef production system. This information will provide valuable insight toward development of feed and water liver abscess mitigation strategies alternatives for future use in beef production.

Materials and Methods

Sample Population

As part of a blinded, randomized, controlled trial, commercial steers (N = 5,481) were sourced and placed at a feedyard in Texas. Ten 4-pen blocks (40 pens) of steers, each consisting of one pen from each of four treatment groups, were assembled beginning in March 2016. Pens contained 60 to 250 head of cattle, with each pen within a block containing an equal number of steers at the onset of the trial. Upon arrival, cattle were weighed and received a standard processing regimen, which included the following: drench dewormer, IBR and BVD vaccines, lot tag, and ear implant (Revalor-XS; Merck Animal Health, Madison, NJ). Following randomization of cattle, pens within each block were randomly assigned to one of four treatments. Cattle weighing less than 341 kg or greater than 398 kg were excluded from the trial. Treatment groups (described below) were all provided corn-based rations ad-libitum with one of the following modifications: a) tylosin (90 mg/hd/d, Elanco Animal Health, Indianapolis, IN) fed until harvest (**TYL**); b) no tylosin (**CON**); c) without tylosin, but with an essential oil (1 g/hd/d of CRINA-L; source of limonene) fed until harvest (**EO**); and d) without tylosin but with a *Saccharomyces cerevisiae* fermentation product (18 g/hd/d) fed until harvest (**SCP**). Before beginning treatment rations, cattle were acclimated to the feedyard

through a standard step-up feeding program consisting of a three-ration transition. Rumensin (Elanco Animal Health) was included in the intermediate and finishing rations, and Optaflexx (Elanco Animal Health) was included in the final 28 to 42 d of the finishing period.

Sample Collection

Feces. Composite fecal samples were obtained from each pen floor within four weeks of placement in the feedyard and again two to four d prior to harvest, following the method described by Yang et al. (2016). Samples were placed into individual WhirlPak bags (Nasco, Modesto, CA) and transported on ice to the Center for Meat Safety & Quality Food Safety Microbiology Lab at Colorado State University (Fort Collins, CO) for analysis within 24 to 36 h.

Cattle Processing. Cattle were harvested at a commercial beef processing facility in Texas during a three-week period of time in August and September 2016. Carcass and pen identity were maintained throughout harvest and fabrication.

Liver Abscesses. Livers (N = 5,042) were evaluated for the presence, and severity of liver abscesses and liver abscess samples (n = 5/pen) were removed from condemned livers at the time of evisceration. From designated livers, abscesses were collected using a sterile scalpel to remove the tissue containing the purulent material. Abscess samples were placed into sterile sample bags, chilled, and transported on ice to the Center for Meat Safety & Quality Food Safety Microbiology Lab at Colorado State University (Fort Collins, CO) for processing within 24 h.

Carcass Swabs. Before application of any antimicrobial interventions, carcass swabs were used to sample the plate and brisket of carcasses (n = 15/pen) using two

sterile sponge-sticks (3M®, Maplewood, MN) which were pre-moistened with 10 ml of Phosphate Buffered Saline (PBS). Samples were obtained by making vigorous up/down and left/right manual swabbing motions. One sponge each was utilized to swab the left and right sides of the carcass at a rapid pace. After sampling, sponges were placed into individual bags, chilled, and transported on ice to the Microbial Ecology Group molecular laboratory at Colorado State University for processing within 24 h.

USDA Grading and Beef Trimmings. At the time of carcass grading (30 to 36 h post-harvest), all USDA Choice cattle were diverted to specified carcass rails to facilitate sample collection during fabrication. Carcass and pen identity were maintained during grading and fabrication, and carcasses were fabricated by pen and pens within a block were fabricated sequentially. At fabrication, trimmings were collected from the chuck, loin, and round of carcasses from each treatment group. Approximately 5 kg were collected from each primal, totaling approximately 15 kg per pen. Trimmings were composited into one sample per carcass primal (i.e., chuck, loin, and round) per pen and placed into individual sterile sample bags and transported on ice to the Center for Meat Safety & Quality Food Safety Microbiology Lab at Colorado State University for processing within 24 h. One block (4 pens) was not sampled due to logistical challenges in the carcass cooler.

Sample Processing and Cultural-Based Detection of Pathogenic Bacteria

Feces. Upon arrival to Colorado State University, composite fecal samples were manually homogenized, and 25 g sub-samples were weighed into filtered, sterile sample bags before the addition of 225 ml of Tryptic Soy Broth (TSB, Acumedia-Neogen, Lansing, MI). Samples were subsequently homogenized for two minutes using

a Stomacher (Seward, West Sussex, United Kingdom), before serial dilution in 0.1% Buffered Peptone Water (BPW; Difco, Becton Dickinson and Company, Sparks, MD). Appropriate serial dilutions were plated in duplicate onto MacConkey and Enterococcosel agars (Difco, Becton Dickinson and Company) for enumeration of generic *E. coli* and *Enterococcus* spp., respectively. MacConkey and Enterococcosel plates were incubated at 35°C for 24 and 48 h, respectively before visual counting of representative colonies.

Following the removal of sample aliquots (1 to 3 ml) for enumeration purposes (as described above), fecal samples were enriched to facilitate isolation of generic *E. coli*, *Enterococcus* spp, and *Salmonella enterica*. Homogenized fecal samples were incubated in TSB at 35°C for 18 to 24 h. Following incubation, samples were manually homogenized and, using an inoculating loop, 10 µL of the enrichment was streaked onto MacConkey agar for isolation of generic *E. coli*. Following a 24 h incubation at 35°C, three isolated colonies were selected from each plate and subsequently streaked onto individual MacConkey plates and again, incubated at 35°C for 24 h. Isolated colonies were repetitively streaked onto fresh agar to facilitate isolation of *E. coli*. A final isolated colony was selected from each plate and transferred to a final MacConkey plate to achieve a pure culture. *Enterococcus* spp. were isolated in the same manner utilizing Enterococcosel agar and a 48 h incubation period between transfers. Isolates were frozen in duplicate in a 16% glycerol solution by combining 800 µL of a TSB culture of the isolate and 200 µL of 80% glycerol (in TSB) and stored at -80°C.

For detection and isolation of *Salmonella enterica*, fecal samples were incubated in TSB at 35°C for 18 to 24 h. Samples were manually homogenized and 100 and 500

µL of enrichment were transferred to Rappaport Vassiliadis (RV) Broth (Difco, Becton Dickinson and Company) and Tetrathionate (TT) Broth Base, Hajna (Difco, Becton Dickinson and Company), respectively. Both broths were incubated at 42°C for 24 h. After incubation, 10 µL of TT and RV were streaked onto a Brilliant Green Agar with Sulfadiazine (BGS) agar and Xylose-Lysine-Tergitol-4 (XLT4) agar split plate (Hardy Diagnostics, Santa Maria, CA). Plates were incubated at 35°C for 24 h and 3 isolated colonies from each agar type streaked onto subsequent BGS and XLT4 plates to facilitate colony purification. Isolates were frozen in duplicate in a 16% glycerol solution by combining 800 µL of a TSB culture of the pure isolate and 200 µL of 80% glycerol (in TSB) and stored at -80°C.

Liver Abscesses. Upon arrival to Colorado State University, liver abscess samples were held at 4°C for up to 48 h to await processing. Individual tissue samples were submerged in 100% ethanol and flamed to sterilize the outer tissue. Using a sterile scalpel, abscess capsules were sliced open to access the purulent material. A small amount of purulent material was streaked onto two Blood Agar plates (Hardy Diagnostics, Santa Maria, CA) before aerobic and anaerobic incubation at 35°C for 24 h. After incubation, a single isolated colony was selected from each plate and placed into Brain Heart Infusion (BHI) broth and incubated at 35°C for 24 h under the same aerobic or anaerobic conditions. This process was repeated through two more cycles of Blood Agar and BHI broth to facilitate colony isolation of a pure culture. Isolated colonies were frozen and stored in duplicate as described above.

Beef Trimmings. Upon arrival to Colorado State University, trimmings were held at 4°C for up to 12 h until assessment for prevalence of *S. enterica* and generic *E. coli*.

Samples of trimmings from the chuck, loin, and round of each pen were composited, by pen, to formulate a 25 g sample for each pen. Microbiological assessment followed beef trimming procedures described in the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) Microbiology Laboratory Guidebook (MLG; USDA-FSIS, 2016). Briefly, after the formulation of trim composites, 75 ml of modified Tryptone Soya Broth (mTSB; ThermoFisher Scientific, Waltham, MA) was added. Samples were subsequently homogenized for two minutes using a Stomacher before incubation at 42°C for 15 to 25 h. After incubation, samples were manually homogenized and 10 µL of enrichment streaked onto MacConkey agar before incubation at 35°C for 24 h. Further processing subsequently followed the procedure previously described for isolation of generic *E. coli*. Additionally, trimming samples were assessed for the presence of *Salmonella* using the methods described above. All isolates were frozen and stored, in duplicate, in a 16% glycerol solution as described above.

DNA Isolation

Feces. At the time of microbiological evaluation, composited fecal samples were manually homogenized and 10 g aliquots removed and stored at -80°C for use in assessment of the fecal microbiome. DNA was isolated from fecal samples using the PowerMax Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) with modifications as previously described (Yang et al., 2016). Briefly, 10 g of sample were thawed at 4°C prior to isolation. A sedimentation step to remove large particulates and inhibitors was performed, where the sample was mixed with 30 ml BPW and allowed to separate into two layers. The top layer containing most of the bacteria was centrifuged, and the pellet rinsed with molecular-grade sterile PBS (ThermoFisher Scientific) and

centrifuged again to pellet cells. The supernatant was removed, the resulting pellet was re-suspended in PowerBead solution, and the remainder of the standard protocol followed. 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. Two replicates were extracted for each fecal sample. Replicate extraction occurred on separate days, and the aliquots of the second replicate underwent an additional thaw cycle to weigh aliquots in preparation for extraction. The remainder of the extraction methods were identical between the first and second replicate.

Liver Abscesses. Liver abscess samples were held at 4°C for up to 48 h to await processing for DNA extraction. After sterilization of the external surface as described above, purulent material was placed in a sterile conical tube and stored at -80°C for later DNA extraction. DNA was isolated using the PowerFecal DNA Isolation Kit (Mo Bio Laboratories) with some modifications to the manufacturer's protocol. Sample input was 0.1 to 0.4 g of purulent material. The Mini-Beadbeater-16 was used for the bead beating step, where samples were processed for 3 pulses of 30 s each. 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.

Carcass Swabs. Carcass swabs (two per carcass, one per each side as previously described) were immediately processed by adding 15 ml of PBS to rinse each individual sponge in their sample bag. Following PBS addition, the sponge was manually agitated, the liquid expressed, and the sponge removed from the bag. Afterwards, the PBS solution from the two carcass sponges collected per carcass was

pooled (~40-50 ml total) and centrifuged at 4300 x g for 20 min at 4°C. The supernatant was poured off and the remaining 500-750 µl pellet stored at -80°C. For carcass sponge pellets, DNA was isolated from the whole pellet using the PowerFecal DNA Isolation Kit (Mo Bio Laboratories) with some modifications. Each pellet was re-suspended in 1500 µl of Bead Solution. The re-suspended pellet was then separated into two dry bead tubes (~900 µl each) and processed as two separate DNA isolations until being combined back into one during DNA elution. The Mini-Beadbeater-16 (Biospec Products, Bartlesville, OK) was used for the bead beating step, where samples were bead beat for 3 pulses of 30 s each. During DNA elution from the spin filter, a single 75 µl volume of C6 elution buffer was passed twice through each of the two spin filters used per sample, resulting in a single 75 µl DNA sample per carcass sponge pellet. Three blank samples (where the kit protocol was followed with reagents only) were extracted alongside carcass sponges to serve as a negative control for the extraction procedure.

Beef Trimmings. Upon arrival to Colorado State University, trimmings were held at 4°C for ~12 h to await initial processing. Approximately 1.3 kg of trimmings from each of the chuck, loin, and round per pen was sub-sampled and stored at -20°C for approximately 2-3 weeks to await rinsate recovery. The trimmings were thawed at 4°C, and 270 ml of BPW was added. The trimmings and BPW were mixed, making sure that all meat surfaces were rinsed, while minimizing blood exudation. The trimming rinsate (200-250 ml) was then recovered and pelleted by centrifugation at 4300 x g for 10 min at 4°C. The supernatant was poured off and the remaining 2-5 ml pellet was stored at -80°C. DNA was isolated from entire trimming rinsate pellets using the PowerMax Soil

DNA Isolation Kit (Mo Bio Laboratories) according to the standard protocol. DNA was eluted in 3 ml of the C6 elution buffer.

16S rRNA Gene Sequencing

16S rRNA gene amplification and sequencing for fecal, carcass swab, trimming rinsate, and liver abscess DNA samples were performed by a commercial sequencing company (Novogene Corporation, Beijing, China). Replicates were shipped and analyzed in distinct sequencing runs. The V4 region of the 16S rRNA gene was amplified using the Earth Microbiome Project primer set 515F/806R (Caporaso et al., 2010), with reverse primers containing unique barcode sequences. Library sequencing (paired-end, 2 x 250 base pairs) was performed on an Illumina HiSeq 2500 platform (Illumina, Inc.). Raw data were demulitplexed and quality filtered by Novogene prior to dissemination to Colorado State University.

