

THESIS

EFFECTS OF ANTIBIOTIC TREATMENT STRATEGIES ON FEEDLOT CATTLE
RESISTOME AND MICROBIOME

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2016

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ABSTRACT

EFFECTS OF ANTIBIOTIC TREATMENT STRATEGIES ON FEEDLOT CATTLE RESISTOME AND MICROBIOME

The objective of this study was to evaluate resistome and microbiome changes in feedlot cattle exposed to commonly used antimicrobials. Sixteen pens of cattle (N=16) were randomly assigned to one of four antimicrobial treatments (n=4) resulting in a complete 2x2 factorial arrangement. The first factor was to treat Ceftiofur crystalline free acid (CCFA) to either the entire pen of animals (high dosage) or to one animal in the pen (low dosage). The second factor was the subsequent feeding of chlortetracycline (CTC) in feed to the entire pen of cattle or not administering CTC to a pen of cattle. Rectal fecal samples were collected from individual cattle within each pen on days 0 and 26. Deoxynucleic acid was extracted from individual fecal samples and pooled by DNA mass, so each pen had one composite sample on day 0 and day 26. Deoxynucleic acid was sequenced on an Illumina HiSeq 2000. Sequencing data (as known as reads) were aligned to a comprehensive antimicrobial resistance gene database and assigned to taxonomic labels. Sixty-eight antimicrobial resistance genes and 431 species were identified across all samples. Resistance to tetracycline was identified as the primary resistance at class level (66.9%) with resistance to Macrolide-lincosamide-streptogramin B (MLS) making up the majority of the remainder (26.2%). Resistance to tetracycline and aminoglycoside in the feces decreased ($P < 0.05$) in relative abundance from day 0 to day 26 when the cattle were fed CTC regardless of CCFA exposure. Beta-lactams were the only class of resistance affected by the CCFA treatment, with low exposure CCFA pens exhibiting a smaller ($P < 0.05$) resistome on day

26 than those steers in high exposure CCFA pens, regardless of CTC treatment. These results indicate that the exposure to tetracycline for cattle may not be directly associated to the resistance to tetracycline in their feces. Further research is needed to explore more about this. Additionally, the decrease in resistance to aminoglycoside with no cattle exposed to anyaminoglycosides during the study raises the possibility of co-selection of resistant genes. Overall, the relative abundance of microbiome did not differ ($P > 0.05$) between pens of cattle with CCFA or CTC treatments but differed ($P < 0.05$) between day 0 and day 26. Overall microbiome relative abundance did not differ ($P > 0.05$) due to CCFA or CTC treatments but differed ($P < 0.05$) between day 0 and day 26. Changes in the microbiome over time affected all 19 phyla identified when all treatments were pooled together. It has been well established in humans that antimicrobial treatment changes in the microbiome (Khoruts et al., 2010; Preidis and Versalovic, 2009). While these findings are not as robust livestock, there is ongoing investigation establishing these same results that may lead to further understanding of how the microbiome of livestock responds to antibiotics.

ACKNOWLEDGMENTS

Without the guidance and support of my committee members, faculty, fellow graduate students, friends, and family I would have never been able to complete my degree.

Thank you to my advisor Dr. Keith Belk for allowing me to take ownership of my research but always being there to support me. Thank you to Dr. Dale Woerner for sparking my interest in meat science and helping me navigate to graduate school. To my committee members Dr. Paul Morley and Dr. Jennifer Martin thank you for supporting me and pushing me forward. Thank you Dr. Ifigenia Geornaras for training me in the lab and encouraging me when I was down. Dr. Noa Roman-Muniz thank you for nurturing a love of science I did not know I had.

To all of my fellow graduate students, thank you for being there through the long nights and early mornings, the days were long but the years were short. Thank you to Crystal Yang, Pablo Rovira, Enrique Doster, and Noelle Noyes for your patience in helping with my data and analysis. Thank you to the entire Microbial Ecology Group at CSU for allowing me to be a part of such a diverse, supportive, and keen group of people.

Finally thank you to my family and friends who were there to support me through the highs and the lows.

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CHAPTER 1

Literature Review

Antibiotic resistance has been characterized as a "worldwide crisis" and "a threat to disease control throughout the world" (Center for Disease Control and Prevention, 2015; World Organization for Animal Health, 2015). With a growing concern of resistance in human health, there has been increased scrutiny regarding use of antimicrobials in food animal production. Recently, there has been a push to decrease antibiotic use in food animal production, especially antibiotics deemed important to human health (World Health Organization, 2011). How antibiotic resistance is transferred from food animals to humans is beginning to be characterized but there is still a great deal unknown (United States House of Representatives, 2010).

While culture based studies have enhanced understanding of the nature of antimicrobial resistance (AMR) in a single class of organisms, the relatively new tool of metagenomics can provide new insight. Metagenomics is not limited to culturable bacteria—it allows for the direct study of all Deoxyribonucleic acid (DNA) present in a sample regardless of culturing technique. Metagenomics allows for the ecological study of samples directly from the environment without enrichment.

1.1. Antibiotic Resistance

Bacteria are classified as antibiotic resistant when they are non-susceptible to at least one antibiotic class. Multidrug resistance (MDR) includes bacteria that are resistant to three or more antimicrobial classes. Pan-drug resistance (PDR) is bacteria not susceptible to any antimicrobial categories (Magiorako et al., 2011).

1.11. History

While there is documentation of the unintentional antibiotic use in alternative medicines, the start of the antibiotic surge can be traced to Salvarsan, a sulfa drug, used in the 1910's to treat syphilis (Aminov, 2010). In 1928, Alexander Fleming first studied penicillin with the antimicrobial properties realized by Howard Florey in 1940 (Zaffiri et al., 2012). From there, the 'golden era' of antibiotic discovery from 1945–1960 commenced during which most of the chemical classes of antibiotics now in clinical use were first characterized (Wright, 2007). Screening soil organisms was the main method of producing antibiotics during this time period (Ling et al., 2015). Synthetic synthesis of antibiotics allowed for discovery of additional antibiotics after the initial surge of naturally discovered antibiotics began to dwindle. However, though proven effective, these techniques of producing antibiotics were limited to culturable organisms. Recently, Ling et al. (2015) described a new class of antibiotic, teixobactin, using a culture free approach. Due to a decrease in recent novel antibiotic discovery, culture free detection holds promise for future discoveries.

Detection of resistance to antibiotics has closely followed their introduction. Resistance was first widely noticed when it began to impact human healthcare. Introduced in 1937, sulfonamide resistance was discovered prior to 1940. Similarly, Penicillin was discovered in 1928 and resistance was reported in 1940 (Davies and Davies, 2010). Resistance to more than one drug was first found in enteric bacteria such as *Escherichia coli* and *Salmonella* spp. in the 1950's and 1960's, though this phenomenon is likely much older (Levy and Marshall, 2004). Antibiotic resistance did not begin with the use of antibiotics. In fact, antibiotic resistance has been part of the microbial world for as long as the microbial world has existed. D'Costa et al. (2011) concluded that antibiotic resistance occurs naturally by dating resistance genes found

alongside flora from the late Pleistocene age. Bhullar et al.'s (2012) conclusions were similar when they characterized resistance to antibiotics in bacteria dated to be over 4 million years old collected in a New Mexico cave. While resistance is accident, selection pressure from antibiotic and environment can increase the phenomena. Use and misuse of antibiotics (such as not completing a course of antibiotics in human medicine) has been recognized as a driver of increased resistance (Goossens et al., 2005).

1.12. Modes of Action

Antibiotics are considered efficient when they inflict harm on a targeted pathogen while inflicting minimal damage to the host (Sefton, 2002). The most efficient toxicity is achieved when an antibiotic inhibits a metabolic pathway not found in the host cell or one that differs drastically from the host, known as selective toxicity. This toxicity can be achieved by targeting either different organelles or structures not present in eukaryotic cells, but present in prokaryotic cells, or by targeting variable regions of shared DNA sequences. An organism is said to be resistant when there is growth of microorganisms in the presence of antibiotics (Wright, 2007). Resistance can be classified several ways, including intrinsic versus acquired, permanent versus temporary, and by function.

Resistance mechanism can be already present or acquired (obtained through some other organism or exposure to a different climate or antibiotic) (Sefton, 2003). Some bacteria are naturally resistant to one or more classes of antibiotics. Organisms in which resistance is not the primary goal sometimes possess natural resistance. For example, some insect gut biomes that have not been exposed to antibiotics have efflux pumps that confer resistance to antibiotics when transferred to *E. coli* (Allen et al., 2010). The public health field is concerned with acquired resistance because once effective antibiotics become ineffective (Tenover, 2006).

Acquired resistance, the acquisition of DNA from an outside source, can be obtained through transformation, transduction, or conjugation (Sefton, 2002). Conjugation is the transfer of exogenous DNA through direct contact with other bacteria, either through plasmid or whole chromosome transfer. Transformation is the direct uptake of DNA fragments by a recipient cell where they recombine with the host cell to form a recumbent cell. Transduction is the transfer of genetic material through bacteriophages (Willey et al., 2013).

Mechanisms of resistance remain the same regardless of the method of acquisition. Enzyme modification, target modification, target by-pass systems, and efflux and influx, or a combination of these traits confer resistance (Wright, 2007). Some mechanisms, such as efflux pumps can confer resistance to multiple antibiotics. Enzyme modification is either plasmid mediated or chromosomal, with chromosomal occurring more frequently in nature, such as β -Lactamases (Sefton, 2002). This modification is effective through alteration of cell wall synthesis (Tenover, 2005). Modification of an existing target is another example of a mechanism modification that relies upon a mutation. An example is the change of a single amino acid through mutation in the S12 protein of the 30S subunit of the ribosome rendering Streptomycin resistance (Sefton, 2002). A target by-pass system is resistance without point modification but, instead, an addition of a process, as seen in MRSA. In this method of resistance, there is an additional penicillin binding protein that does not bind to β -lactams, rendering the organism resistant to all β -lactams. Finally, tetracycline resistance is an example of efflux and influx, which actively removes the drug through pumps in the cell wall (Sefton, 2002).

1.13. Other Drivers of Antibiotic Resistance

Other concerns in antibiotic resistance include co-selection of resistant bacteria in relation to metals and biocides, as well as interactions among different resistant bacteria. In the

case of metals and biocides, the metals function as a selective agent in increasing antibiotic resistance. This occurrence has been documented by multiple studies including Barker-Austin et al. (2006) that pointed out the structural and functional characteristic shared by metal ions and antibiotics, such as efflux pumps shared by copper and zinc, as well as tetracyclines and β -lactams. Pai et al. (2015) conducted a more recent study aiming to understand this co-selection by reviewing sequenced genomes known to contain both AMR and metal and biocide resistance. Findings included that bacteria containing metal and biocide resistance more often carried AMR compared to bacteria without metal and biocide resistance. Furthermore, while metal and biocide resistance genes were common in bacteria genomes (86% occurrence with 17% AMR co-occurrence), they were much more rare in plasmids; though plasmids originating from domestic animals did have a higher rate of co-occurrence than the average environmental plasmid--less than 0.7% vs. 7%. In addition to co-selection, Braoudaki and Hilton (2004) found evidence that some types of biocide resistance could provide cross-protection in certain organisms. They observed *E. coli* O157 cross-resistance between antibacterial agents and biocides. For example, the cross-resistance expressed moved strains from the category of “sensitive” to “resistant” according to the NCCLS guidelines on antimicrobial susceptibility testing.