Shotgun Metagenomics Sequencing

Pooled samples from the replicates of fecal samples collected prior to harvest were 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 Denver Genomics and Microarray Core for sequencing. Genomic libraries for all samples were prepared using the Illumina TruSeq DNA PCR-Free Library Prep Kit, following the protocol to obtain a mean insert size of 350 base pairs. Next generation sequencing was completed on the Illumina HiSeq 4000 with 17 samples/lane, Mode V4 chemistry and paired-end reads of 150 nucleotides in length.

Bioinformatics and Statistical Analysis

All statistical analyses were performed with pen as the experimental unit and an alpha level of 0.05 with statistical trends acknowledged at alpha levels between 0.051 and 0.10.

Liver Abscess Prevalence. Statistical analyses of liver abscess prevalence were performed using the PROC GENMOD procedure of SAS (v. 9.4; Cary, NC) and adjusted relative risk values were determined to control for population structure in Poisson regression models. Treatment differences were detected using the PDIFF statement, and 95% Confidence Limits were produced.

Culture-Based Data. Statistical analyses of *Salmonella* spp. prevalence in feces and trim and counts of generic *E. coli* and *Enterococcus* spp. were performed using the PROC MIXED procedure of SAS with block and treatment as fixed effects. Generated LSmeans were separated using the PDIFF statement and an alpha of 0.05.

16S rRNA Gene Sequencing. Raw data 16S rRNA gene sequencing data received from Novogene were trimmed of primers using cutadapt. Quality was checked using the FastQC tool and Phred Scores and Sequence Length distribution were examined for each read. Reads were trimmed until the average Phred score was > 20. Forward and reverse reads for each successfully amplified sample were concentrated using PEAR with a minimum fragment length of 187 and a maximum target fragment length set to 310. All further analyses were performed using QIIME 1.9 (Caporaso et al., 2010).

Merged reads were combined into a single fastq file and open reference OTU picking was performed. Operational taxonomic units (OTUs) were assigned taxonomic classification. OTUs present in less than 0.005% of samples were filtered from the OTU

table (Bokulich et al., 2013). If applicable, OTUs representing host mitochondria or plant chloroplasts rRNA sequences were removed. Rarefaction curves were produced for each sample matrix (feces, liver abscess, carcass swabs, trim) in order to compare OTU diversity within samples at various sequencing depths. OTU tables were subjected to rarefaction at a level deemed adequate to capture alpha diversity. In the case of liver abscesses, rarefaction resulted in loss of four of 126 samples. In the case of carcass swabs, rarefaction resulted in the loss of 18 of 489 samples.

Alpha diversity tables and boxplots were produced from the rarefied OTU tables. An unweighted UniFrac distance matrix was generated to compare OTU diversity between treatment groups, blocks, harvest dates, sampling of feces at placement and harvest, and location on the carcass of trim samples. Results were visualized via Principle Coordinate Analysis to observe differences. Statistical comparisons were made using the unweighted UniFrac distance matrices through the anosim function from the “vegan” package in R. Anosim uses permutations to perform non-parametric comparisons between groups. Additionally, a Kruskal-Wallis test was performed in R to compare alpha diversity between treatment groups.

Shotgun Metagenomics Sequencing. Sequencing data were quality controlled using Trimmomatic (Bolger et al., 2014). Host (bovine) 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); reads aligning to the *B. taurus* genome were removed (Li and Durbin, 2009). The remaining reads were aligned to the MEGARes antimicrobial resistance database (v1.01) and select, hand-annotated genes from the BacMet database using BWA (Pal et al., 2014; Lakin et al., 2017).

Microbiome profiles were obtained for each sample using the quality controlled reads processed by Kraken (v0.10.5-beta) (Wood and Salzberg, 2014).

Both the Antimicrobial Resistance (AMR) and microbiome count data obtained from the bioinformatics pipeline were analyzed using the following statistical analyses. Measures for rarefied and alpha diversity were calculated using the "vegan" package 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. Counts obtained as output from the bioinformatics methods were normalized using Cumulative Sum Scaling as implemented in the "metagenomeSeq" package for the R programming language (Paulson et al., 2013). Log-fold changes and *P*-values for regression coefficients were calculating using multivariate Zero-inflated Gaussian Mixture Model regression as implemented in the "metagenomeSeq" package. *P*-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (Benjamini and Hochberg, 1995). Database files, analytic data files, and analytic code are available at a public GitHub repository).

Results and Discussion

Traditional Microbiological Culture

Feces. Overall prevalence of generic *E. coli* and *Enterococcus* spp. was 100% at placement and at harvest for all treatment groups. Across all treatment groups, populations of generic *E. coli* (Table 1) and *Enterococcus* spp. (Table 2) were greater at placement than at harvest ($P < 0.001$). However, there were no differences in *E. coli* populations among treatment groups at either placement ($P = 0.3447$) or at harvest ($P = 0.0757$). When a direct comparison was made between inclusion of tylosin and the

three non-tylosin treatment groups at harvest, populations of *E. coli* in feces of tylosin-supplemented cattle were lower ($P = 0.0285$); but the difference was less than a 1.0 log cfu/g and this effect was not considered to be biologically important. Furthermore, *Enterococcus* populations did not differ among treatment groups at placement ($P = 0.1057$) or at harvest ($P = 0.8158$). Similarly, there were no differences ($P = 0.4507$) at harvest in *Enterococcus* populations between the feces of tylosin-supplemented cattle when compared to the three non-tylosin treatment groups.

At the time of cattle placement, *Salmonella enterica* was isolated from the pen-floor feces of 85% of treatment pens, with 95% of pens harboring *Salmonella* at harvest. However, *Salmonella* prevalence did not differ ($P = 0.1395$) from placement to harvest. At placement, initial *Salmonella* prevalence was lower ($P = 0.002$) for the SCP treatment group than the other treatment groups, however, at harvest there was no difference ($P = 0.5780$) among treatment groups. As samples collected in this study were pen-composites, it was not surprising that *Salmonella* prevalence at placement and harvest were considerably higher than those found in studies which collected individual fecal grabs. One such study, which evaluated *E. coli* O157:H7 and *Salmonella* prevalence in the Southern Plains region of the United States, found an average *Salmonella* prevalence of only 22.5% across four feedlots, compared with the current study (Callaway et al., 2006). However, a study of feedlot cattle in Mexico reported fecal *Salmonella* prevalence of 55.6% (Narvaez-Bravo et al., 2013). Additionally, fecal shedding of *Salmonella* in feedlot cattle differs by season. *Salmonella* prevalence in feces tends to increase in the spring months, peak in the summer, and taper off in the

fall, with the lowest prevalence recorded during the winter months (Van Donkersgoed et al., 1999; Barkocy-Gallagher et al., 2003).

Beef Trimmings. The prevalence of *Salmonella* and generic *E. coli* were not different ($P > 0.05$) among treatment groups. Nonetheless, the overall prevalence of generic *E. coli* in beef trimmings was 100%, while the prevalence of *Salmonella* across all treatment groups was 27.78%. *Salmonella* prealence differed ($P < 0.001$) among blocks and collection dates, with blocks 1 and 2 being 100% positive for *Salmonella*, and block 3 being 50% positive. Overall, *Salmonella* was isolated from 10 of 36 (28%) samples collected; however, 8 of those samples (22%) were collected during the single collection day in which blocks 1 and 2 were collected. The isolation of generic *E. coli* from all sampled beef trimmings is surprising given that all carcasses were subjected antimicrobial interventions during the harvest process.. However, a previous study conducted by United States Department of Agriculture Food Safety Inspection Service (USDA FSIS), found 15.7% of beef trimming samples to be positive for generic *E. coli*. The same study reported a *Salmonella* prevalence of 1.3% (FSIS, 2008).

Traditionally, the prevalence of *Salmonella* on post-intervention carcasses is nonexistent or extremely low (Barkocy-Gallagher et al., 2003; Koohmaraie et al., 2012). However, generally, an increase in *Salmonella* prevalence is observed in beef trimmings or ground beef. For example, Koohmaraie et al. (2012) reported a *Salmonella* prevalence of 7.14% in beef trimmings and 1.67% in ground product, while Bosilevac et al. (2009) reported a 4.2%prevalence of *Salmonella* in ground beef samples. The prevalence reported in this study is numerically greater than others reported. It is possible that the higher prevalence of *Salmonella* in beef trimmings in this study is

related to an environmental source during fabrication—especially since most of the *Salmonella* positive samples were collected during a single shift. Environmental presence of *Salmonella*, which poses the risk of contamination to meat products, has been previously documented. Rivera-Betancourt et al. (2004) isolated *Salmonella* from swabs taken of post-production conveyor belts on a commercial fabrication floor which had previously passed pre-processing inspection and testing, suggesting that *Salmonella* could be spread to additional beef trimmings through contact with a belt contaminated by beef earlier in production.

Since 1973, 99 *Salmonella* outbreaks have been attributed to beef, in recent years, primarily from ground beef (Laufer et al., 2015; CDC, 2018d). Some studies have given rise to the thought that *Salmonella* in the peripheral lymph nodes of cattle may be responsible for the presence of *Salmonella* in ground beef as they are often embedded in the peripheral fat this trimmed from beef carcasses (Koohmaraie et al., 2012; Webb et al., 2017).

Liver Abscess Prevalence

The overall liver abscess prevalence across all treatment groups was 21.32%. As expected, TYL cattle had a lower prevalence (14.97%) of liver abscesses than the three other treatment groups (Table 3). Prevalence of abscesses in carcasses of cattle from all pens not exposed to tylosin was 23.50%. Cattle from pens exposed to tylosin were approximately 1.48 times less likely ($P < 0.05$) to develop a liver abscess than cattle from the CON, EO, or SCP treatment groups (Table 4), whereas cattle that received diets excluding tylosin were all equally likely to develop liver abscesses. There was a lower prevalence (6.8%) of severely abscessed livers, or A+ livers, for the TYL

treatment group than the three remaining treatment groups (12.4%; $P = 0.0052$). Overall liver abscess prevalence was slightly greater than that reported by the 2016 National Beef Quality Audit average of 17.8% (Eastwood et al., 2017). Results of this study were similar to those of several other studies that have highlighted the utility of tylosin for reducing liver abscess prevalence (Brown et al., 1975; Potter et al., 1985; Meyer et al., 2009; Brown and Lawrence, 2010). However, Scott et al. (2017) reported similar abscess prevalence among cattle exposed to tylosin and those supplemented with SCP.

16S rRNA Gene Sequencing

Feces. All 80 fecal samples (40 at placement, 40 at harvest) were successfully amplified and sequenced. After quality filtering, trimming, and merging forward and reverse reads, 39,873,243 sequences were clustered into 122,334 OTUs during open-reference OTU picking. After further filtering of rare OTUs, 1209 OTUs remained, and the resulting OTU table was rarefied at a level of 153,331 sequences per sample and used for downstream analysis.

A rarefied taxonomic plot displaying relative abundance of taxa at the phylum level is shown in Figure 1. There were 13 phyla represented across all treatment groups at harvest, with the two most relatively abundant phyla being Firmicutes (45.3%) and Bacteroidetes (40.7%). Proteobacteria, the phylum to which *Salmonella* and *E. coli* belong, was present at 5.6% relative abundance. Principle Coordinates Analysis (PCoA) plots were constructed to aid in visualization of detected differences, or lack thereof. Differences in alpha diversity were detected using the Kruskal-Wallis test to compare Shannon alpha diversity scores.

Overall, the fecal microbiome was different ($P < 0.001$) between samples collected at cattle placement in the feedyard versus those collected harvest, evidenced by the way samples clustered in the PCoA by sampling period (Figure 2). At harvest, the microbiome was different ($P = 0.002$) among blocks (Figure 3) and tended ($P = 0.052$) to be different among treatment groups as well as among blocks (Figure 4). There were no differences ($P = 0.872$) in alpha diversity within treatment groups at harvest.

To determine if there were particular OTUs driving differences between sample collection time (i.e., at placement or harvest), differences among blocks, or the trend in differences among treatments, the rarefied OTU table was further filtered to exclude any OTU not present in 25% of samples and then analyzed for OTU significance. From placement to harvest, more than 75% of the remaining OTUs (654 of 1204) differed ($P < 0.05$) with their mean abundance changing drastically between samplings. For block and treatment, there were no significant OTUs driving the differences ($P > 0.05$).

Overall, results were consistent with previous findings. Similar to the current study, the primary gut phyla identified in two recent studies of the bovine gastrointestinal system were Bacteroidetes, Firmicutes, and Proteobacteria across all gut locations sampled (Mao et al., 2015; Thomas et al., 2017). One study took samples from the rumen, cecum, and colon of cattle exposed to tylosin and found that tylosin-exposed cattle had a less diversified gut microbiome than did cattle that had no tylosin exposure. However, this study used a smaller samples size (n = 5) compared with the current study (Thomas et al., 2017). Although the gut microbiome of feedlot cattle has not been widely characterized, the human gut microbiome is well known. Similar to ruminants,

Firmicutes and Bacteroidetes are also the primary gut phyla of a healthy adult; however, an increased ratio of Firmicutes to Bacteroidetes (F:B) has been recently associated with weight gain (Chakraborti, 2015). The same F:B principle may not be applicable with regard to ruminants, due to the physiological necessity of ruminants to rely on gluconeogenesis for the majority of their glucose requirements.

Liver Abscesses. Of 128 liver abscess samples collected, 126 successfully amplified and sequenced resulting in 39,470,663 sequences, which were clustered into 21,195 OTUs during open-reference OTU picking. After filtering of rare OTUs, chloroplasts, and mitochondria, 92 OTUs remained, and the OTU table was rarefied at a level of 75,075 sequences per sample. Rarefaction at this level resulted in the loss of four samples which fell below the threshold. Again, rarefaction curves were constructed to determine the point at which diversity no longer increased with additional sequences (Figure 5).

A rarefied taxonomic plot displaying the relative abundance of taxa at the phylum level is shown in Figure 6. There were 10 phyla represented across all treatment groups, with the two most relatively abundant phyla being Fusobacteria (80.8%) and Bacteroidetes (16.0%), both of which are commonly found in the rumen of bovine animals. The phyla Fusobacteria consisted of a single genus, *Fusobacterium*. As *Fusobacterium necrophorum* is considered to be the primary etiologic agent of liver abscesses and is commonly isolated from bovine liver abscesses through traditional culture techniques, it was not surprising to find an increased relative abundance (Amachawadi et al., 2017). Surprisingly, the phyla Actinobacteria was only present at 0.3% relative abundance. *Trueperella pyogenes* is often named as the second most

common causative bacterium of liver abscess, making the almost complete absence of the organism in this study somewhat unusual (Nagaraja and Chengappa, 1998). PCoA plots were again constructed to aid in visualization of beta diversity differences, and alpha diversity differences within treatment groups were detected using the Kruskal-Wallis test to compare Shannon alpha diversity scores.

Overall, the microbiome of liver abscesses was affected by block ($P < 0.001$; Figure 12). There were no differences ($P = 0.1164$) in alpha diversity within treatment groups. To determine if particular OTUs were driving the differences between block, the rarefied OTU table was further filtered to exclude any OTU not present in 25% of samples and analyzed for the significance of individual OTUs. There were 23 individual OTUs which were different ($P < 0.05$) among blocks. The OTUs were spread over several phyla, with no single group indicated to be a driver of differences among the blocks.

These results were similar to those presented by Weinroth et al. (2017) wherein the primary phyla identified also were Fusobacteria and Bacteroidetes. However, in contrast with the current study, the *Fusobacterium* genus only accounted for 15.1% of the microbial community. The two most abundant phyla, Fusobacteria and Bacteroidetes, accounted for almost 97% of the microbial community of the liver abscesses and both comprise gram-negative bacteria. Tylosin is a macrolide antibiotic and is primarily effective against gram-postive bacteria. As *F. necrophorum* is gram-negative, this may aid in explaining why tylosin is not consistently effective in preventing liver abscesses. However, applying the principles outlined by Rothman and Greenland (2005) may give rise to new thought regarding the efficacy of tylosin.

The formation of liver abscesses may be the result of component causes, a principle wherein there is the potential for many underlying factors to contribute to the occurrence of liver abscesses. Additional component causes, which may not be considered in the ruminitis-liver abscess complex, include genetic pre-disposition to liver abscess formation and environmental stress to the animal. Environmental stressors could manifest in the form of extreme hot or cold weather, additional illness such as BRD, excessive movement and processing of cattle within the feedlot, and more. Under this model, it is possible that *F. necrophorum* may not be the primary causative agent of bovine liver abscesses, but rather an opportunistic bacterium which proliferates in an environment which has been voided of competing Gram-positive bacteria.

Carcass Swabs. Of the 600 carcass swabs collected, 489 were successfully sequenced and resulted in 53,248,197 sequences which clustered into 104,571 OTUs, during open-reference OTU picking. After subsequent filtering, 673 OTUs remained, and the OTU table was rarefied at 50,080 sequences per sample, resulting in the loss of 18 samples which fell below the threshold. Rarefaction was performed at this level as the alpha rarefaction curve produced (Figure 8) indicates that diversity continues to increase with increased number of sequences prior to approximately 50,000 sequences.

A rarefied taxonomic plot displaying relative abundance of taxa at the phylum level is shown in Figure 9. There were 11 phyla represented across all treatment groups, with the two most relatively abundant phyla being Proteobacteria (68.0%) and Firmicutes (23.4%). Family *Enterobacteriaceae* represented 4.1% of the microbial community. These data did not yield specific genera belonging to *Enterobacteriaceae;* however, it was notable that *Salmonella* and *E. coli* both belong to this family. PCoA

plots and alpha diversity differences were constructed as previously described for both fecal and liver abscess matrices.

Overall, the carcass microbiome was impacted by block ($P < 0.001$; Figure 10). There was also a strong tendency toward differences in treatment ($P = 0.058$; Figure 11). Additionally, there was a difference in the within-sample diversity as represented by Shannon alpha diversity scores ($P < 0.001$; Figure 12).

To determine if there were particular OTUs driving differences between block or treatment, the rarefied OTU table was further filtered to exclude any OTU not present in 25% of samples and analyzed for OTU significance. Nearly all (639) of the 642 remaining OTUs differed ($P < 0.001$) among blocks. Only 82 of the 642 OTUs differed ($P < 0.001$) among treatment groups, but those 82 were spread over several different phyla, indicating that one group of OTUs was not responsible for driving the trend toward differences among treatments.

Trimmings. Of the collected 120 beef trimmings samples, 70 were successfully amplified and sequenced. Those 70 samples were spread across all treatment groups and trim collection locations. A total of 10,092,436 sequences were clustered into 39,948 OTUs during open reference OTU picking. Following subsequent filtering, the resultant table containing 891 OTUs were rarefied at a level of 42,065 sequences per sample and used for all downstream analyses.

A rarefied taxonomic plot displaying the relative abundance of taxa at the phylum level is shown in Figure 13. There were 17 phyla represented across all treatment groups, with the two most abundant being Proteobacteria (44.2%) and Firmicutes

(28.9%). PCOA plots and alpha diversity differences were utilized to depict data as previously described for all sample matrices.

Overall, the microbiome of beef trimmings differed ($P < 0.001$) among block (Figure 14) and collection date (Figure 15). Similar to the carcass swabs, family *Enterobacteriaceae* was present, representing 3.8% of the total microbial community. Unlike the carcass swabs, however, traditional culture methods were applied to the trimmings samples and resulted in isolation of *E. coli* and *Salmonella* indicating that although genera were not identified via 16S rRNA gene sequencing, they were present in a portion of the samples. Furthermore, traditional meat spoilage bacteria such as genera *Lactobacillus* (2.3%) and *Pseudomonas* (6.7%) and family *Shewanellaceae* (2.3%) were detected across all trim samples.

Due to the nature of the fabrication floor, the belt from which trimmings were collected may have been previously contaminated by carcasses that were not part of this study; therefore, collection date was analyzed in addition to block only. There were no differences in treatment group ($P = 0.812$) or trim collection location (chuck, loin, or round; $P = 0.567$). There was a trend for alpha diversity to differ ($P = 0.063$) within treatment groups (Figure 16). With regard to both carcass swabs and trim, little research has been published at this time regarding the microbiome of beef. As the search for tylosin alternatives continues, it will be imperative to understand and characterize the microbiome of beef carcasses and finished products. As results of the current study indicated, the microbiome of beef carcasses and trim did not differ with and without tylosin exposure. This information will be critical in the development of

future strategies aimed at mitigation of liver abscess while maintaining beef safety and quality.

Shotgun Metagenomics Sequencing

Across the 40 fecal samples sequenced, 1,125,338,252 paired-end reads were generated. The mean number of reads generated was 28,133,456, with a maximum of 67,995,656 reads and a minimum of 3,128,744 reads. Average quality (phred) score was 38, indicating that 1.6 of every 10,000 bases were assigned incorrectly by the sequencer. Across all samples, 176 individual genes were identified using a gene fraction threshold of 80%, with 1,865,654 reads mapping to the MEGARes database, indicating that the resistome was approximately 0.17% of all DNA extracted..

Following normalization, 795,044 reads remained; most of which displayed resistance to antimicrobial drugs (99.72%). Of the 792,854 reads that were classified to antimicrobial drug resistance genes, 66.74% aligned to tetracycline resistance ribosomal protection proteins, while 24.26% aligned to macrolide resistance efflux pumps. Of the 1,446 reads that aligned to metal resistant genes, 23.94% were attributed to metal resistance proteins, 16.04% to metal efflux regulators, and 13.91% to zinc specific resistance regulators. Of the 628 reads that aligned to biocide resistant genes, biocide resistance proteins were most prevalent (65.28%; Table 5).

The fecal resistome at the time of harvest was characterized using richness (reverse Simpson's index) and abundance at the gene, class, and mechanism levels. Differences were visualized through non-metric multidimensional scaling (NMDS) plots. Direct comparisons were made among treatment groups, with block as a random effect. The relative abundance of tetracyclines, aminoglycosides, betalactams, and macrolide-

lincosamide-streptogramin (MLS) resistance genes, the most abundant classes of resistance, were not different ($P > 0.05$) among treatment groups (Figure 17). Additionally, there were no differences among treatment groups for antimicrobial resistance of the macrolide phosphotransferase mechanism ($P > 0.05$). NMDS ordination at the class, mechanism, group, and gene levels were not separate between treatment groups (Figure 18).

Treatment differences were detected within metal (Tellurium, Mercury, Silver, Copper, Zinc, and Nickel), biocide and antimicrobial drug (Phenicol, and Bacitracin) classes of resistance genes ($P < 0.01$), but the average expression for most of these differences was < 1, and therefore not considered to be biologically significant. Zinc, Nickel, and biocide resistance all displayed average expressions >1. Zinc resistance was greater ($P < 0.01$) for CON and EO than TYL and SCP, and Nickel resistance was greater ($P < 0.01$) for TYL, CON, and EO than Treatment SCP. Biocide resistance was greater ($P < 0.01$) for EO than for SCP. Overall, these results indicated very little difference between treatment groups..

In a study conducted by Alexander et al. (2011), PCR detection was used to identify resistance genes in fecal samples from cattle exposed to subtherapeutic levels of antibiotics or no antibiotics. Similar to the current study, feces from both exposed and non-exposed cattle exhibited resistance to tetracycline and erythromycin, a macrolide. Noyes et al. (2016) also identified genes encoding for tetracycline and macrolide resistance in a study of the beef production system. Overall, results of the present study suggested that removal of tylosin from feedlot systems will not upend the safety of finished beef products but will impact liver abscess prevalence of feedlot cattle.

Summary and Conclusions

The objective of this study was to determine the effect of tylosin alternatives on the prevalence of liver abscesses, and the microbial communities of feces, carcasses, liver abscesses, and beef trimmings of feedlot cattle. Results indicated that dietary treatments used in this project largely had no impact on microbial populations of any of the targeted matrices. Mean liver abscess prevalence of 21.3% and was slightly higher than those presented in the 2016 National Beef Quality Audit (Eastwood et al., 2017). Effectiveness of tylosin phosphate in reducing prevalence of liver abscesses is well documented (Nagaraja and Chengappa, 1998; Reinhardt and Hubbert, 2015) and this study found that cattle receiving tylosin supplementation were, on average, 1.48 times less likely to develop abscesses. Although the evidence of this study continues to highlight efficacy of tylosin at reducing the prevalence of liver abscesses, it did not prevent them altogether.

Following characterization of feces, liver abscess, carcass, and beef trimming samples via 16S rRNA gene sequencing, differences among pen blocks were often detected. Although the experimental design should have accommodated for any initial differences in cattle, such that cattle were randomly assigned to pens and each pen was randomly assigned to one of the four treatments, any differences attributed to pen block or harvest date are likely environmental in nature. As cattle were similarly sourced, analysis of alternative matrices (i.e., soil) may yield information regarding the variation in the microbiome. However, it is still pertinent to note that there were no differences among treatment groups in any of the matrices that were analyzed.

As alternatives to tylosin continue to be explored, it is imperative that the alternatives not only effectively reduce liver abscesses, but also do not negatively alter the microbial populations related to beef production—particularly with regard to the presence of pathogens. The information gained in this study will assist in the identification of antimicrobial alternatives which have the desired benefits to cattle performance and liver abscess rates, but do not sacrifice beef quality or safety.

Table 1. Populations (log CFU/g) of generic *Escherichia coli* in composited fecal samples obtained from the floors of feedlot pens at the time of cattle placement and prior to shipping for harvest.

Sampling Period	TYL[1]	CON	EO	SCP
Placement	6.27	6.43	6.60	6.50
Harvest	5.68	6.02	6.23	5.94

[1]Treatments included Tylosin, Control, Essential Oil, *Saccharomyces cerevisiae* fermentation product

Table 2. Populations (log CFU/g) of *Enterococcus* spp. in composited fecal samples obtained from the floors of feedlot pens at the time of cattle placement and prior to shipping for harvest.

Sampling Period	TYL[1]	CON	EO	SCP
Placement	4.23	4.89	4.60	4.77
Harvest	4.18	3.98	4.08	3.89

[1]Treatments included Tylosin, Control, Essential Oil, *Saccharomyces cerevisiae* fermentation product

Table 3. The influence[1] of supplementation with tylosin (90 mg/hd/d), an essential oil (1 g/hd/d), or a *Saccharomyces cerevisiae* fermentation byproduct (18 g/hd/d) on the prevalence of liver abscesses in feedlot cattle.

Treatment	Abscessed Livers	Total Livers Scored	Mean % Abscessed
TYL[2]	212	1353	14.97[a]
CON	311	1359	23.72[b]
EO	314	1349	24.48[b]
SCP	313	1354	22.32[b]

[ab] Within column, mean values with the same letter do not differ.

[1] *P*-value: 0.0036; SEM = 1.879.

[2]Treatments included Tylosin, Control, Essential Oil, *Saccharomyces cerevisiae* fermentation product

Table 4. Relative risk of liver abscesses compared by treatment.