Interactions among antibiotics and bacteria resistance to antibiotics is another avenue being studied to mitigate resistance. Resistance to antibiotics in a certain population is intrinsic with each population having a baseline resistant profile or “resistome.” Sun et al. (2013) hypothesized that resistance occurs at a relative fitness cost. Commensal bacteria that do not carry resistance to antibiotics may outcompete resistant bacteria immediately after antibiotic treatment, or at least mitigate an increase of resistant bacteria immediately post treatment. Preliminary research conducted by Platt et al. (2008) supports this theory with their work in

feedlot cattle. Feeding chlortetracycline decreased the likelihood of recovering ceftiofur-resistant (β -lactam) *E. coli* isolates, though there was also an increase in temporary resistance to tetracyclines.

1.14. Antibiotic Use in Animal Agriculture

Antibiotics are used to treat disease and increase growth in animal agriculture. Some antibiotics are used to increase feed efficiency, animal growth, and prevent diseases. Additionally, over 100 antibiotics in the United States have been used in production to treat and control pathogens such as *E. coli*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (Hao et al., 2014). Livestock can harbor several pathogens that are detrimental to human health including *E. Coli* O157:H7, *Campylobacter* spp., and *Salmonella* spp. When animals are treated with antimicrobials, it reduces the amount of pathogenic bacteria present, reducing the overall likelihood of transmission to humans. For example, significant reductions of *E. coli* O157:H7 were seen in live cattle that were fed Neomycin (Elder et al., 2002).

Antibiotics can increase animal performance. In the 1940's, farmers noticed higher performance in pigs administered antibiotics. Upon investigation, Hewes (1955) recorded a significant difference in weight gain among animals treated with chlortetracycline. In 1951, FDA approved antibiotics for growth promotion for food animals (Hao et al., 2014). Why antimicrobials increase growth was immediately studied, but even today, exact modes of action are still being investigated. Common assumptions for why antimicrobials increase growth include: (1) reducing colonization of intestinal bacteria and inhibiting the growth of pathogenic microorganisms; (2) decreasing the thickness of mucous membrane, leading to more absorption of nutrients and reduced fermentation; (3) directly neutralizing the host immune response; (4) and shifting to more energy efficient conversion (an example of this would be ionophores that

increase the propionate:acetate ratio) All of these modes of action manipulate host intestinal flora, intestinal physiology, and immune system which contributing to the prevention of disease and changes in microbiome equilibrium (Niewold, 2007).

The method of administration of antibiotics varies depending on the desired outcome of the treatment. While administration to treat animals exhibiting clinical illness is typically targeted to specific animals, application for herd prevention or growth promotion is traditionally applied to whole pen or herd levels. The dosage, duration, mode of administration, and withdrawal times are some of the considerations when administering antimicrobials to animals intended for the food supply (or for animals that produce a product for the food supply such as milk and eggs). Routes of administration vary depending desired outcomes and labor costs. Common administration forms for feedlot cattle include parenteral therapy and oral therapy. Parenteral administration of antibiotics is recommended treatment for severe infection in ruminants, especially in the case of long acting preparations (Gigère et al., 2013). Long acting preparations, antibiotics formulated to have long half-lives, are common in commercial feedlot settings due to the cost and convenience of only needing one injection. An example of a long acting preparation is cephalosporin; while the drug itself does not have a long half-life, the way it is prepared extends its half-life (Gigère et al., 2013). The other method of administration in livestock is orally, typically applied as a top dressing to food in a commercial feedlot setting.

As of 2013, antibiotic classes currently used in food-producing animals are: aminocoumarins, aminoglycosides, amphenicols, diaminopyrimidines, fluoroquinolones, glycolipids, lincosamides, macrolides, penicillins, pleuromutilins, polymyxins, polypeptides, quinoxalines, streptogramins, sulfonamides (sulfas) and tetracyclines (FDA, 2013). The World Health Organization (WHO) considers cephalosporins (3rd/4th generation), fluoroquinolones, and

macrolides of “highest priority critical important;” aminoglycosides and polymyxins “critically important,” and amphenicols, cephalosporins (1st/2nd generation), lincosamides, penicillins, pleuromutilins, streptogramins, sulfonamides, and tetracyclines “highly important” to human health (WHO, 2011). As of 2011, the USDA Animal and Plant Health Inspection Service National Animal Health Monitoring System estimated that 90.1% of cattle in feedlots received an ionophore and 71.25% received tylosin (USDA–APHIS–VS–CEAH–NAHMS, 2013). While prescriptions and feed mill data are sometimes used to estimate the amount of antibiotics used, they are not accurate measurements because some feed is not used directly after purchase. Two antibiotics commonly used in cattle production are Cephalosporins and Chlortetracycline.

1.141. Cephalosporins

Cephalosporins, part of the β -lactam class, were first isolated from fungus in 1945 and used in medicine in the 1960's (Flynn, 1972). The WHO considers 3rd and 4th generation cephalosporins critically important to human health (WHO, 2011). Cephalosporins are critically important because they are one of the few classes that can treat acute bacterial meningitis, disease due to *Salmonella* spp. in children, and infections due to MDR *Enterobacteriaceae*. *Enterobacteriaceae* infections can stem from *E. coli* and *Salmonella* spp. from non-human sources (WHO, 2011). While third and fourth generation Cephalosporins are used primarily in a hospital setting, first and second generation make Cephalosporins one of the most widely used in human medicine making up 40% of outpatient prescriptions (Powers, 2015). In agriculture, the FDA estimates cephalosporins make up around 1% of antibiotics used in animal agriculture (FDA, 2013). Beta-lactams halt cell division by inhibiting the development of phospholipids in the cell wall unique to prokaryotes. Cephalosporins pass through this outer layer and inhibit the

enzyme that catalyzes the crosslinking of oligopeptides to form long-chain polymers that become the cell wall (Weil et al., 1995).

Escherichia coli is commonly resistant to ampicillins, typically acquired by β -lactamases through plasmid acquisition of TEM-1, TEM2, or SHV-1, which inactivates the drugs through hydrolase. A case study by Tenover (2006) illustrated that resistance to β -lactam can develop in a short time frame. A child was admitted to a hospital exhibiting bacteremia with an initial culture positive for *E. coli* isolates that were resistant some antibiotics but susceptible to third-generation cephalosporins. Over 3 weeks, the child received various antibiotics including third-generation cephalosporins. During the fourth week of hospitalization, *E. coli* isolates showing increasing resistance to third-generation cephalosporins. This suggested that the *E. coli* isolates had acquired a new resistance mechanism in a short time frame. (Tenover, 2006).

In US animal agriculture, Ceftiofur and Cephapirin are the two forms of cephalosporins approved for animal use (FDA, 2012). Currently, a Ceftiofur crystalline free acid is trade named Excede™ marketed by Zoetis to treat acute postpartum metritis, bovine respiratory disease due to *Mannheimia* spp., and bovine interdigital necrobacillosis (Zoetis, 2015). Both forms are considered extended spectrum and typically only require one administration of the antibiotic.

1.142. Chlortetracycline

Tetracyclines were discovered in the 1940s and act as a broad-spectrum antibiotic used to treat gram-positive and gram-negative bacteria, atypical organisms, and protozoan parasites (Chopra and Roberts, 2001). Currently, Tetracyclines are considered highly important to human health due to the limited therapy options for infections from *Brucella*, *Chlamydia* spp. and *Rickettsia* spp., as well as the risk of transmission of *Brucella* spp. from non-human sources (WHO, 2011). In 2011, chlortetracycline was feed to 18.4% of all feedlot cattle (USDA–APHIS–

VS–CEAH–NAHMS, 2013). Chlortetracycline, oxytetracycline, and tetracycline are the tetracyclines used in food animal production (FDA, 2013). Tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome by binding to the 30s subunit (Chopra and Roberts, 2001).

Chlortetracycline is fed to treat bacterial enteritis caused by *E. coli* and to control bacterial pneumonia with label claims citing *Pasteurella multocida* as the organism of concern (Alliance Animal Health, 2015). Injectable oxytetracycline is used for treatment of pneumonia and shipping fever complex associated with *Pasteurella* spp. and *Hemophilus* spp; infectious bovine keratoconjunctivitis (pinkeye) caused by *Moraxella bovis*; foot-rot and diphtheria caused by *Fusobacterium necrophorum*; bacterial enteritis (scours) caused by *E. coli*; wooden tongue caused by *Actinobacillus lignieresii*; leptospirosis caused by *Leptospira pomona*; liver abscesses, wound infections and acute metritis caused by strains of *staphylococci* and streptococci organisms sensitive to oxytetracycline in cattle (Kahn and Line, 2010).

Tetracycline is also associated with increase weight gain. Wieser et al. (1966) conducted a study with 300 early-weaned calves in which half were fed the control diet of no chlortetracycline and the other half were fed the same diet with 20mg of chlortetracycline added. The calves fed chlortetracycline had an increase live-weight gain of close to five percent and improved feed utilization of nearly five percent increased efficiency.

1.15. Human and Animal

The risk of transmitting AMR from animals to humans has always been a concern. Levy (1976) demonstrated the ability of antibiotic resistant plasmids to be transferred from both animal-to-animal and animal-to-human through direct contact. He established this by inoculating a chicken with a marked tetracycline resistant plasmid and cohousing it with uninoculated bird

and sampling feces for resistance. After co-housing, he found other birds in the house began to shed the marked plasmid. Recently, pathways of resistance other than direct contact with animal production have been examined, such as meat consumption and particulate matter in the air. In 2001, multi-drug resistant *Salmonella* spp. was found in ground meats (beef, pork, chicken, and turkey) sold at retail. Twenty percent of 200 samples contained *Salmonella* spp., with 84% of those resistant to at least one antibiotic (Walker et al., 2001). Aerial transmission in particulate matter (PM) has also been associated with antibiotic resistance, though at a level that needs more investigation. McEachran et al. (2015) collected PM downwind of several cattle feedlots and analyzed the material. While the material was found to contain antibiotic resistant genetic material, the study was conducted only over a small duration of time and in one area. Multi-drug resistant pathogens have also been of growing concern. Voss (2005) concluded that MRSA could be transmitted between animals and humans, between family members, and between caretakers and patients. Furthermore, those in direct contact with swine had a 760X higher prevalence of MRSA than the general population (Voss et al., 2005). The link of passage between species through zoonotic pathogens, as well as other vectors, has been well established.

Animal agriculture's contribution to resistance first came to light with the publication of two 1960's United Kingdom reports; those of the Netherthorpe committee and the Swann committee, though they reached different conclusions. The Netherthorpe committee concluded that there were no negative effects on resistance from agriculture while the Swann report concluded there were substantial risks (Netherthorpe Committee, 1962; Swann Committee, 1969). The Swann report recommended antibiotics used in human medicine not be used for growth promotion in agriculture and urged more oversight; though the report's concerns were not acknowledged in the United Kingdom until a committee was formed 30 years later (Wise,

2007). In 1975, the US Food and Drug Administration (FDA) formed an advisory committee to study the consequences of sub-therapeutic use of antibiotics in animal agriculture (Ahart, 1977). In the 1980s and 1990s, many groups, including the Institute of Medicine and the Council for Agricultural Science and Technology, pushed for the suspension of antibiotics in animal feeds. However, these groups' arguments were based on conceptual risk, not proven risk (Dibner and Richards, 2005).