Comparison	Adjusted Relative Risk	CI	*P*-value
CON vs TYL[1]	1.4605	(1.06, 2.02)	0.0223
EO vs TYL	1.4854	(1.08, 2.04)	0.0144
SCP vs TYL	1.4754	(1.13, 1.92)	0.0039
EO vs CON	1.0171	(0.76, 1.36)	0.9080
SCP vs CON	1.0101	(0.80, 1.27)	0.9310
SCP vs EO	0.9931	(0.80, 1.23)	0.9504

[1]Treatments included Tylosin, Control, Essential Oil, *Saccharomyces cerevisiae* fermentation product

Table 5. Number and percentage of normalized reads aligning to each category of mechanism resistance (antimicrobial drug, metal, biocide). Before normalization, a total of 1.9 million reads matched to the MEGARes database out of 1.1 billion. After normalizing, 795,044 reads were characterized by MEGARes.

Mechanism by Category	Total Reads Assigned	Percent of Resistance within Category	Percent of Total Resistance
Antimicrobial drug resistance			
Tetracycline resistance ribosomal protection proteins	530603	66.92%	66.74%
Macrolide resistance efflux pumps	192892	24.33%	24.26%
Class A betalactamases	21717	2.74%	2.73%
Lincosamide nucleotidyltransferases	10255	1.29%	1.29%
Aminoglycoside O-nucleotidyltransferases	6953	0.88%	0.87%
23S rRNA methyltransferases	5859	0.74%	0.74%
Tetracycline resistance major facilitator superfamily MFS efflux pumps	5244	0.66%	0.66%
Multi-drug efflux pumps	4501	0.57%	0.57%
Tetracycline inactivation enzymes	4293	0.54%	0.54%
Macrolide phosphotransferases	4241	0.53%	0.53%
MDR regulator	1775	0.22%	0.22%
Aminoglycoside efflux pumps	1136	0.14%	0.14%
Aminoglycoside O-phosphotransferases	970	0.12%	0.12%
Lipid A modification	878	0.11%	0.11%
Penicillin binding protein	498	0.06%	0.06%
Polymyxin B resistance regulator	336	0.04%	0.04%
Aminoglycoside N-acetyltransferases	231	0.03%	0.03%
Aminoglycoside efflux regulator	179	0.02%	0.02%
Class C betalactamases	153	0.02%	0.02%
Undecaprenyl pyrophosphate phosphatase	47	0.01%	0.01%
Class D betalactamases	42	0.01%	0.01%
Chloramphenicol acetyltransferases	39	0.00%	0.00%
Phenicol efflux pumps	14	0.00%	0.00%

Table 5 (cont.)

Mechanism by Category	Total Reads Assigned	Percent of Resistance within Category	Percent of Total Resistance
Metal Resistance			
metal resistance protein	346	23.94%	0.04%
metal efflux regulator	232	16.04%	0.03%
zinc resistance regulator	201	13.91%	0.03%
nickel ABC efflux pump	178	12.29%	0.02%
metal ABC efflux pump	150	10.41%	0.02%
copper resistance protein	120	8.31%	0.02%
metal resistance regulator	100	6.89%	0.01%
zinc ABC efflux pump	44	3.03%	0.01%
nickel ABC efflux regulator	38	2.60%	0.00%
metal efflux pump	19	1.29%	0.00%
metal efflux protein	10	0.71%	0.00%
silver RND efflux pump	4	0.30%	0.00%
mercuric resistance protein	2	0.15%	0.00%
tellurium resistance protein	2	0.14%	0.00%
Biocide Resistance			
biocide resistance protein	410	65.28%	0.05%
biocide resistance regulator	161	25.61%	0.02%
biocide RND efflux regulator	57	9.12%	0.01%
Cross-category			
metal and biocide resistance protein	105	91.32%	0.01%
drug metal and biocide RND efflux pump	10	8.68%	0.00%

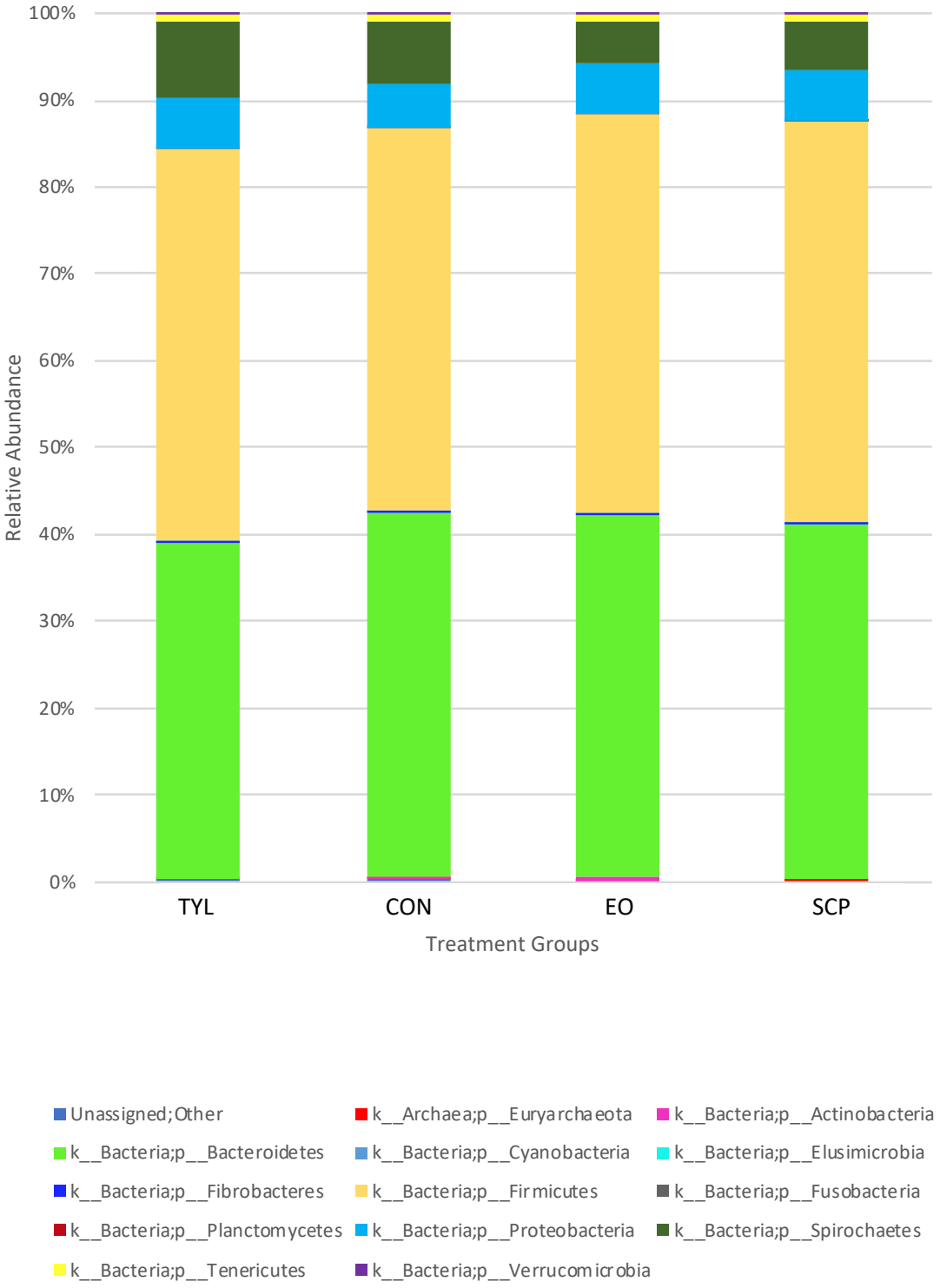

Figure 1. Phylum-level classification summary of fecal sequence reads obtained through 16S rRNA sequencing, by treatment.

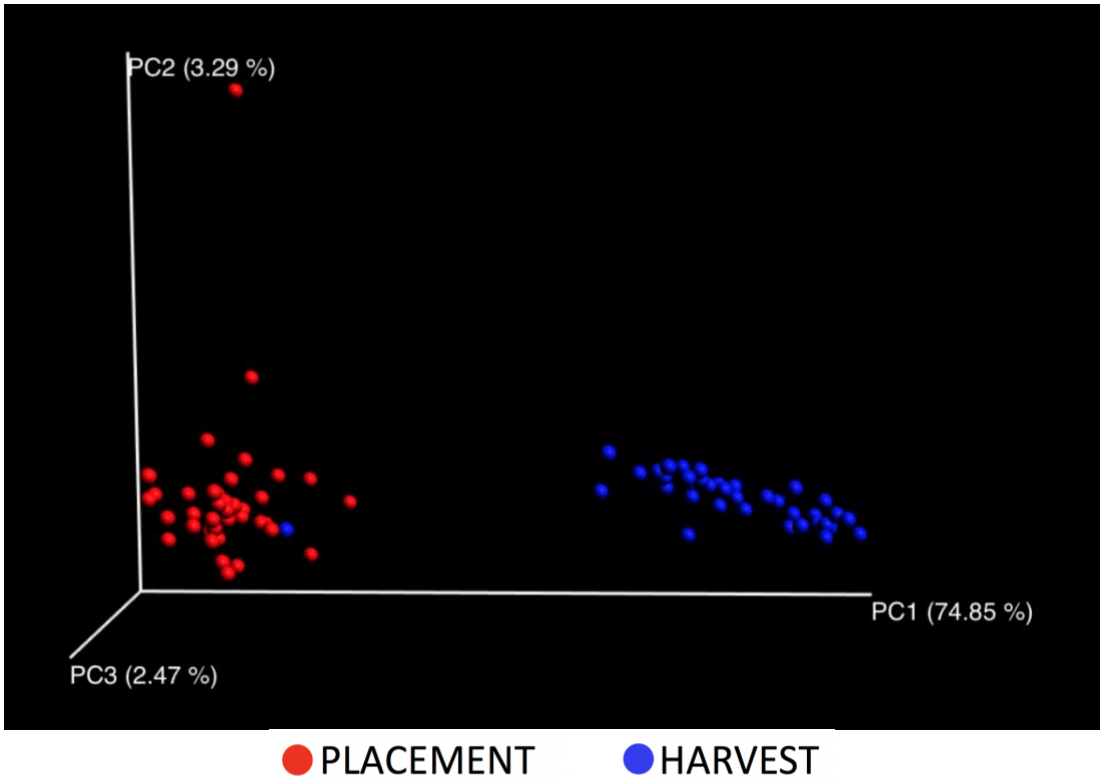

Figure 2. Principle Coordinate Analysis plot based on unweighted UniFrac distance of fecal samples subjected to 16S rRNA gene sequencing, by sampling period ($P < 0.001$).

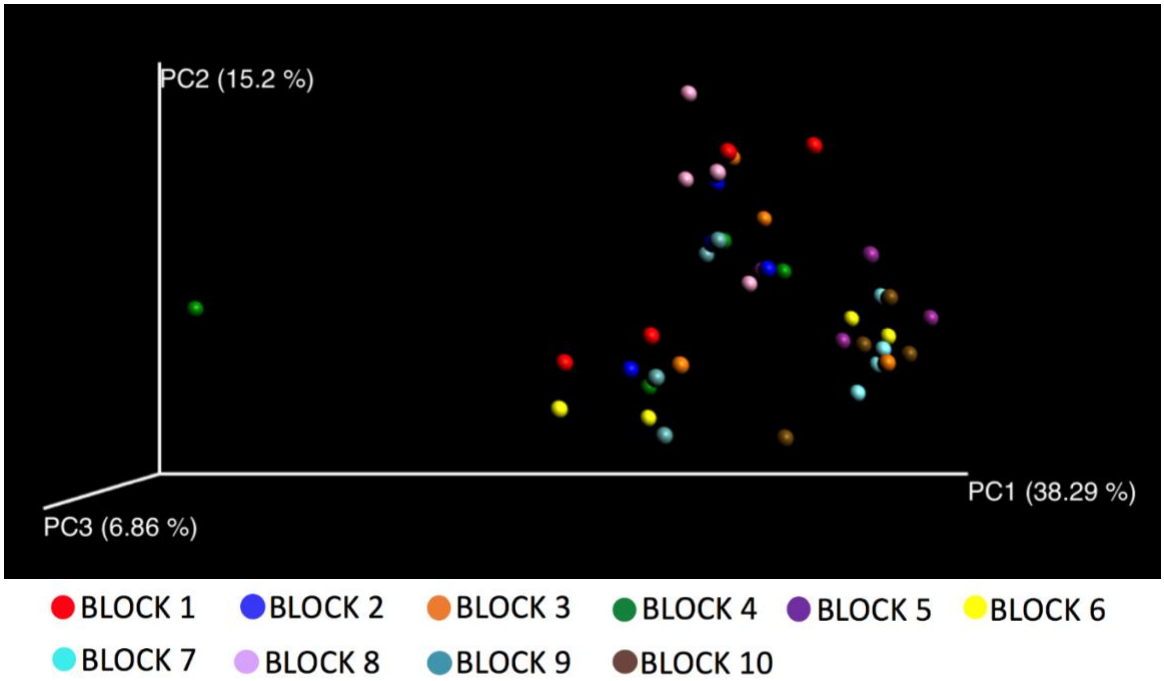

Figure 3. Principle Coordinate Analysis plot of fecal samples collected at harvest subjected to 16S rRNA sequencing, by block ($P = 0.002$).