The United States, in comparison to other countries, focused on other policy issues throughout the next few decades. It was not until 1996 that the National Antimicrobial Resistance Monitoring System was founded as a collaboration between the Centers for Disease Control and Prevention (CDC), FDA, and the United States Department of Agriculture (USDA). The program monitors susceptibility and resistance of select bacteria by establishing baselines and monitoring trends. *Salmonella* spp. was the original bacteria monitored through human, animal, and later retail meat sampling. This was later expanded to *Campylobacter* in 1998 and *E. coli* in 2000 (White, 2007). In 1999, a task force comprised of the CDC, National Institutes of Health (NIH), and FDA released the *Health Action Plan to Combat Antimicrobial Resistance*. The plan covered surveillance, prevention and control, research, and product development related to antimicrobial resistance (Matthew et al., 2007). During this time, Guidance for Industry 152 was released which raised the concern of animal agriculture's impact on antibiotic resistance in consumers' intestinal microflora and ecology (FDA, 2003).

Internationally, Sweden was the first to ban antibiotics as growth promoters in 1986 (Aarestrup, 2003). In the following 12 years, bans throughout the European Union prohibited some antibiotics in animal feed over concern of resistance in human medicine. Avoparcin, a glycopeptide, was banned in Denmark and Germany over concerns of glycopeptide resistance;

spiramycin, a macrolide, was prohibited in Finland due to human medical use; and virginiamycin, a streptogramin, was prohibited in Denmark because some streptogramins were clinically important in human medicine (Castanon, 2007). Denmark banned all non-prescription use of growth promotants in animal feed in 2000 (DANMAP, 2013). In 2003, the EU, as a whole, banned the use of antibiotics as growth promoters in Regulation 1831/2003 (European Parliament, 2003). Many other countries then began to investigate antibiotics in food productions such as Australia's 1999 report, *The use of antibiotics in food-producing animals: antibiotic-resistant bacteria in animals and humans*, published by their Joint Expert Advisory Committee on Antibiotic Resistance and Canada's 2002 report, *Final Report of the Advisory Committee on Animal Uses of Antimicrobials and Impact on Resistance and Human Health*, from Health Canada (JETACAR, 1999; Health Canada, 2002).

After these bans in the EU, there were studies conducted to assess the ban on antibiotics on resistance in human healthcare and animal agriculture, though the results were mixed and inconclusive. In human healthcare, there were small changes to various indicators of AMR. Three years after the ban, there was a decrease in acquired resistance in enterococci from human feces carriers attributed to the legislation (Casewell et al., 2003). A decrease in resistance in some classes of antibiotics served as the foundation for some countries banning non-therapeutic antibiotics in animal agriculture (Marshall and Levy, 2011).

Animal agriculture also saw decreases in antibiotic resistance isolates with the banning of certain antimicrobials. Vancomycin-resistant enterococci frequency was above 70% prior to the Avoparcin ban, but fell to 5% after implementation. Similarly, in Denmark, there was an up to 70% prevalence of *E. faecium* resistance--attributed in part to the broiler industry--that saw a 36% decline following the ban (Marshall and Levy, 2011). However, animal agriculture did see

adverse effects associated with the ban including increased animal illness coupled with decreased productivity. Specifically, an increase in weight loss and mortality due to *E. coli* and *Lawsonia intracellularis* in post-weaning pigs and clostridial necrotic enteritis in broilers. As a result, there was an increased use of antibiotic treatment of clinically sick animals (Casewell et al., 2003). Marshall and Levy (2011) have attributed a decrease in negative outcomes of the ban to producers having adopted management practices to combat disease resulting in less outbreaks.

1.16. Recent United States agricultural interest

Antimicrobial resistance has seen increased policy attention in the United States. The 111th Congress (2009/2010) renewed an AMR agenda with 15 policy recommendations related to antibiotics and stewardship (Guidos, 2011). During the 111th session, the House of Representatives held hearings on antibiotic resistance with one hearing devoted to agriculture's effect (United States House of Representatives, 2010). The concern was not only impact, but also the lack of scientific literature directly relating AMR use in animal agriculture to human health (US House of Representatives, 2010).

The CDC brought the issue of AMR into the spotlight in the 2013 report: *Antibiotic Resistance Threats in the United States* (Centers for Disease Control and Prevention, 2013). The Obama administration became involved with the issue with the release of Executive Order 13676: *Combating Antibiotic-Resistant Bacteria* in September of 2014. The order called for a task force and national action plan to address antimicrobial resistance (Obama, 2014). *National Strategy for Combating Antibiotic Resistance* (September 2014), *National Action Plan for Combating Antibiotic Resistant Bacteria* (March 2015), and the Presidential Advisory Council on Combating Antibiotic-Resistant Bacteria (Chartered in March 2015 and filled in September of 2015) were outcomes of the executive order. The national strategy focuses on organizing

research to address the pathogens of most concern in the CDC report, while the action plan focuses on more specific goals to counter the growing trends of AMR infections (White House, 2014; White House, 2015).

In addition to the action plan, two Food and Drug Administration Guidance for Industry (GFI) documents were published on the issue. Guidance 209, *The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals*, sought to define judicious use and determine how to properly use antibiotics that are medically important to human health. The guidance defines the term ‘judicious’ broadly, stating unnecessary or inappropriate use should be avoided (FDA, 2012). The two goals of GFI 209 were an increase in veterinary oversight and appropriate use of antibiotics classified as "important to human and animal health" (FDA, 2012). Guidance 213, *New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food-Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions with GFI #209*, was released in 2013 as a more specific follow up plan to the ideas outlines in GFI 209. Guidance For Industry 213 proposed a specific timeline for decreased use of medically important antibiotics in animal production and the phasing out of those products within a three-year period starting in December of 2013 (FDA, 2013).

1.2. Culture Free Approaches

One percent of the microbial world is accessible for study through traditional culture-based methods (Steit and Schmitz, 2004). With such a large number of unculturable microorganisms, culture-free methods have clarified previously unknown aspects of microbial communities (Stahl et al., 1985).

1.21. History

Culture free approaches began with the original studies of heritability conducted by Mendel. From Mendel's Law of Segregation and the Law of Independent Assortment, first published in 1865, to Theodor Boveri's findings on haploid chromosomes, DNA is intertwined with many disciplines of science (Balmain, 2001). Avery–MacLeod–McCarty and Hershey–Chase began DNA exploration by establishing that DNA is the carrier of genetic material. Avery used *Pneumococcus* to demonstrate that DNA causes bacterial transformation while Hersey and Chase infected a cell by inserting DNA into a cell sans protein (Avery et al, 1944; Hersey and Chase, 1952). Subsequently, Watson and Crick determined the double helix shape (1953) and Sanger developed rapid sequencing for DNA (1977) (Smith, 1986; Watson and Crick, 1953).

Due to interest in human genetics, The Human Genome Project (HGP) officially began in 1990 through the National Institutes of Health (NIH), though an early version was started in 1987 by the United States Department of Energy (National Human Genome Research Institute, 2015). The first full version of the human genome was published in February of 2001 with a completed version published in September of 2004. While the genome was a monumental milestone in science, it was the methods developed and refined during this process that matured the field of genomic sciences. The HGP created tools now fundamental to the –omic fields, such as shotgun sequencing with paired end reads, automation, quality, and throughput of collecting raw DNA sequences, and bioinformatics software (International Human Genome Sequencing Consortium, 2001).

At the same time as the HGP, scientists began to understand the limitations of traditional culture based methods (Handelsman, 2004). Plate count anomaly, a discrepancy between the viable plate count and total direct microscopic count of bacteria, especially in aquatics and soil,

was a known concern (Staley and Konopka 1985). Additionally, organisms known to be in samples could not be cultured in a laboratory setting for a variety of reasons, including temperature, pH, and other growth conditions. Because of these limitations, there began to be a focus on nucleic acid methods due to the unchanging nature of DNA independent of growth or cell condition (de Boer and Beumer, 1999).

Polymerase Chain Reaction (PCR), first developed by Kary Mullis and Cetus Corporation in the 1980's, was a major advancement in the study of nucleic acid and is still the gold standard (Bartlett and Stirling, 2003). Using PCR, it is possible to amplify specific DNA sequences more than a million fold in less than an hour (Eisenstein, 1990). In one PCR run, several cycles are ran with target DNA strands exponentially replicating. Major steps in each PCR cycle are: (1) heated denaturing of DNA resulting in a double strand being separated (2) reduction of temperature and attachment of DNA primers (3) synthesizing of complement strand of DNA with *Taq* polymerase (4) Replication of cycle 28-25 times (Eisenstein, 1990). In the 1980s, PCR advancement in primer technology allowed for whole genes to be amplified leading to the discovery of many new phyla (Weisburg et al., 1991). During this period, use of the 16S Ribosomal Ribonucleic acid on the 30S subunit of prokaryotic ribosomes was used because it is a “component of all self-replicating systems; it is readily isolated; and its sequence changes but slowly with time—permitting the detection of relatedness among very distant species” (Woese and Fox, 1977). While PCR is specific with the correct primers, there is no way to differentiate viable and non-viable cells without an enrichment step prior to amplification (Velusamy et al., 2010).

1.22. Metagenomics

Metagenomics, the characterization of microbial communities and cataloging microbial diversity and distribution without isolating or culturing organisms, is an emerging discipline (Doolittle and Zhaxybayeva, 2010). In 1985, the first use of metagenomics was sequencing microorganisms living in a 91°C hot spring in Yellowstone National Park from direct sampling without enrichment (Stahl et al., 1985). Pace completed the sequencing by direct work with the 5S and 16S rRNA genetics as opposed to culturing (Stahl et al., 1985). This work was further advanced when the same research group isolated and cloned large amounts of environmental DNA (Schmidt et al., 1991). This methodology, along with a decrease in sequencing costs, pioneered the approach for culture free application and made it more readily assessable to researchers.

1.22.1. Uses

Metagenomics can complement culture-based methods or be utilized as a stand-alone research tool. If a culture-based method requires manipulation of a variable outside the normal parameters, metagenomics can be used. An example of this is *Helicobacter pylori*, a cause of gastric cancer and ulcers, which was cultured by accidentally incubating plates for five days instead of three (Handelsman, 2004). While an accident, this illustrates that there are many growth parameters that can be manipulated which is often financially prohibitive. With the advancement in culture-independent methods, there has been progress in identifying previously un-cultured organisms. Pelagibacterales, an order in Alphaproteobacteria, belong to the group that contains one-fourth of all ribosomal RNA genes contained in seawater, and was not cultured until it was first identified by sequencing--at which point nucleic acid probes allowed for

quantitative assessment of enrichment and growth, which was previously impossible (Rappé et al., 2002).

As a stand-alone scientific tool, metagenomics can be used to study diversity, extreme environments, and changes over time. One of the first diversity studies was sampling of the Sargasso Sea and the characterization of 148 previously undiscovered bacterial phylotypes (Venter et al., 2004). After this, a 2002 study on seawater found 65% of the virus sequences obtained from two different marine viral communities were not previously reported, meaning the majority of viruses found were novel and had never been characterized before (Breitbart et al., 2002). With the ability to circumvent broths and amplifications, metagenomics' ability to study microbiomes and ecosystems, which have been difficult to study due to the limited ability to culture them, is unique. Another example of this is the characterization of thermophilic viruses in Yellowstone National Park hot springs. The result was identification and function classification of many viruses that were previously unculturable (Schoenfeld, 2008). Finally, changes in microbiomes are also an area where metagenomics differs from traditional culture based methods. Jami et al. (2013) sequenced rumen fluid from cattle in five different stages of life to see how the organisms in the rumen developed and changed over time.