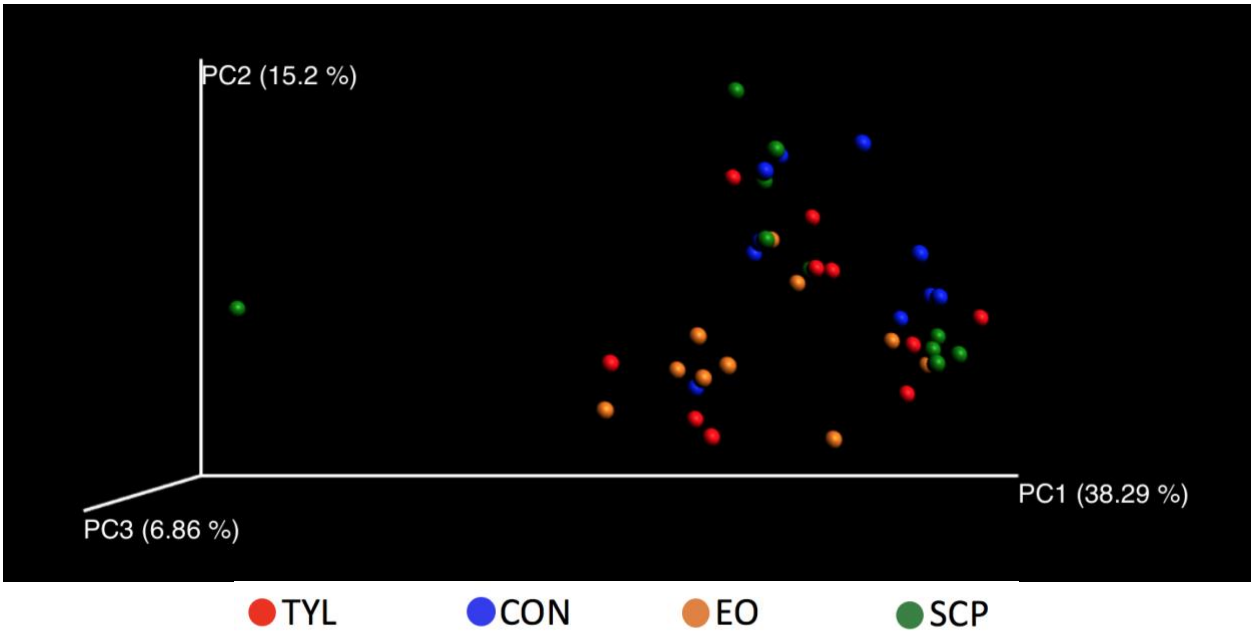

Figure 4. Principle Coordinate Analysis plot of fecal samples collected at harvest subjected to 16S rRNA sequencing, by treatment ($P = 0.052$).

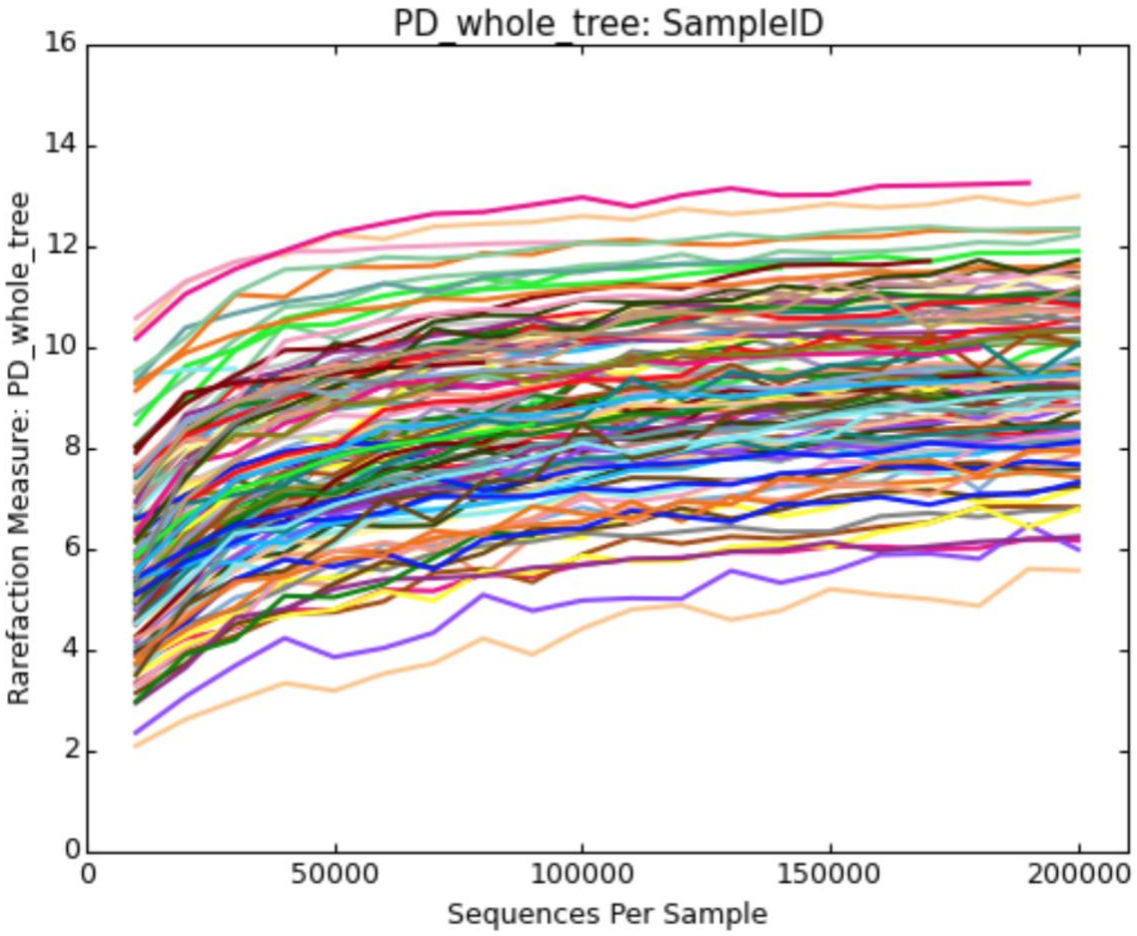

Figure 5. Rarefaction curve indicating alpha (within-sample) diversity vs. sequences per sample for all liver abscess samples subjected to 16S rRNA sequencing, by sample.

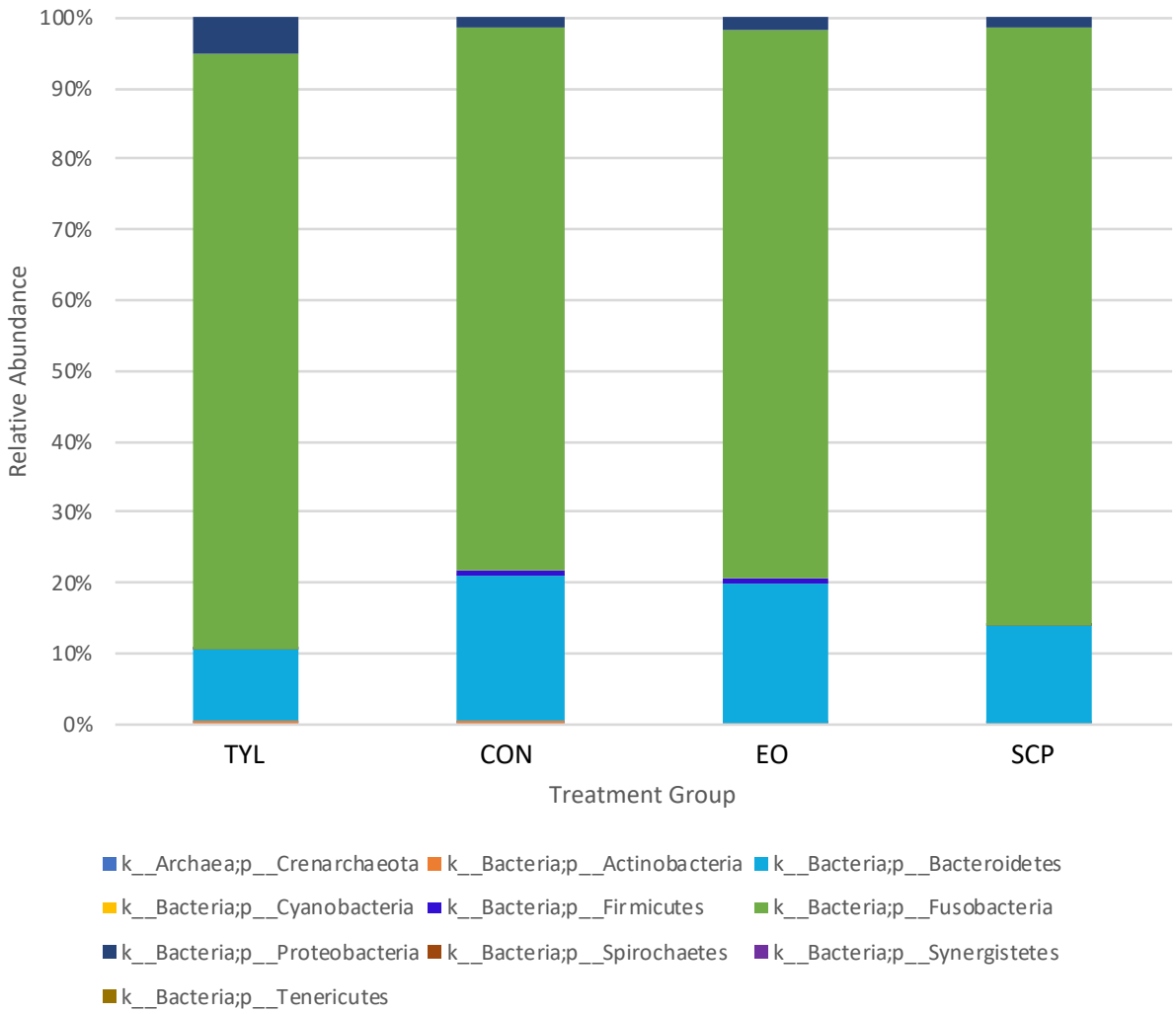

Figure 6. Phylum-level classification summary of liver abscess sequence reads obtained through 16S rRNA gene sequencing, by treatment.

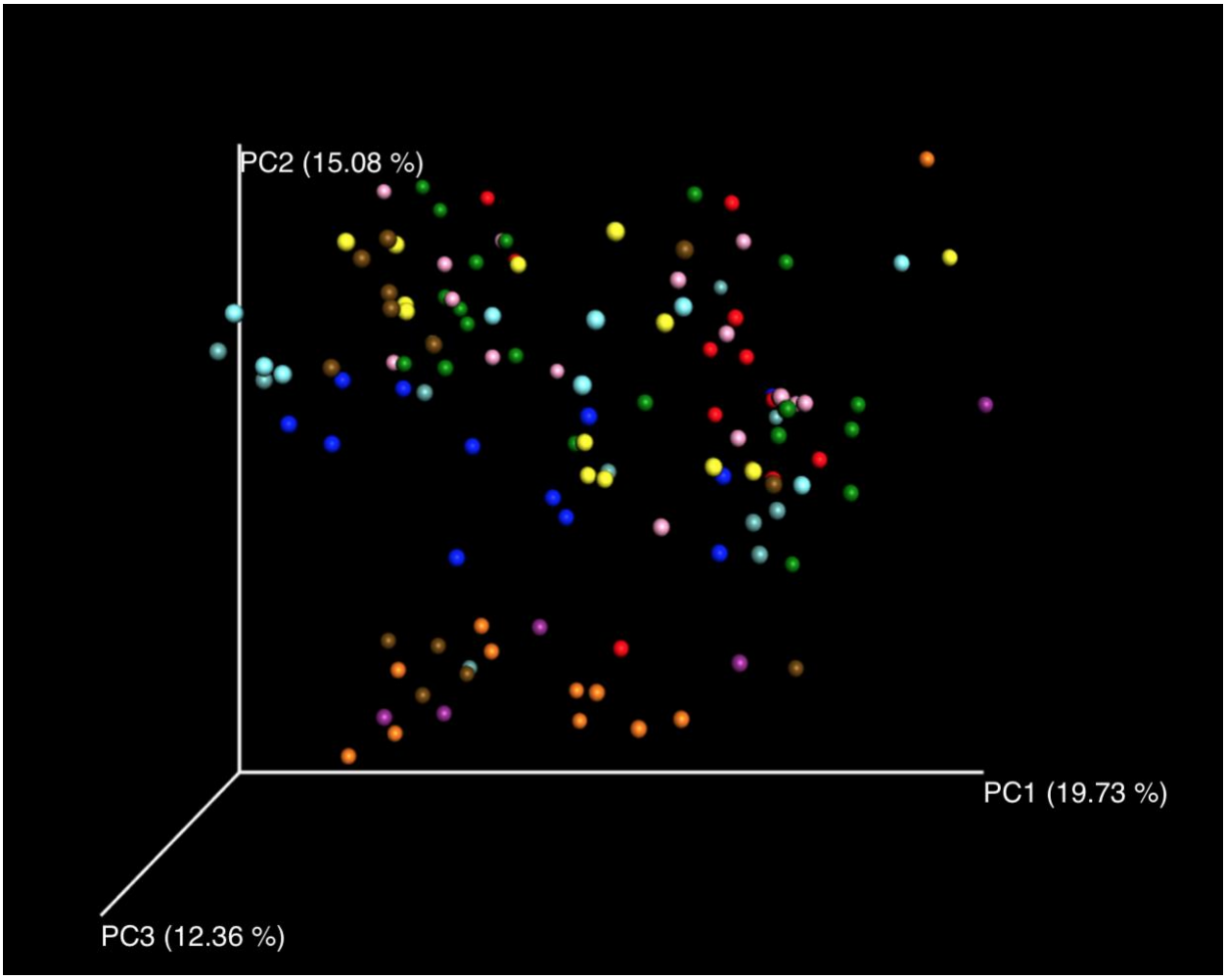

Figure 7. Principle Coordinate Analysis plot of liver abscesses subjected to 16S rRNA sequencing, by block ($P < 0.001$).

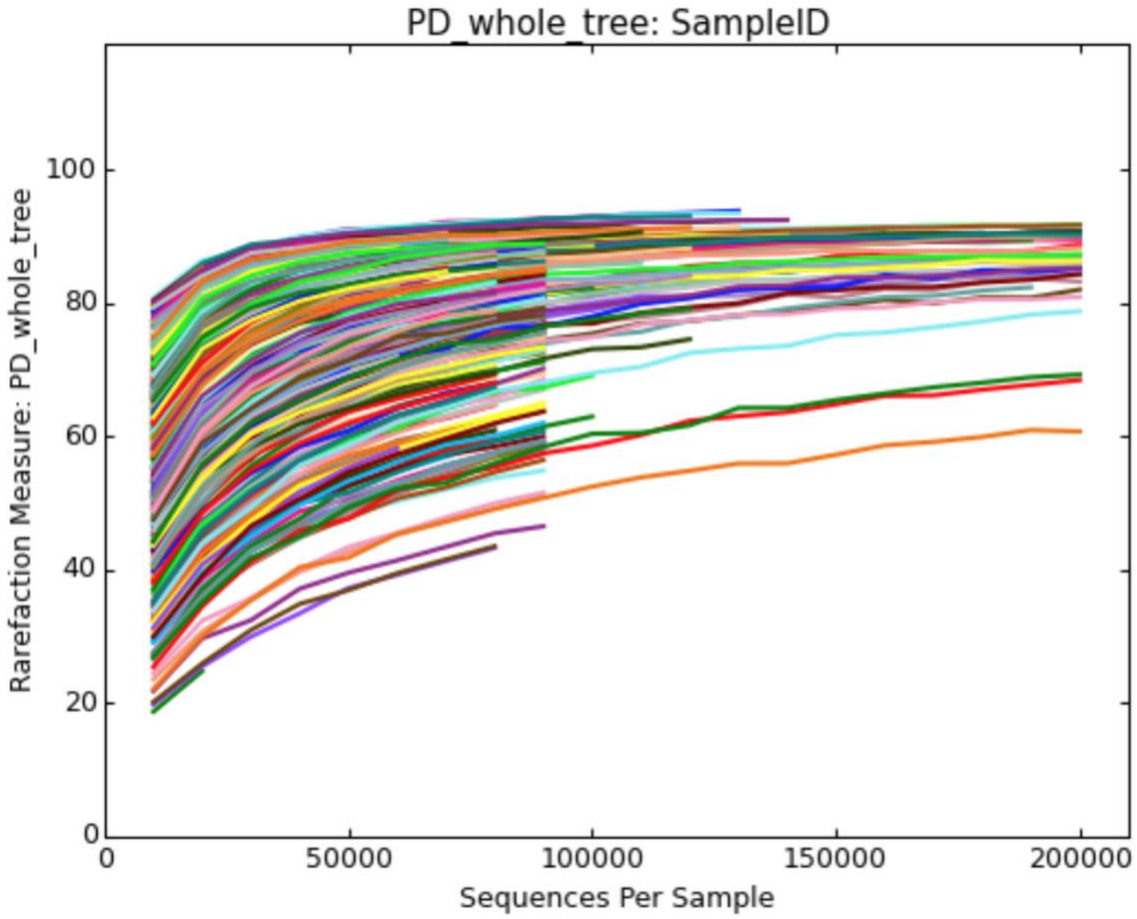

Figure 8. Rarefaction curve indicating alpha (within-sample) diversity vs. sequences per sample for all carcass swab samples subjected to 16S rRNA sequencing, by sample.

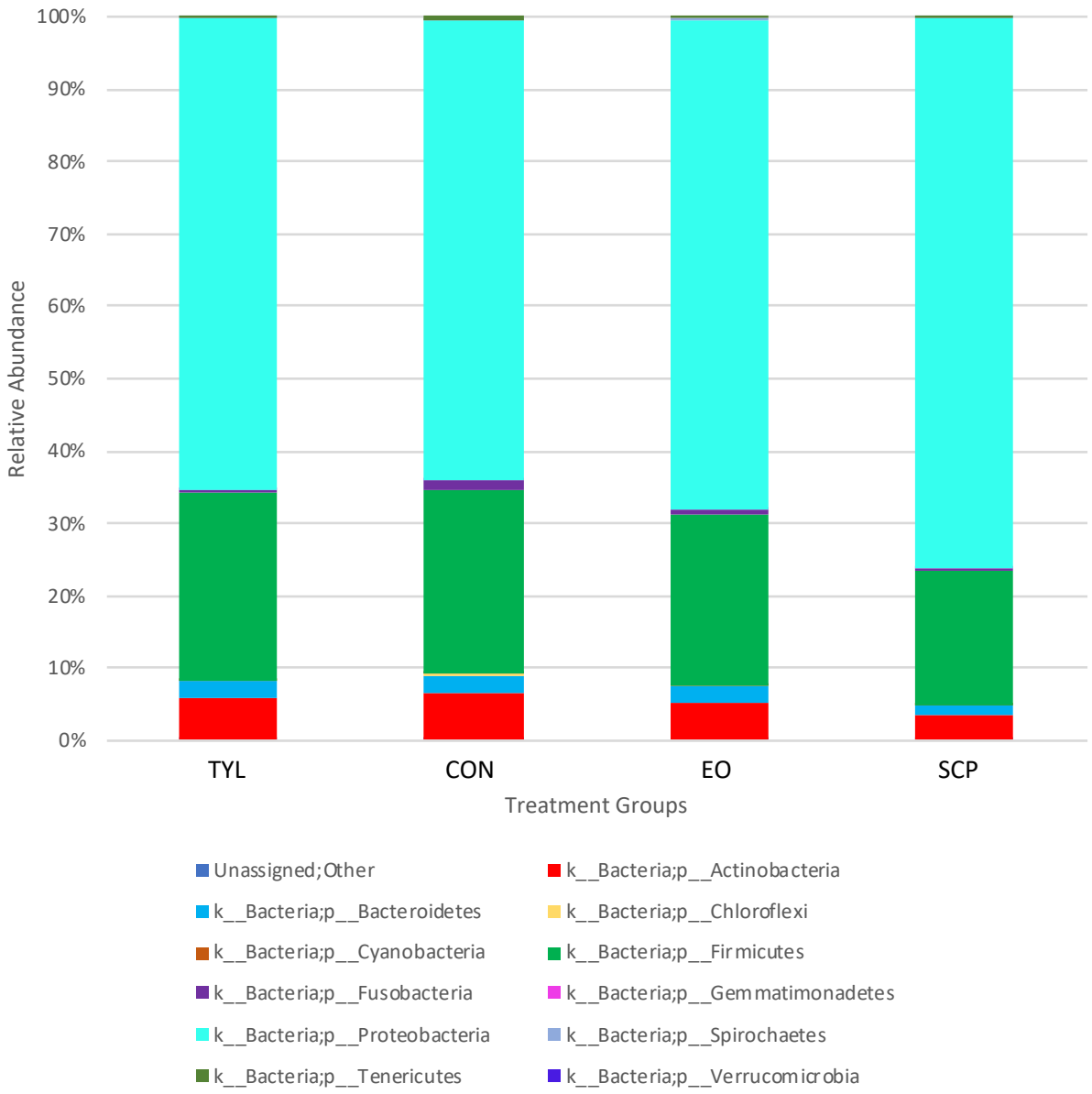

Figure 9. Phylum-level classification summary of carcass swab sequence reads obtained through 16S rRNA sequencing, by treatment.

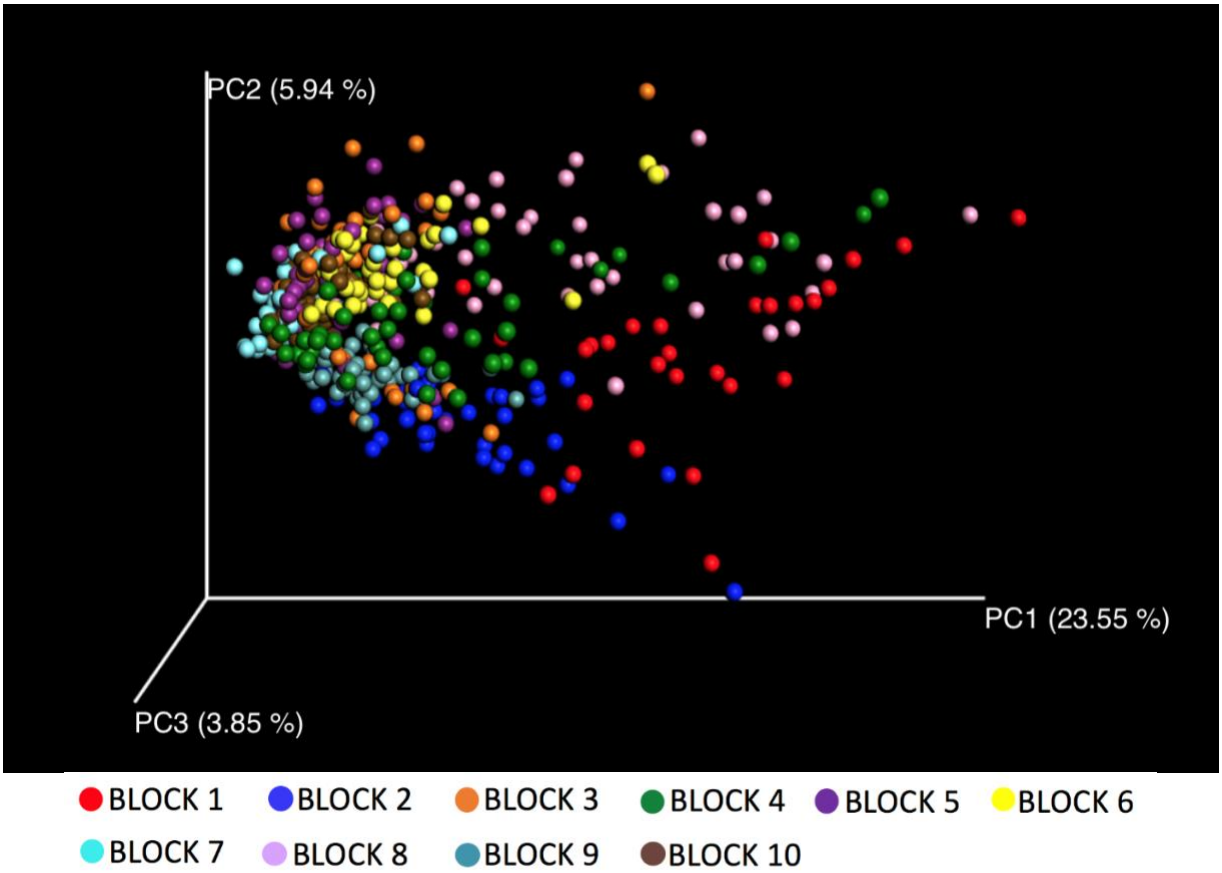

Figure 10. Principle Coordinate Analysis plot of carcass swabs subjected to 16S rRNA sequencing, by block ($P < 0.001$).

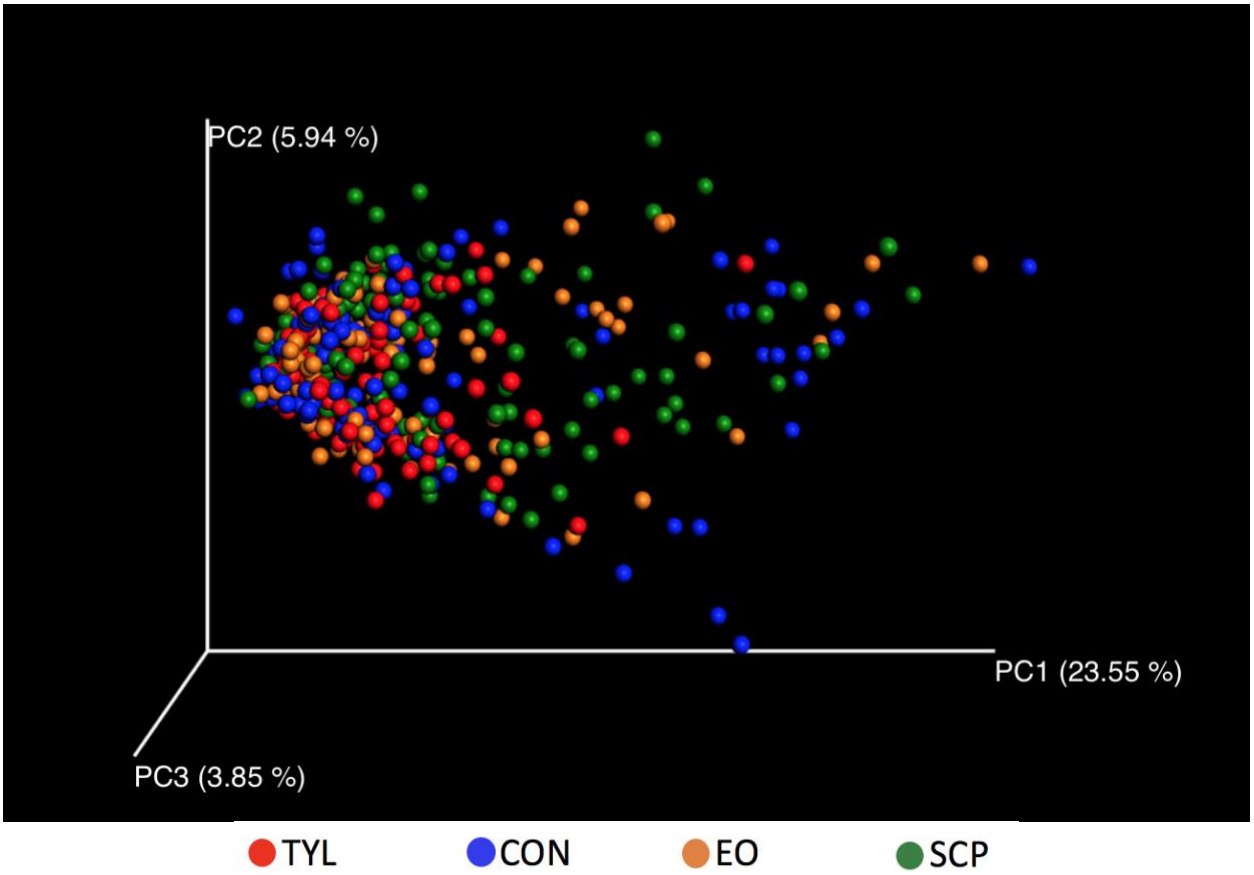

Figure 11. Principle Coordinate Analysis plot of carcass swabs subjected to 16S rRNA sequencing, by treatment ($P = 0.058$).