Metagenomics also brings unique advantages to different disciplines. An area of study especially important to food science is the ability of metagenomics to study sub-lethally injured bacteria. Some bacteria survive antimicrobials or other interventions that are aimed at decreasing bacterial populations, but survivors are too injured to be cultured. This issue was first highlighted by Colwell and his work with *Vibrio cholerae* that were alive and capable of causing illness, but not cultivable until passed through an intestine (Handelsman, 2004).

1.22.2. Techniques

Unique challenges that arise in the metagenomics field require tailored techniques, including unique sampling design, unbiased extraction and sequencing, and large data analysis. Because of this, experimental design, processing, and analysis must be considered.

The first consideration is design and size of an experiment. Sample processing is significantly more costly than culture-based experiments, so each sample must be handled with care. When collecting samples, DNA contamination, from individuals collecting the samples and from outside sources, must be kept to a minimum to maintain the integrity of the sample. Additionally, metadata, such as location and sampling conditions, must be collected at each sampling location. If samples are not immediately sequenced, they should be stored at -80 degrees Celsius (Hale et al., 2015). Sequence depth is another consideration before sampling. Sequencing depth is the “average number of times that each nucleotide is expected to be sequenced given a certain number of reads of a given length and the assumption that reads are randomly distributed across an idealized genome” (Sims et al., 2014). If sequencing is not deep enough, the target genes of interest may be overlooked. However, sequencing too deeply can be cost prohibitive.

Extraction of DNA is commonly performed with kits, depending on the type and amount of sample collected. If the study goal is to investigate a community's dependence on a host, methylation can be used to decrease the amount of host DNA (Feehery, 2013). Once DNA is extracted, a nanodrop or qPCR run can determine concentration and quality of DNA; with the concentration increasable through ethanol precipitation (Zeugin and Hartley, 1985).

Processing can be broken down into two phases: library preparation and sequencing. The collection of DNA fragments is termed library preparation. Libraries can either be paired-end or

fragmented, with paired-end being more desirable due to a double amount of DNA. Once DNA is fragmented, sequencing can begin. The Sanger method of sequencing was the gold standard for many years, but there has been a shift to Next Generation Sequencing (NGS), which is quicker and cheaper than the Sanger method (The European Bioinformatics Institute, 2015). Next Generation Sequencing is the basis of widespread, affordable, metagenomic studies. First introduced by 454 Life Sciences, NGS achieves a 100-fold increase in overall throughput compared to the Sanger Method (Margulies et al., 2005). Next Generation Sequencing is done by arranging hundreds of thousands of sequencing templates in either picotiter plates or agarose thin layers and analyzing all of them at the same time; a process known as “parallel sequencing” (Schuster, 2008). The commercial companies that compete in this market include Illumina/Solexa, 454/Roche, Oligo Ligation Detection (SOLiD) by Applied Biosystem/Life Technologies (Thomas et al., 2012).

Another unique component of metagenomics is the analysis a large amount of data, which requires an interdisciplinary field of study. Bioinformatics is "conceptualizing biology in terms of molecules and applying 'informatics techniques' (derived from disciplines such as applied maths, computer science and statistics) to understand and organize the information associated with these molecules, on a large scale” (Luscombe et al., 2001). Data can be directly analyzed or assembled computationally by reference-based or *de novo* assembly. Reference-based is used when a reference genome is available and requires little computational power. *De Nova* assembly, on the other hand, is computationally costly but can be used for diverse samples or those that differ from reference genomes (Thomas et al., 2012). Sharing sequence data across different scientific fields benefits both collaborators. However, due to the large size of most sample sequence reads, there are three DNA archives currently used to share sequence data

funded by three different governments: GenBank funded by NIH, EMBL funded by European Molecular Biology Lab, and the DNA Database of Japan funded by the Japanese Ministry of Education (National Research Council, 2007). These databases require metadata, along with nucleotide sequences, as environmental variables influence these data.

Because metagenomics is still a new field, there are few certainties in analysis; however, there are some generally accepted methods for managing data: clustering, binning, and gene annotation. Clustering divides the dataset into subsets based on specific characteristics such as evolutionary origin. Binning is a type of clustering method that uses composition or other characteristics of contigs to divide them into clusters that belong to specific genomes or groups. Gene annotation classifies predicted genes into well-characterized gene families (National Research Council, 2007).

After quality control measures, such as removal of unwanted DNA, normalization must be performed on metagenomic samples. Normalization adjusts for biases including sequencing depth and gene length (Oshlack and Wakefield, 2009). Normalization is needed because, in many instances, determining if an observed difference in read hits is significant is the objective of a study. In the past, Poisson distributions have determined this significance. However, the Poisson distribution is too restrictive and type-I error is not correctly controlled for. Therefore, Anders and Huber proposed to model count data with negative binomial (NB) distributions, which is now widely used as DESeq in the R Bioconductor package (2010). Another commonly used normalization is cumulative sum scaling, which addressed heteroscedasticity and the detection of differential abundance of species (McMurdie and Holmes, 2014).

Metagenomics, genomics, and other -omic fields are a potential tool for all life science disciplines. Any discipline that incorporates any microbial community and their interactions

could benefit from metagenomics as a companion tool to current research or as a new method to explore an old problem. Metagenomics offers a new approach to conventional research, and also opens the door research questions we would not even have posed without next generation sequencing.

CHAPTER 2

Effects of antibiotic treatment strategies on feedlot cattle resistome and microbiome

Summary

The objective of this study was to evaluate resistome and microbiome changes in feedlot cattle exposed to commonly used antimicrobials. Sixteen pens of cattle (N=16) were randomly assigned to one of four antimicrobial treatments (n=4) resulting in a complete 2x2 factorial arrangement. The first factor was to treat Ceftiofur crystalline free acid (CCFA) to either the entire pen of animals (high dosage) or to one animal in the pen (low dosage). The second factor was the subsequent feeding of chlortetracycline (CTC) in feed to the entire pen of cattle or not administering CTC to a pen of cattle. Rectal fecal samples were collected from individual cattle within each pen on days 0 and 26. Deoxynucleic acid was extracted from individual fecal samples and pooled by DNA mass, so each pen had one composite sample on day 0 and day 26. Deoxynucleic acid was sequenced on an Illumina HiSeq 2000. Sequencing data (as known as reads) were aligned to a comprehensive antimicrobial resistance gene database and assigned to taxonomic labels. Sixty-eight antimicrobial resistance genes and 431 species were identified across all samples. Resistance to tetracycline was identified as the primary resistance at class level (66.9%) with resistance to Macrolide-lincosamide-streptogramin B (MLS) making up the majority of the remainder (26.2%). Resistance to tetracycline and aminoglycoside in the feces decreased ($P < 0.05$) in relative abundance from day 0 to day 26 when the cattle were fed CTC regardless of CCFA exposure. Beta-lactams were the only class of resistance affected by the CCFA treatment, with low exposure CCFA pens exhibiting a smaller ($P < 0.05$) resistome on day 26 than those steers in high exposure CCFA pens, regardless of CTC treatment. These results

indicate that the exposure to tetracycline for cattle may not be directly associated to the resistance to tetracycline in their feces. Further research is needed to explore more about this. Additionally, the decrease in resistance to aminoglycoside with no cattle exposed to anyaminoglycosides during the study raises the possibility of co-selection of resistant genes. Overall, the relative abundance of microbiome did not differ ($P > 0.05$) between pens of cattle with CCFA or CTC treatments but differed ($P < 0.05$) between day 0 and day 26. Overall microbiome relative abundance did not differ ($P > 0.05$) due to CCFA or CTC treatments but differed ($P < 0.05$) between day 0 and day 26. Changes in the microbiome over time affected all 19 phyla identified when all treatments were pooled together. It has been well established in humans that antimicrobial treatment changes in the microbiome (Khoruts et al., 2010; Preidis and Versalovic, 2009). While these findings are not as robust livestock, there is ongoing investigation establishing these same results that may lead to further understanding of how the microbiome of livestock responds to antibiotics.

Introduction

Antimicrobial resistance (AMR) has become a global health concern that transcends traditional scientific disciplines. Resistance to antibiotics in human healthcare can originate from several sources, with animal agriculture playing a role. American beef is not only widely consumed domestically, but the US is a large beef producer and exporter in the world market (Daniel et al., 2010). The US beef industry relies on antibiotics not only for clinically sick animals, but also to prevent disease and increase growth. Some of antibiotic classes used in production, such as third-generation cephalosporins and tetracyclines, are considered important to human health (World Health Organization, 2011). Several studies have demonstrated that use of antimicrobials in food animals can correspond with increased risk of resistant bacteria being

transmitted to in humans (Marshall and Levy, 2011; Voss et al., 2005; Threlfall et al., 2000). Recently, there have been several changes to historical antimicrobial practices to mitigate the spread of AMR. Guidance For Industry 213, proposed by the Food and Drug Administration (FDA), created a specific timeline for decreased use of medically important antibiotics in animal production and the phasing out of those products within a three-year period starting in December of 2013 (FDA, 2013). Others have proposed a stronger stance on use of antibiotics in food animals, such as complete bans. However, if all antibiotics were to be eliminated from animal production this would negatively affect animal welfare, which, in turn, would affect animal health, thus negatively impact human health (National Academy Press, 1999). The need to understand all factors that influence AMR bacteria has expanded beyond simply administering or not administering an antibiotic to understanding how the resistant genes interact with other antibiotics and host microbiomes.

Two antibiotics were used in this study, ceftiofur crystalline free acid (CCFA) and chlortetracycline (CTC). Ceftiofur crystalline free acid, or Ceftiofur (a third-generation cephalosporin) is used in cattle to treat acute postpartum metritis, bovine respiratory disease due to *Mannheimia* spp., and bovine interdigital necrobacillosis (FDA, 2008). The World Health Organization (WHO) considers 3rd and 4th generation cephalosporins critically important to human health (2011). Cephalosporins are critically important because they are one of the few classes that can treat acute bacterial meningitis, disease due to *Salmonella* spp. in children, and infections due to MDR *Enterobacteriaceae*. In agriculture, the FDA estimates cephalosporins make up around 1% of antibiotics used in animal agriculture (FDA, 2013). In 2011, chlortetracycline was feed to 18.4% of all feedlot cattle in US feedlots (USDA–APHIS–VS–CEAH–NAHMS, 2013). Tetracyclines are considered highly important to human health due to

the limited therapy options for infections from *Brucella*, *Chlamydia* spp. and *Rickettsia* spp. as well as the risk of transmission of *Brucella* spp. from non-human sources (WHO, 2011).

Chlortetracycline is fed to treat bacterial enteritis caused by *E. coli* and to control bacterial pneumonia (Kahn and Line, 2010). Tetracyclines also have properties associated with increased weight gain (Wieser et al., 1966).

The objective of this study was to evaluate changes to feedlot cattle resistomes and microbiomes as a result of commonly used antimicrobial exposure. Feces from cattle exposed to commonly used antimicrobials in a controlled research feedlot was used in this study. While the study was conducted over 26 days with multiple samplings throughout, day 0 and day 26 were used as the two points. This study's aim was to understand the long-term effects of antimicrobial resistance that could be disseminated through the food system and possibly transmission to humans. Therefore, the last day sampling (day 26) was used as the closest point to slaughter. Microbiome and resistome changes due to antimicrobial treatment have been well established (Noyes et al., 2016; Kanwar et al., 2013). However, a study that has specifically controlled for antimicrobial treatment through a shotgun metagenomic lenses has not yet been conducted, which is what this study provides. Also an added component was the ability to compare previously obtained PCR data from the same study design (Kawar et al., 2013 and Kanwar et al., 2014) to newly acquired shotgun metagenomic results.