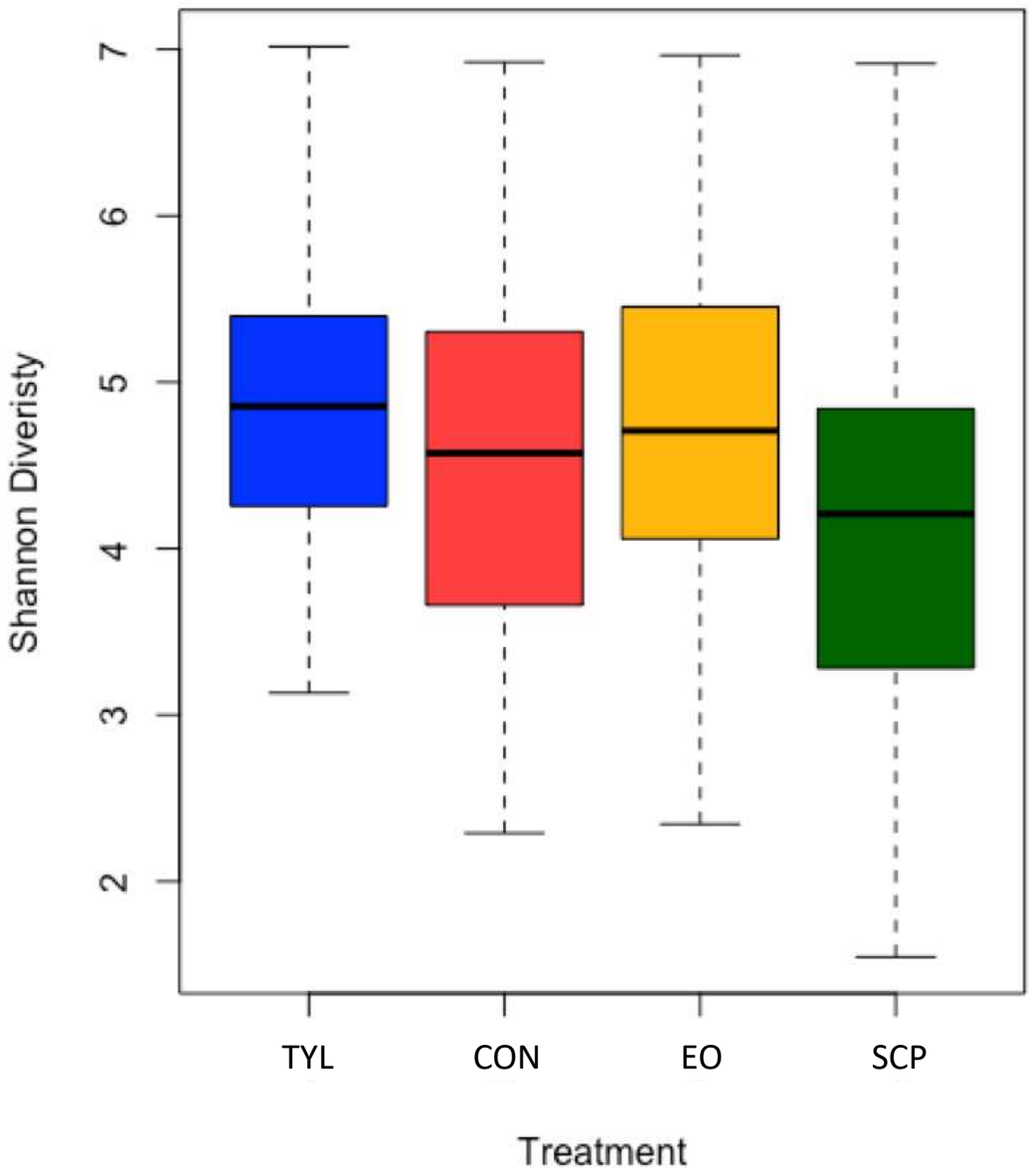

Figure 12. Shannon alpha diversity of carcass swabs subjected to 16S rRNA gene sequencing, by treatment ($P < 0.001$).

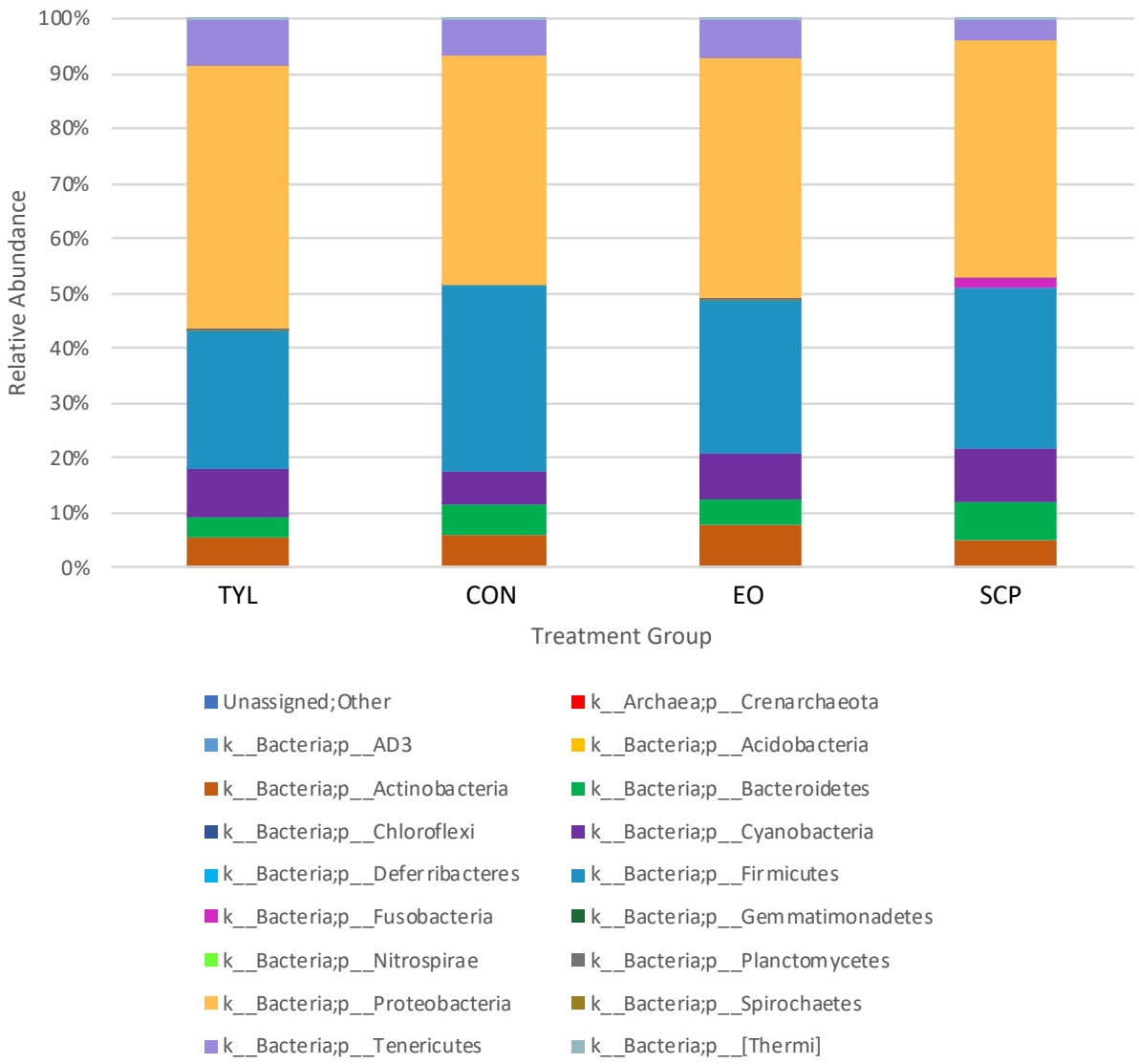

Figure 13. Phylum-level classification summary of beef trim sequence reads obtained through 16S rRNA gene sequencing, by treatment.

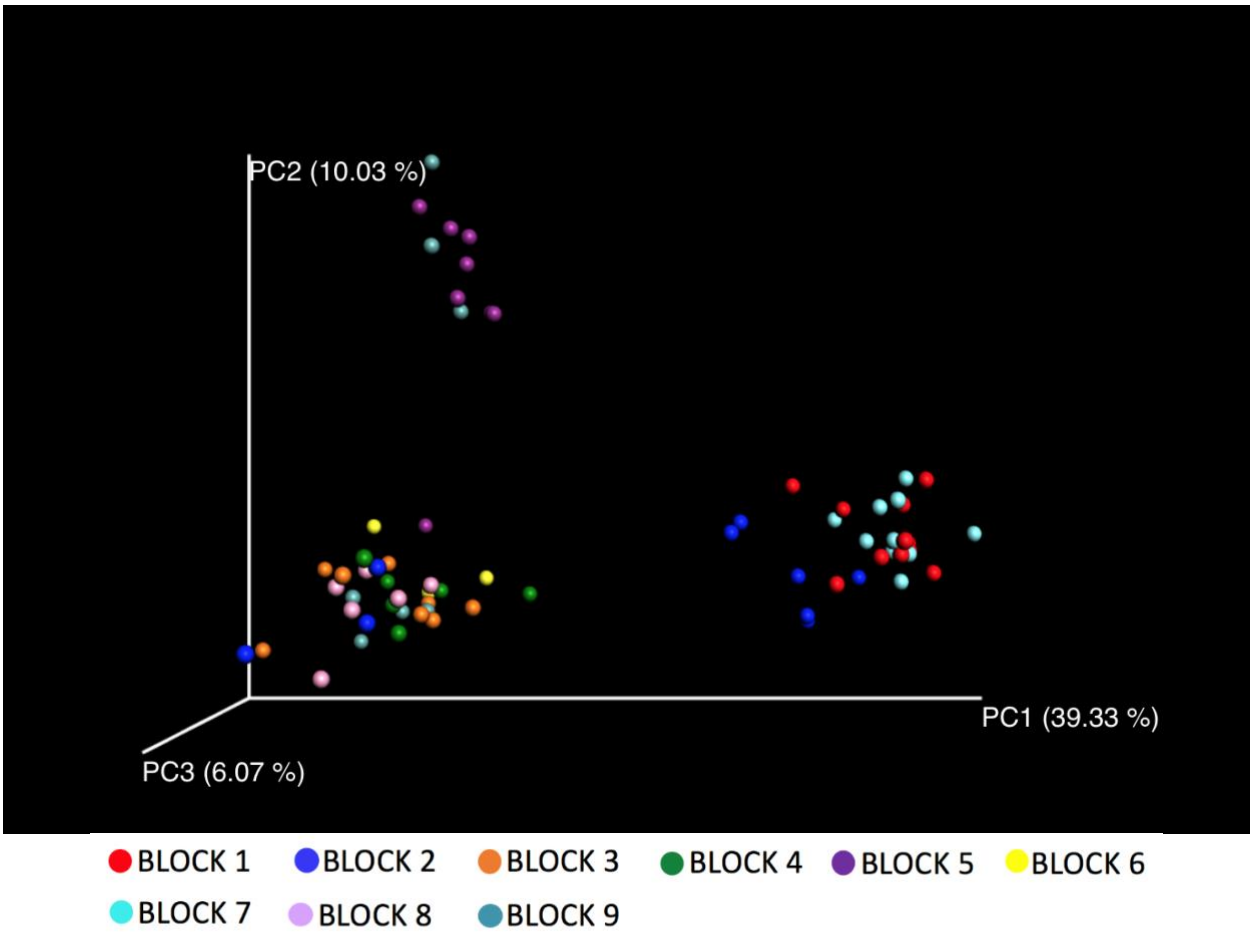

Figure 14. Principle Coordinate Analysis plot of beef trim subjected to 16S rRNA sequencing, by block ($P < 0.001$).