Materials and Methods

Study Design

Bovine fecal samples were collected on two sampling days over a 26-day feeding period. Eighty-eight steers were blocked by weight into 8 pens of 11 steers each. The study was conducted twice with 88 cattle in each duplicate for a total of 16 pens enrolled in the study (N =

16). In each replication, the study factors and antibiotics administration remained the same. The two antibiotic treatments at two levels were combined, resulting in four combinations and a complete 2x2 factorial. The first factor was low exposure or high exposure to ceftiofur crystalline free acid (CCFA). Low exposure was classified as one steer in each pen out of 11 received parenteral CCFA, while high exposure was defined as all 11 steers in the pen received CCFA. The second factor was if an entire pen received in-feed chlortetracycline or did not. Day, with fecal samples collected on day 0 and day 26, was considered a repeated measure. The statistical model was a 2x2 factorial design with a repeated measure ($n = 4$).

On day 0, long acting CCFA (Excede®, Zoetis Animal Health, NJ, USA) was injected subcutaneously at the base of the ear at a dose rate of 6.6mg/kg. In eight pens, one steer in the pen was treated (low exposure) and in the other eight pens, all steers in the pen were treated (high exposure). In the same 16 pens, Chlortetracycline treatment (Aureomycin®, pre-mix complex equivalent to 220.5 g of chlortetracycline/kg, Alpharma, Bridgewater, NJ, USA) was either fed not fed. Chlortetracycline was administered during three separate 5-day periods, with a one-day break in between feeding periods, starting on day 4 with the final treatment on day 23. It was delivered via top dressing after the morning feeding.

Sample collection

Rectal fecal grabs were collected on day 0 and day 26 from individual animals, after animals were restrained in a squeeze chute. In total, 352 fecal samples were collected. Samples were stored at -70°C until further processing.

DNA extraction

Individual fecal samples were thawed for DNA extraction. DNA was extracted from 200 mg of feces from each sample using the QIAamp DNA Stool Mini Kit™ (Qiagen, Valencia, CA,

USA) according to the manufacturer's instructions. The extraction was performed in the QIAcube robot™ (Qiagen, Valencia, CA, USA). Once DNA was extracted, samples were pooled by DNA mass. From each pen, 11 individual DNA samples (one sample from each steer) were pooled, in equal parts, into one composite sample. This was done for each pen on each day for a total of 16 pens on each day and 32 composite samples total. Quality and concentration of the DNA was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Library preparation and Sequencing

After DNA extraction, samples were delivered to the Genomic and Microarray Core at the University of Colorado Denver for sequencing (Aurora, CO, USA). A library preparation kit made for a smaller quantity of DNA was used. Sample libraries were constructed using the Nugen Ultralow System V2 (NuGen Technologies Inc., San Carlos, CA, USA). Library sequencing (paired-end, 2x100 base pairs) was performed on four lanes of an Illumina HiSeq 2000 (Illumina, Inc. San Diego, CA, USA) with eight samples per lane.

Quality Control of metagenomic sequence data

Raw sequence data was trimmed and filtered using Trimmomatic to remove low quality reads (Bolger et al., 2014). The “ILLUMINACLIP” command was used to remove Illumina TruSeq adaptors. Each read’s first and last three base pairs were removed. Then, starting at the 3’ end of the read, a four nucleotide sliding window calculated the average Phred score and, if the score was lower than 15, that window was removed until the average quality score rose above 15. Finally, any reads with less than 36 nucleotides and their mates were removed from the dataset. Trimmed reads were aligned to the *Bos Taurus* (UMD_3.1) and the draft *Bos indicus* genome (Canavez et al., 2012) and these sequences were filtered out of the sample using the

Burrows-Wheeler Aligner (BWA) default settings (Li and Durbin, 2009). The removal of these genomes created a non-host read sample for each of the 32 samples.

Microbiome Analysis

Non-host reads were assigned taxonomic labels by Kraken version 0.10.6 beta (Wood and Salzberg, 2014). Kraken-filter was used at a threshold of 0.20, meaning labels were adjusted up the phylogenetic tree until the label's score meets or exceeds 20%, so that 20% of kmers for a given read must be at or below a taxon to allow the classification of a read. The filter function has been shown to increase classification precision as the threshold is increased (Wood and Salzberg, 2014). For statistical analysis, output from Kraken was converted into raw counts with rows representing taxa, columns containing each sample, and a cell containing the number of reads classified to a taxon. Counts were normalized using cumulative sum scaling with a default percentile of 0.5 for normalization due to the sparseness of count data (Paulson et al., 2013). While there were changes in all levels of the microbiome (phylum, class, order, family, genus, and species), the lower levels of family, genus, and species are not reported as the lower taxonomic units become less reliable (Peabody et al., 2015).

Resistome Analysis

Non-host reads were aligned to a custom AMR gene database described by Noyes (2016) comprised of publically available genes from Resfinder, ARG-ANNOT and CARD (Zankari et al., 2012; Gupta et al., 2014; McArthur et al., 2013). The non-redundant database contained 4309 resistant genes. A custom-developed Java-based script was used to parse the resulting SAM file such that the gene fraction, defined as the proportion of nucleotides in a given reference gene that aligned to at least one read, was calculated for each AMR gene in each sample (accessible at https://github.com/colostatemeg/gene_fraction_script/releases). Per Noyes et al. (2016) only

AMR genes with gene fraction of >80% were considered present in a sample and included in further analyses. This method aimed to decrease the number of false positive identifications (Gibson et al., 2015, Noyes et al., 2016).

The number of hits to each AMR gene was compiled with samples in columns and AMR genes in rows. Additionally, each AMR gene was classified at the mechanism, class, and group level. Analysis at the gene level was not performed to avoid biasing diversity measures as a result of non-uniform gene naming nomenclature (Hall and Schwarz, 2016). Thirty-three AMR genes present in less than 3 samples were removed from the analysis due to the incapability to accurately normalize these counts. The remaining counts were normalized in a two step process, first cumulative sum scaling then accounting for differences in sequence length in AMR genes and bacteria load in samples. First, counts were normalized using cumulative sum scaling with a default percentile of 0.5 for normalization due to the sparseness of count data (Paulson et al., 2013). Li et al.'s (2015) equation was adopted to account for difference in sequence length of AMR genes and bacteria load in samples. First, reads were aligned to the full Greengenes database using BWA with default paired-end settings to identify 16S sequences in all non-host read samples (DeSantis et al., 2006). Then, the abundance of each AMR gene was calculated:

$$Abundance = \sum_1^n \frac{N_{AMR-like\ sequence} / L_{AMR\ reference\ sequence}}{N_{16S\ sequence} \times / L_{16S\ sequence}}$$

with $N_{AMR-like\ sequence}$ as the number of CSS normalized hits to one specific AMR gene; $L_{AMR\ reference\ sequence}$ as the sequence length of the corresponding AMR gene; $N_{16S\ sequence}$ as the number of hits to 16S sequences and $L_{16S\ sequence}$ as the average length of the 16S sequences in the Greengenes database. Li's equation allowed for the expression of AMR counts to be as a "copy

of AMR gene per copy of 16S-rRNA gene.” After normalization, counts were aggregated to the class, mechanism, and group level for statistical analysis.

Statistical Analysis

For statistical analysis, CSS and Li normalized counts were used for the resistome and CSS counts were used for microbiome. In R (version 3.3.0), ordination and log-fold changes in abundance were calculated. Ordination was performed with Hellinger transformed normalized reads on 2 dimensions with “vegan’s” metaMDS function using Euclidean distances (Legendre and Gallagher, 2001). On completed ordination plots, separation between groups was tested with analysis of similarities (ANOSIM; Clarke, 1993). Log-fold changes in abundance between groups was determined by a multivariate, zero inflated, Gaussian model using metagenomesSeq’s “fitZig” function. FitZig output was used in limma’s “makeContrast” function for pairwise comparisons and adjustments for multiple comparisons, were made using the Benjamini-Hochberg procedure with $\alpha = 0.05$ (Benjamini and Hochberg, 1995). Interactions between treatments were determined using the PROC MIXED procedure in SAS (version 9.4). Day, CTC, and CCFA main effects, as well as their interactions, were calculated for each class, mechanism, and group of AMR genes and each phylum, class, and order in the microbiome for relative abundance. These main effects and interactions were also used to study Richness (the number of unique features in a samples, e.g., number of classes of AMR genes) and Shannon’s diversity (the number and proportion of unique features in a sample) Experiment duplication and pen were dropped from the model as they were not significant and lane was used in the RANDOM statement. LSMEANS/PDIFF was used for means separations when a main effect or interaction was significant ($\alpha = 0.05$). All analyses considered pen the experimental unit.

Primer Sequencing Comparison

Due to the identical study design in Kanwar et al. (2013), comparison of their findings to our results was a possibility. Therefore, we searched for the exact primers they used in their PCR analysis to compare our findings. Because the primers (table 1) were small in size (20-24bp) BWA alignment was unsuccessful even after changing the minimum seed length to 10bp. Instead, Tablet (version 1.15.09.01) was used to visually assess primer region to verify AMR aligning results (Milne et al., 2009). Each of the 32 samples was individually visually inspected for the primers tet(A), tet(B), *bla_{CMY2}* using Tablet (version 1.15.09.01). The primary assembly sequence used was the individual sample's "sam" file while the reference file was a "fasta" version of the AMR gene database. Once a file was loaded, the primer region was found on the reference gene and visually inspected for presents of absence of hits in the region.

Results and Discussion

Sequencing Results

Sequencing generated 1.42 billion reads with an average of 44.24 million reads per sample (range 14.62 to 67.87 million). Quality scores (Phred scores) across all samples averaged 35.11 and ranged from 32.6 to 35.77. Phred scores above Q30 indicate that there was a less than 0.1% chance a base was called incorrectly. Trimming resulted in removal of 2.4% of reads across all samples. Of remaining trimmed reads, 0.14% were classified as *Bos taurus*, and were removed.

Resistome

Using an 80% gene cutoff fraction, 1.25 million reads were aligned to 101 AMR genes, though after quality controls were imposed, this number was reduced to 68 genes. The AMR genes were classified into 6 unique classes of resistance, 13 mechanisms and 31 groups.

Tetracycline resistance was the predominant class that reads aligned to, with macrolides-lincosamide-streptogramin (MLS) resistance making up most of the remainder (figure 1). The main mechanism of resistance in the tetracycline class (98.5%) was resistance ribosomal protection proteins (RRPP). Macrolide resistance efflux pumps (MREP) were the main mechanism that conferred resistance in the Macrolides-lincosamide-streptogramin class (88.7%). Besides RRPP and MREP, the other predominant mechanism of resistance was class A β -lactamases which composed 4.6% of total resistance mechanisms and 99.5% of β -lactam resistance. Overall the resistome characterized in this study was similar to other metagenomic and culture based characterizations. Kanwar et al. (2013) characterized isolated, cultured, *E. coli* from the same study design and found the most predominantly resistance was to tetracycline at 61.14%. Noyes et al. (2016) also found tetracycline to be a predominant class of resistance in beef production.

The overall size of the resistome (how many genes present conferred resistance) was not affected ($P > 0.05$) by day or CCFA treatment. However, feces from cattle treated with CTC had a larger ($P < 0.05$) resistome than those not treated with CTC. Additionally, relative abundance of resistance was affected at the class, mechanism, and group level. Two classes of resistance were significantly affected by CTC treatment and one class was affected by CCFA treatment. Tetracycline resistance in the feces decreased ($P < 0.05$) in relative abundance from day 0 to day 26 when the cattle were fed CTC regardless of CCFA treatment. In contrast to our results, Noyes et al. (2016) found an increase in the proportion of samples positive for two mechanism of tetracycline resistance (major facilitator superfamily efflux pumps and ribosomal protection proteins) in feedlot pens where at least one animal was administered tetracycline during feeding (ranged from 117 to 227 days). Though our findings and Noyes et al.'s (2016) offer different

outcomes based on tetracycline exposure, exposure to tetracycline may not be the only driver of tetracycline resistance. For example, Morley et al. (2011) conducted a feedlot study evaluating non-type specific *E. coli* (NTSEC) antibiotic susceptibility cultures from pen floor feces. While some rations feed during the study containing macrolides and ionophores, none contained tetracycline. However, tetracycline resistance prevalence among NTSEC isolates increased as the feeding period progressed. Tetracycline resistance cannot be definitively linked to an increase use or disuse of tetracycline antimicrobials, but instead needs to be further explored. Another consideration when evaluating changes to tetracycline resistance in the length of the present study's time. While this study was designed to evaluate the long-term effects of antimicrobial exposure on resistance, the last sampling day was three days after the final in feed administration of CTC. A more extended timeline, or a longer 'washout' period of the animals not being fed tetracycline may have yielded different results.

Aminoglycoside resistance also declined, ($P < 0.05$) but remained present, in relative abundance from day 0 to day 26 when the cattle were fed CTC, regardless of CCFA treatment. However, no aminoglycoside class antibiotics were administered to any cattle throughout the study. While Noyes et al. (2016) also found aminoglycoside resistance altered in their study sans treatment with this antimicrobial; they did not find the same decreases in relative abundance. Because no aminoglycosides were administered in the course of the study, it is clear direct selection pressure from this antimicrobial is not the cause of the change of aminoglycoside resistance. The changes and persistence of this class of antibiotic could be present for several reasons; including co-selection. Coque et al. (2008) found the proliferation of Extended-spectrum beta-lactamases (ESBLs) resistance has been aided by co-selection with aminoglycosides.

Beta-lactams were the only class of resistance affected by the CCFA treatment, with lower exposure CCFA pens exhibiting smaller ($P < 0.05$) resistance on day 26 than those steers in high exposure CCFA pens, regardless of CTC treatment (table 2). Low exposure CCFA pens more quickly return to their day 0 levels than high exposure CCFA pens. However, this difference was observed across CTC treatments, meaning pens treated with CTC treatment after CCFA treatment did not differ in the time it took to return day 0 levels for CCFA resistance. Kanwar et al.'s (2014) qPCR results of the same study treatment design agreed that CTC treatment did not reduce CCFA resistance in the fecal resistance more quickly. Kanwar et al. (2014) also addressed the CCFA different exposure levels but found that their 16S rRNA gene copies CCFA resistance did not differ between high and low exposure. This does differ from the findings of Kanwar et al. (2013) that demonstrated low exposure cattle exhibited less CCFA resistance than high exposure. While this study observed no changes in CCFA resistance as a result of CTC treatment, other studies drew different conclusions. Platt et al. (2008) demonstrated CTC temporarily decreased CCFA resistance when administered in feed, though this was specific to *E. coli* isolates. Platt et al.'s (2008) study was limited in scope as it only viewed resistance through *E. coli* as oppose to other organisms.

Predictably, many of the mechanisms and groups that further classify and divide classes of antimicrobials that saw significant differences (tetracyclines, aminoglycoside, and β -lactams) in resistance were affected in the similar ways by treatments; an exception to this was tetracycline resistance. Resistant groups within the tetracycline class (a more specific categorization than an a mechanism but encompassing more than a specific nucleotide gene makeup, such as all Tet(A) or TetB) genes) were altered by CTC treatment and/or day (Figure 2). Five groups of tetracycline resistance--Tet32, Tet40, Tet44, TetO, TetW, and TeteX-- increased

($P < 0.05$) in relative abundance of resistance between day 0 and day 26 of cattle fed CTC across CCFA treatments. While these groups of tetracycline resistance increased, other groups, such as TetQ, did not increase (table 3). These changes in tetracycline relative abundance raise another interesting piece of tetracycline resistance because even though overall relative abundance of tetracycline decreased, there were several groups that increased.

There was no difference in richness of the resistome at the class, mechanism, or group level ($P = 0.31$; $P = 0.52$; $P = 0.11$). Pens across CCFA treatments on day 26 fed CTC had, on average, 6 additional AMR genes than those not fed CTC. At the class level, in pens not treated with CTC, cattle feces from low exposure CCFA treatment had more ($P < 0.05$) resistome diversity than those in high exposure pens. At the group level, changes in diversity were more pronounced: regardless of CTC treatment, cattle in low exposure CCFA pens had higher ($P < 0.05$) resistome diversity than cattle in high exposure CCFA pens. These differences in diversity, a combination of richness and relative abundance, could be due to relative fitness costs. Sun et al. (2013) hypothesized that resistance occurs at a relative fitness cost. Commensal bacteria that are not resistant to antibiotics, may outcompete resistant bacteria immediately after antibiotic treatment, or at least mitigate an increase of resistant bacteria immediately post treatment. In this study, high exposure to CCFA (an entire pen of cattle was treated with CCFA) may have had less commensal bacteria to repopulate after treatment thus, the lower diversity findings than pens that had low exposure (only one animal in the pen treated with CCFA).

Microbiome

After quality control measures, 19 bacteria phyla, 30 classes, 73 orders, 135 families, 260 genus, and 431 species were identified across all samples. Four phyla--Bacteroidetes, Firmicutes, Spirochaetes, and Proteobacteria--made up over 99% of all phyla identified across all

samples (46.8%, 25.8%, 18.2%, and 8.5% respectively, figure 3). Predominant classes identified included Bacteroidia (42.2%), Spirochaetia (17.7%) and Clostridia (13.0%). Bacteroidales, Spirochaetales, and Clostridiales made up the majority (73%) of orders with 43.0%, 17.4%, and 12.4%, respectively.

The microbiome described in our study did share several similarities to previously described microbiomes in beef cattle production. Durso et al. (2010) described Bacteroidetes, Firmicutes, and Proteobacteria as major phyla found in beef cattle production using total fecal extraction and PCR. Yang et al. (2016) further described phyla found in beef production by using metagenomics and also found Bacteroidetes, Firmicutes, and Proteobacteria to be main contributors, though observed Spirochaetes at a nominal level. Rumen microbiomes of cattle have also proven to be similar to fecal results. Jami and Mizrahi (2012), though looking at dairy cattle, also found Bacteroidetes, Firmicutes, and Proteobacteria to be predominant phyla, though they also observed Spirochaetes at much lower levels than in the present study. Spirochaetes was found at higher levels in the present study than other work had previously reported. Although Rice et al. (2012) found Spirochaete at a lower level than this study; they determined Spirochaete levels could be influenced by diet. Relative to this study, Spirochaetes followed general trends of the microbiome. Meaning, the phyla did not differ between CTC treatments ($P = 0.933$) or between CCFA exposure levels ($P = 0.926$), but did increase from day 0 to day 26 ($P < 0.05$). Due to the study-wide nature of the Spirochaete proportion, the large proportion likely has to do with systematic feeding or climate and not to do with treatments.

Overall microbiome relative abundance did not differ ($P > 0.05$) due to CCFA or CTC treatments but differed ($P < 0.05$) between day 0 and day 26 (Figure 4). On day 0 and day 26 (Figure 5) treatments did not differ from each other within their day ($P = 0.30$; $P = 0.63$);

meaning there was more dissimilarity within a treatment combination than between treatments on both day 0 and day 26. This finding, that the microbiome was more impacted by time than antimicrobial treatments, is consistent with Doster and Rovira (unpublished manuscript) who saw more separation between time (day 0 vs. day 11) than between their treatment groups of Draxxin (a macrolide) and a control group.

Changes in the microbiome over time affected all 19 phyla identified when all treatments were pooled together. While eight phyla decreased in log-fold changes in abundance from day 0 to day 26, the other 11 phyla increased in log-fold changes in abundance of hits to taxa (Figure 6). Changes in class and order level also saw similar changes with both levels of classification seeing all 30 classes and all 73 orders change due to time. These changes in microbiome appear to be larger in scale than those in Doster and Rovira's (unpublished manuscript) study but this could be due to the fact that this current study was more than twice as long (26 days as oppose to 11 days).

It has been well established in humans that antimicrobial treatment changes in the microbiome (Khoruts et al., 2010; Preidis and Versalovic, 2009). While these findings are not as robust livestock, there is ongoing investigation establishing these same results. Looft et al. (2012) found pigs treated with antimicrobials saw an increase in Proteobacteria as well as microbial functional genes relating to energy production and conversion compared with pigs not treated with antimicrobials. While research is still in earlier stages of development, further understanding of how the microbiome of livestock responds to antibiotics will help better treat disease while minimizing AMR impact and prevalence.

Comparisons of PCR vs. Metagenomics

Because of previously available results on the same study design (Kanwar et al., 2013 and Kanwar et al., 2014), we had the ability to compare our shotgun metagenomic findings with PCR results. Three specific primers for resistance were used in the PCR studies: tet(A), tet(B), *bla_{CMY2}*. Upon initial AMR alignment, no reads were aligned to the tet(B) or *bla_{CMY2}* gene and 1 read was attributed to tet(A). The alignment was consistent with visual inspection of the primers with one sample containing one hit to tet(A) (Figure 7). This indicated that tet(B) was the only gene found in both studies, though it was only in one sample in the present study. This result is not unexpected due to the differing approaches of PCR and metagenomics. While a cornerstone of PCR is amplification of specific nucleotide sequences, either, or both, before PCR in an enrichment broth or actual amplification during PCR cycles, metagenomics is, by definition, not enriching for a specific gene or species. While metagenomics presents a representative DNA pool of an entire sample, PCR is aimed to find or quantify specific primers related to specific pathogens or plasmids of interest. Therefore, PCR or metagenomic analysis is neither better nor worse than one another but different tools to answer different questions. While PCR, through the Kanwar et al. publications (2013, 2014) has been shown to excel in specific primer recognition and quantification, it does not allow the same scope of microbiome or even resistome discovery or comparison as metagenomics. On the other hand, while metagenomics allows for comparison and discussion of the microbiome at a much grander scale (in this particular study 431 species from metagenomic analysis vs. 3 species PCR dictates samples) there are limitations to this approach as well. In this study, on average 98.62% (range 98.16% to 99.00%) of the reads were unclassifiable through Kraken. This classification drawback is not unique to this study. In a beef production metagenomics study using Kraken, Yang et al. (2016) classified, on average, 6.4% of

reads, though this was across multiple sample matrices not just feces. Nonetheless, even a limited scope of the microbiome allows insight into the ecology of feedlot environments that culture based and PCR studies lack. This study illustrates the importance of using the correct genomic tools to answer research objectives. While PCR is still the preferred choice for specific identification, a metagenomic approach allows for ecological and microbiome exploration unrivaled by traditional approaches.