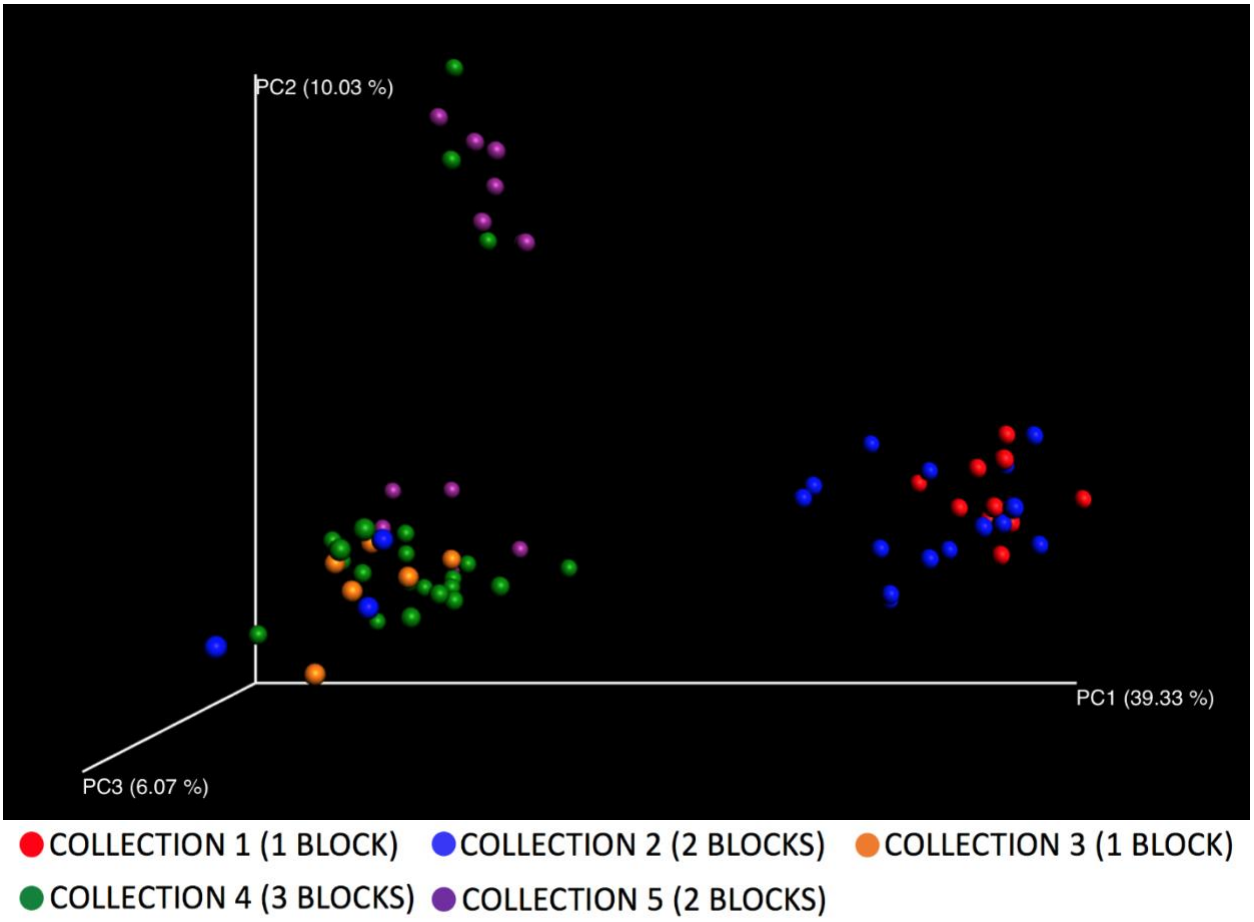

Figure 15. Principle Coordinate Analysis plot of beef trim subjected to 16S rRNA sequencing, by collection date ($P < 0.001$).

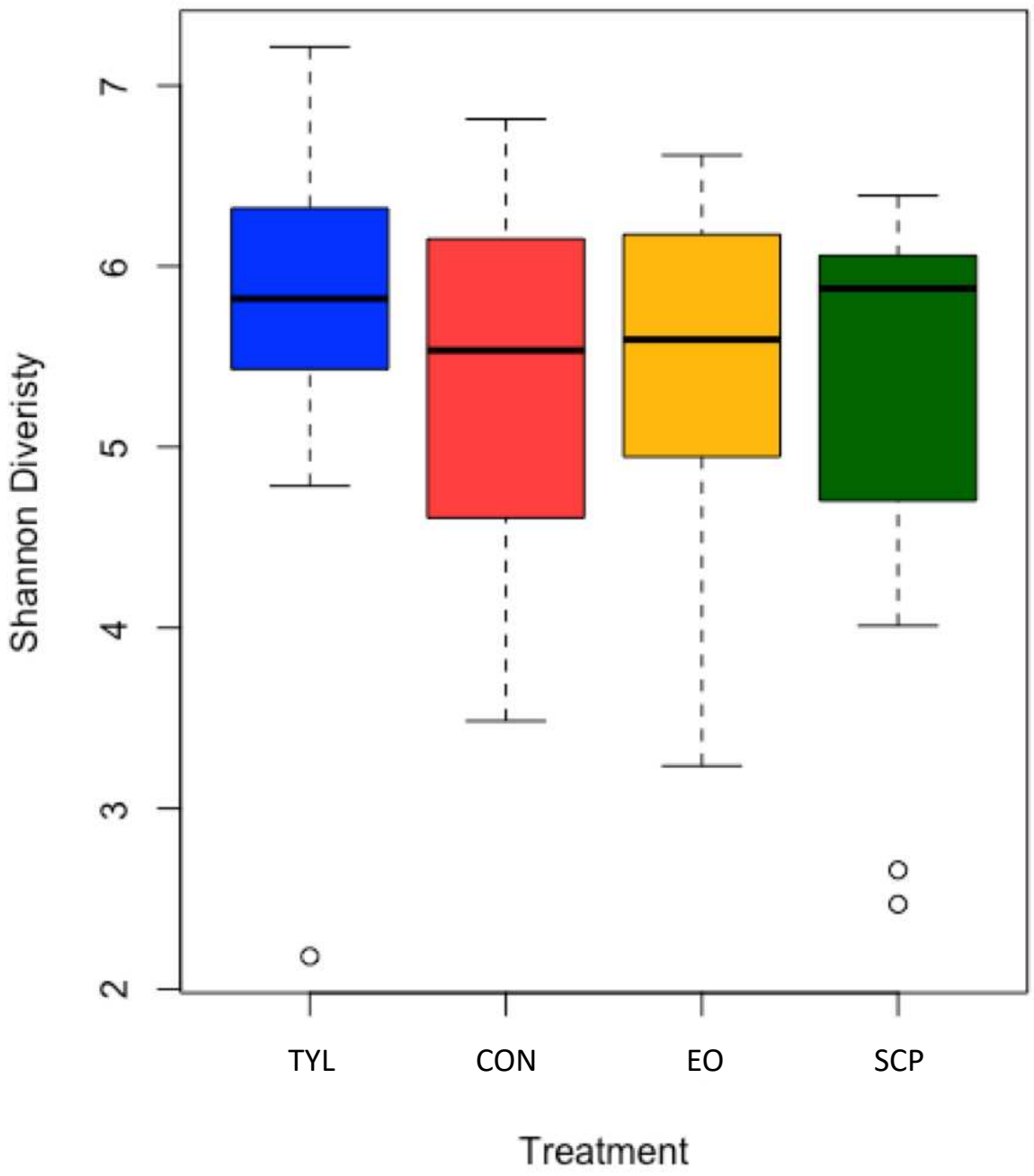

Figure 16. Shannon alpha diversity of trim rinsates subjected to 16S rRNA sequencing, by treatment ($P = 0.063$).

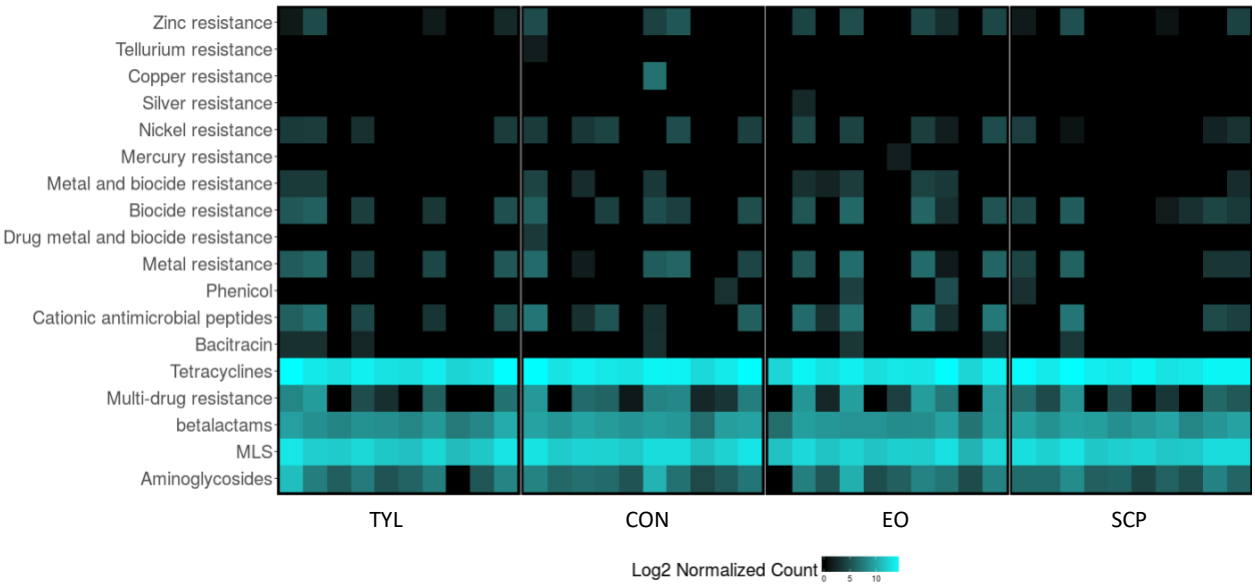

Figure 17. Treatment comparisons of normalized counts of resistance genes, by class.

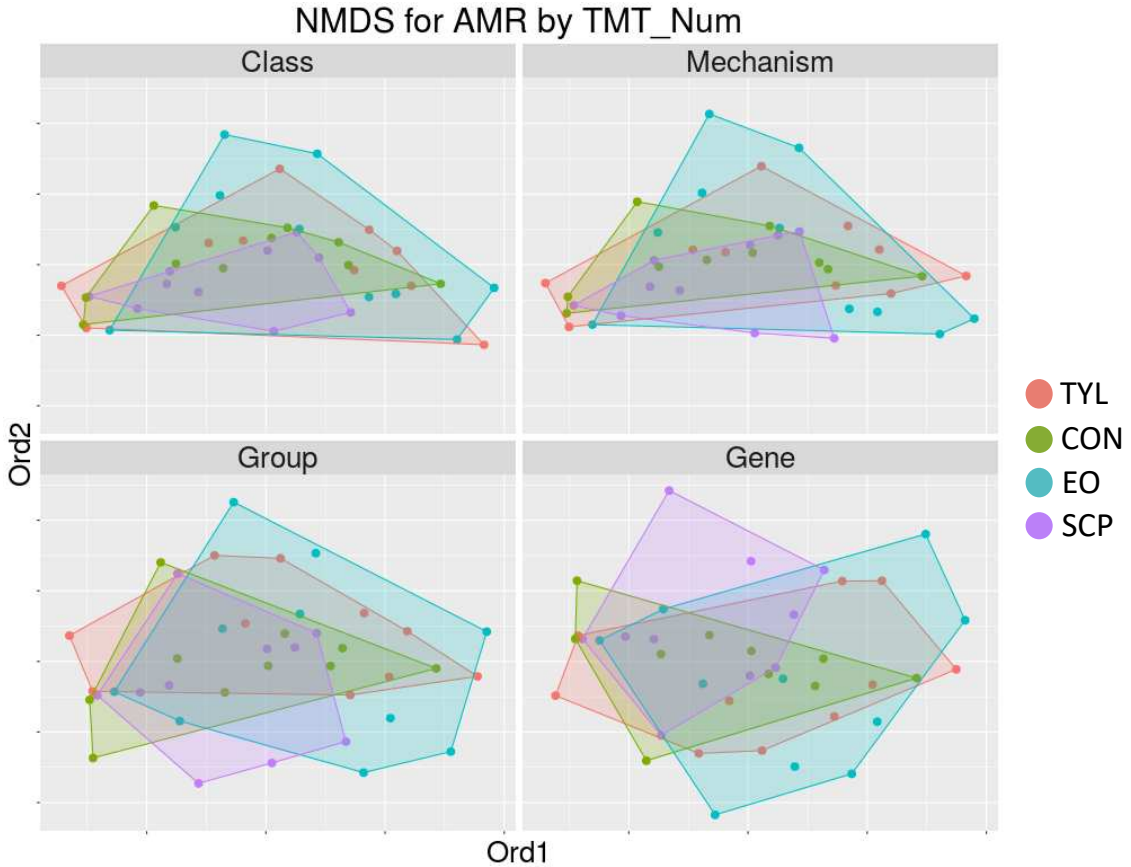

Figure 18. Non-metric multidimensional scaling plot of resistome across all treatment groups, at class, mechanism, group, and gene levels

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