Table 1: Primers used for PCR Reactions

Directly Adopted from Kanwar et. al., 2013

Gene name	Primer	Sequence	GeneBank Accession Number
<i>bla</i> _{CMY2}	585F	5'- CAG ACG CGT CCT GCA ACC ATT AAA -3'	AB212086
	1038R	5'- TAC GTA GCT GCC AAA TCC ACC AGT -3'	
	675F	5'- AGG GAA GCC CGT ACA CGT T -3'	
	738R	5'- GCT GGA TTT CAC GCC ATA GG -3'	
<i>tet</i> (A)	<i>tet</i> (A)(F)	5' -GCTACATCCTGCTTGCCTTC- 3'	X61367
	<i>tet</i> (A)(R)	5' -CATAGATCGCCGTGAAGAGG- 3'	
<i>tet</i> (B)	<i>tet</i> (B)(F)	5' -TTGGTTAGGGGCAAGTTTTG- 3'	J01830
	<i>tet</i> (B)(R)	5' -GTAATGGGCCAATAACACCG- 3'	

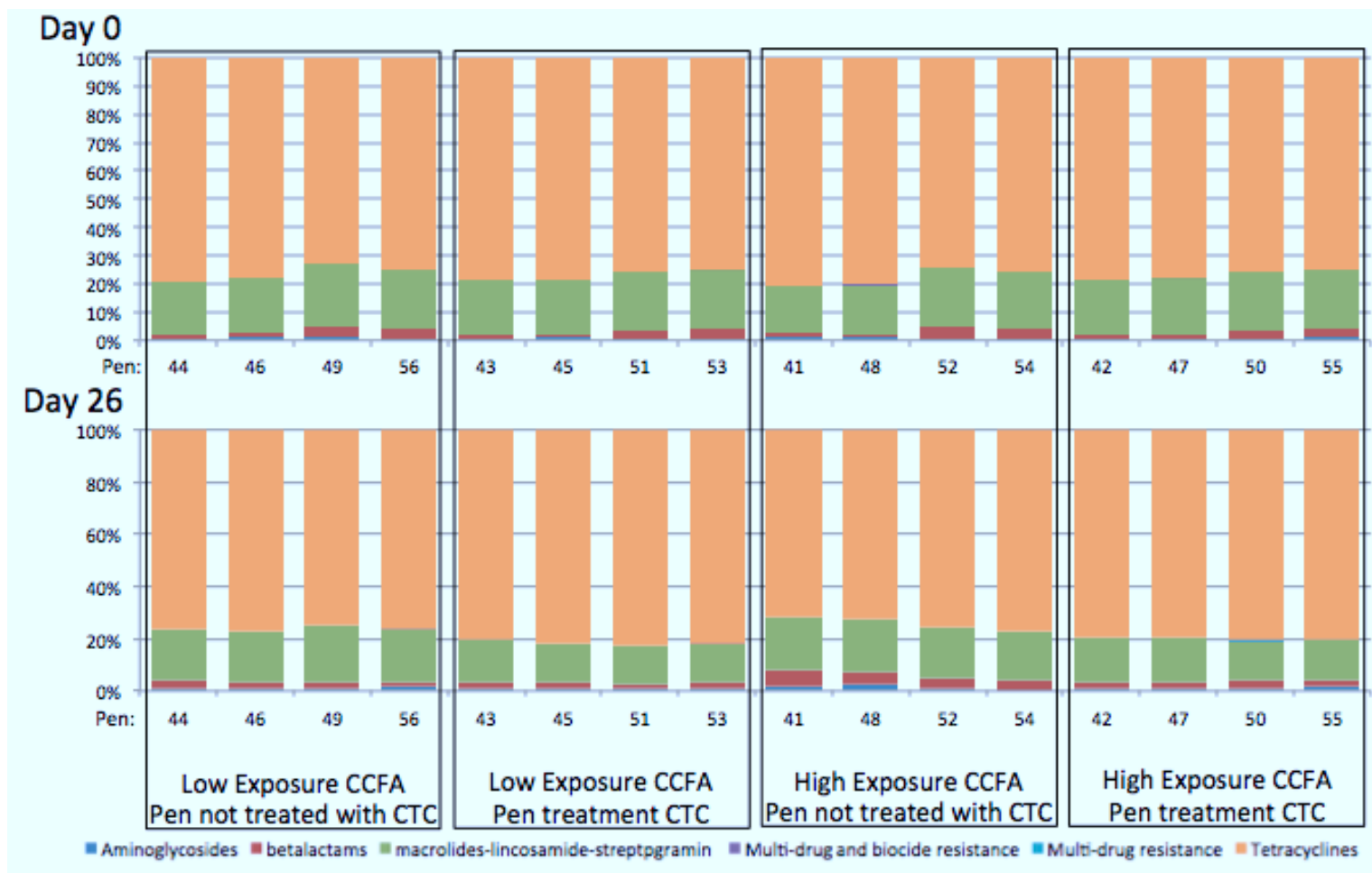


Figure 1: Normalized relative abundance of classes of antimicrobial resistance present in each pen treated in a 2x2 factorial of chlortetracycline (yes or no) or Ceftiofur crystalline free acid (low exposure—one animal treated in the pen or high exposure—all animals treated in a pen). Each column contains a pen on day 0 and day 26 with pens grouped by treatment.

Table 2: Least-square means of Class A β -lactamases resistance per 1000 copies of 16S-rRNA gene on day 26.

Treatment	AMR/16S Ratio
CCFA not cohoused CTC no	13.05 ^b
CCFA cohoused CTC no	30.43 ^a
CCFA not cohoused CTC yes	16.77 ^b
CCFA cohoused CTC yes	18.96 ^b
SEM	3.863

¹Standard error of the means

^{a, b} Means with different superscripts are different ($P < 0.05$).

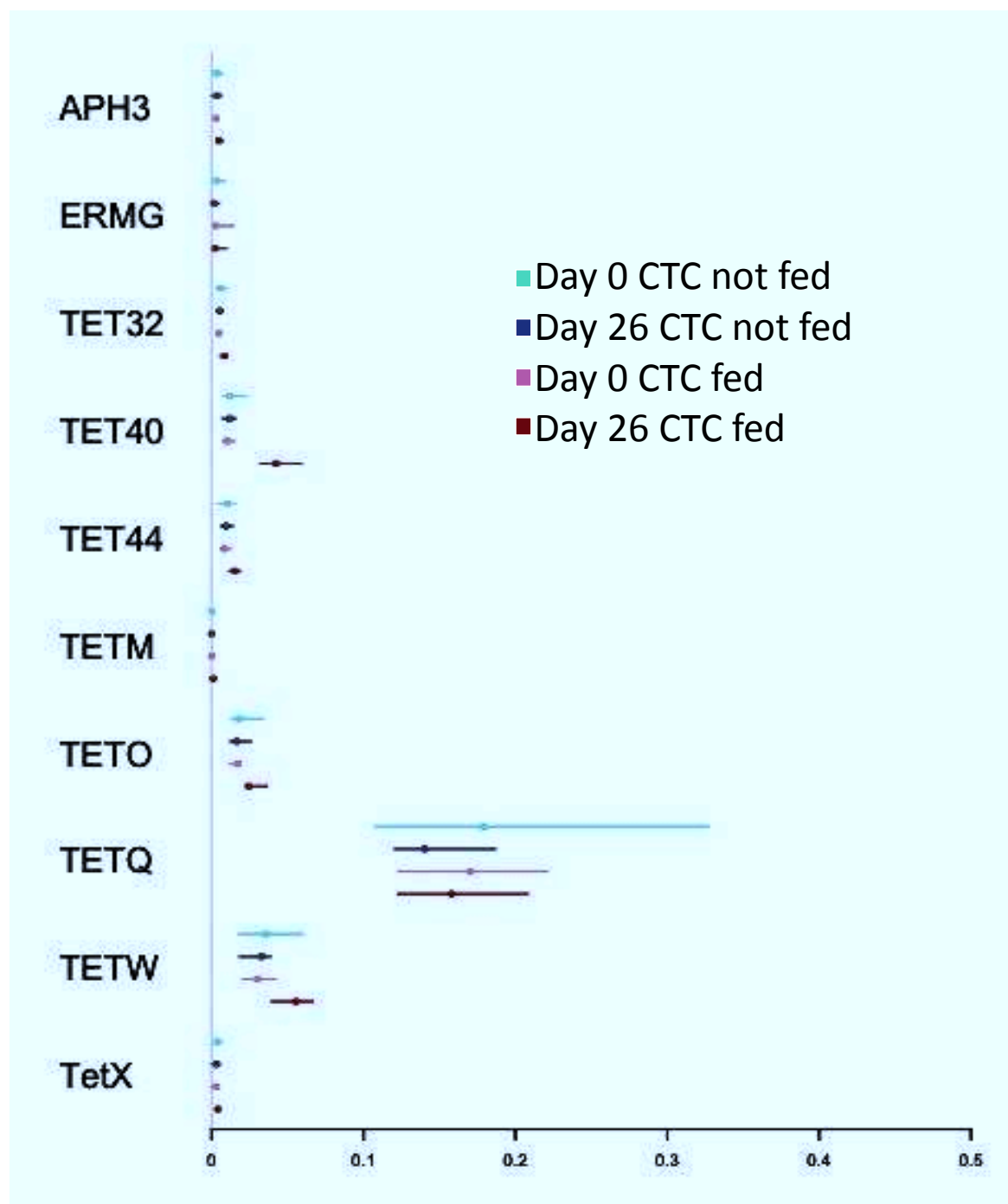


Figure 2: Forest plot of resistant gene group classifications that had significant interactions (all but ERMG which had a main effect significance of $P > 0.05$) between Chlortetracycline treatment and day; pooled across low and high exposure to Ceftriaxone crystalline free acid pen treatments.

Table 3: Least-square means of AMR gene groups per 1000 copies of 16S-rRNA gene of cattle being feed Chlortetracycline; pooled across Ceftiofur crystalline free acid pen exposure.

Group	Day 0	Day 26	SEM ¹
APH3	2.889 ^b	5.300 ^a	0.688
TET32	4.707 ^b	7.950 ^a	0.72
TET40	12.049 ^b	42.940 ^a	4.198
TET44	8.588 ^b	14.810 ^a	2.685
TETM	0.040 ^b	0.844 ^a	0.224
TETO	15.980 ^b	27.480 ^a	2.15
TETW	31.020 ^b	54.060 ^a	7.951

¹Standard error of the means

^{a, b} Means with different superscripts within a row are different ($P < 0.05$)

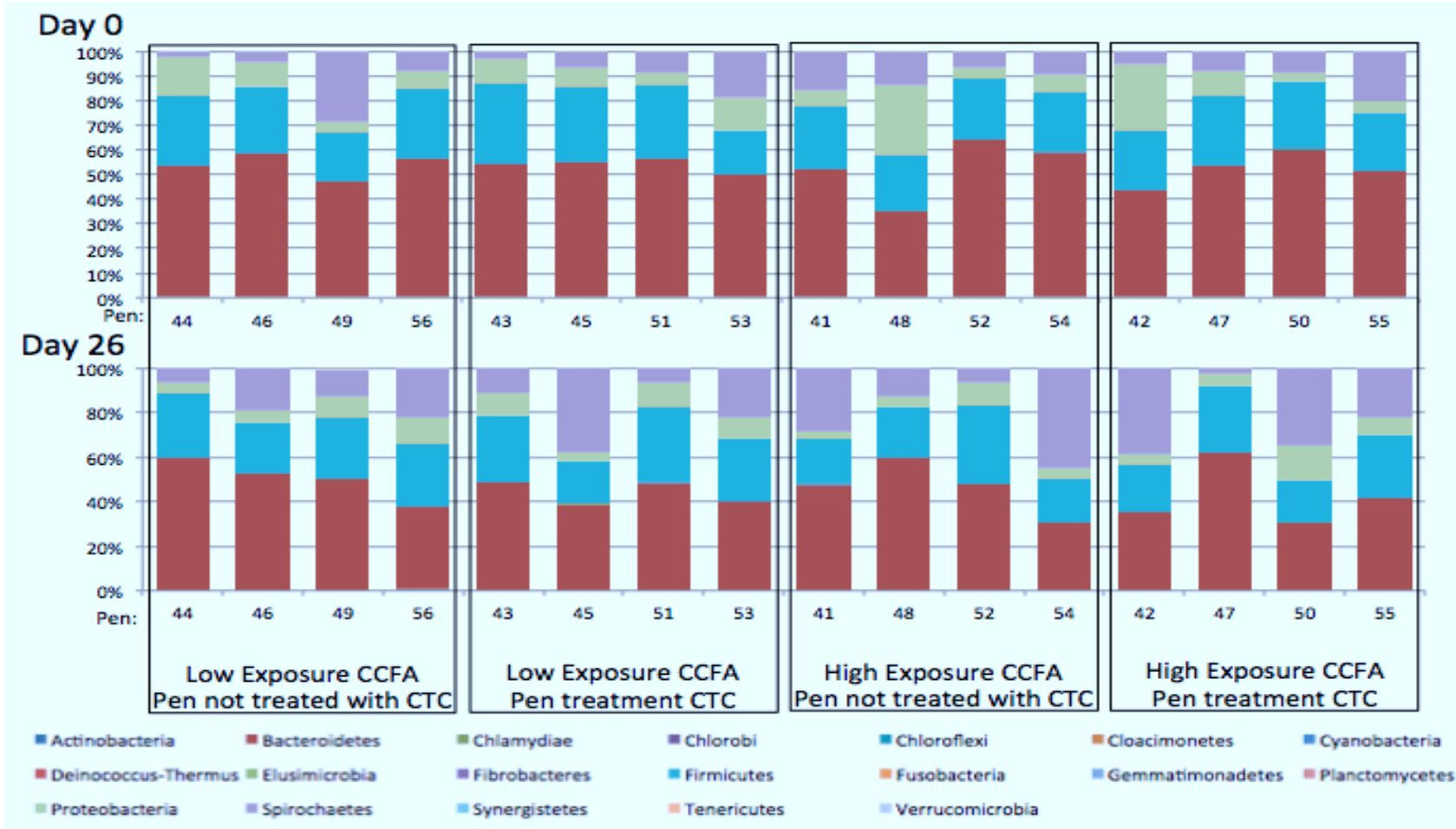


Figure 3: Normalized relative abundance of phyla present in each pen treated in a 2x2 factorial of chlortetracycline (yes or no) or Ceftiofur crystalline free acid (low exposure—one animal treated in the pen or high exposure—all animals treated in a pen). Each column contains a pen on day 0 and day 26 with pens grouped by treatment.

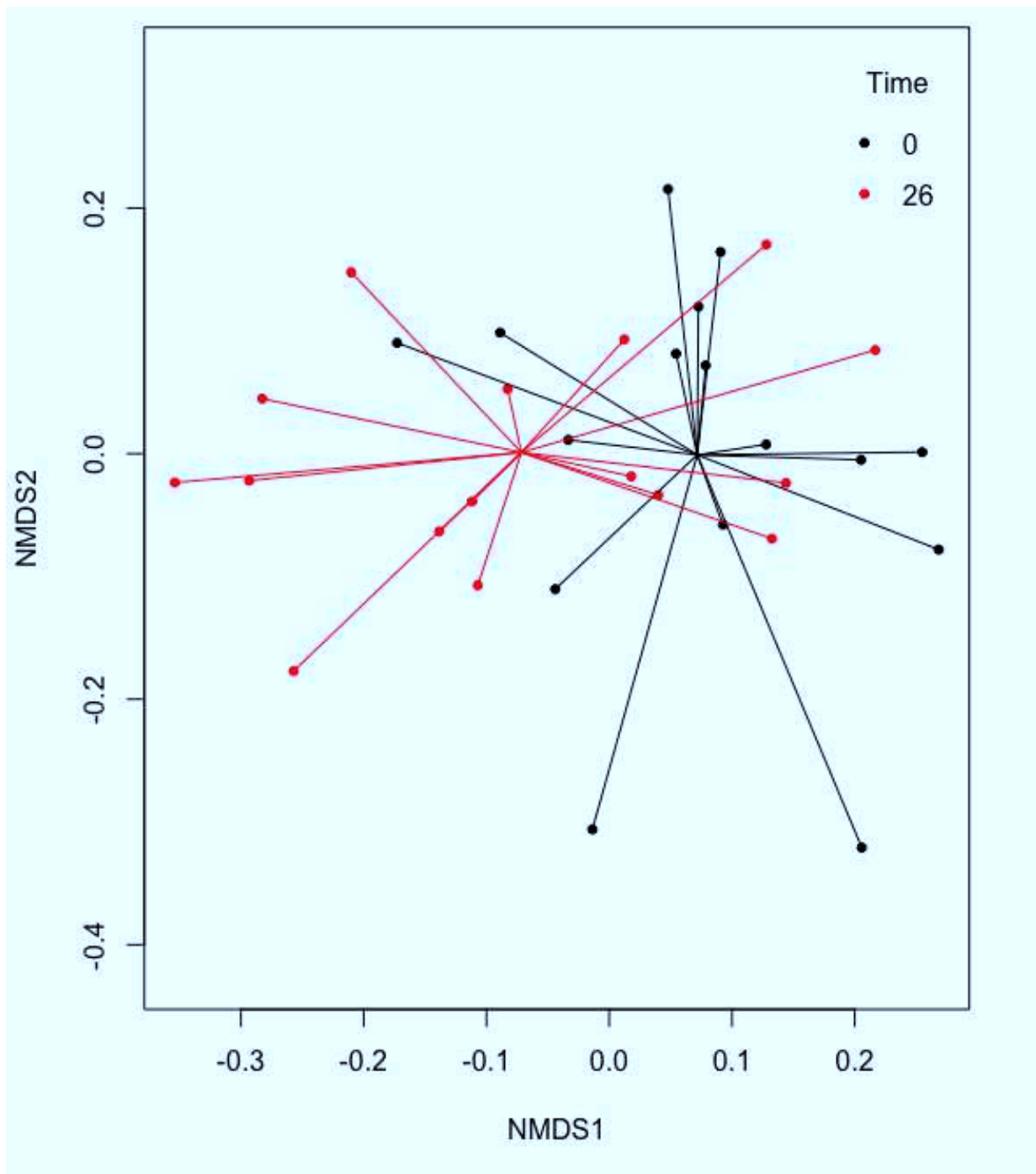


Figure 4: Microbiome (all taxa) non-metric multidimensional scaling (NMDS) ordination for all groups pooled across day. Separation between day 0 and day 26 was different (ANOSIM $P < 0.05$).

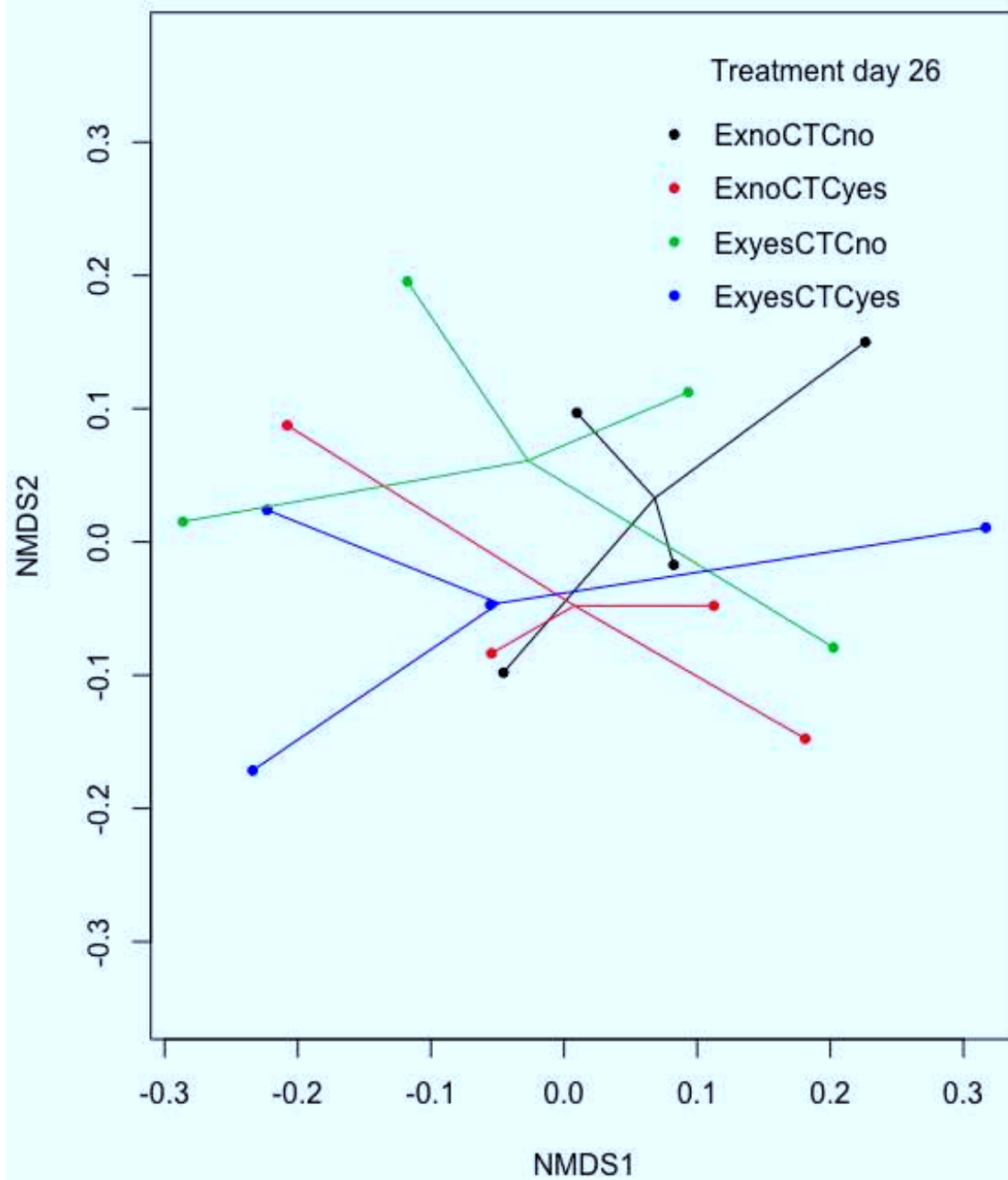


Figure 5: Microbiome (all taxa) non-metric multidimensional scaling (NMDS) ordination for all groups on day 26. Separation between treatment groups was not different (ANOSIM $P = 0.64$).

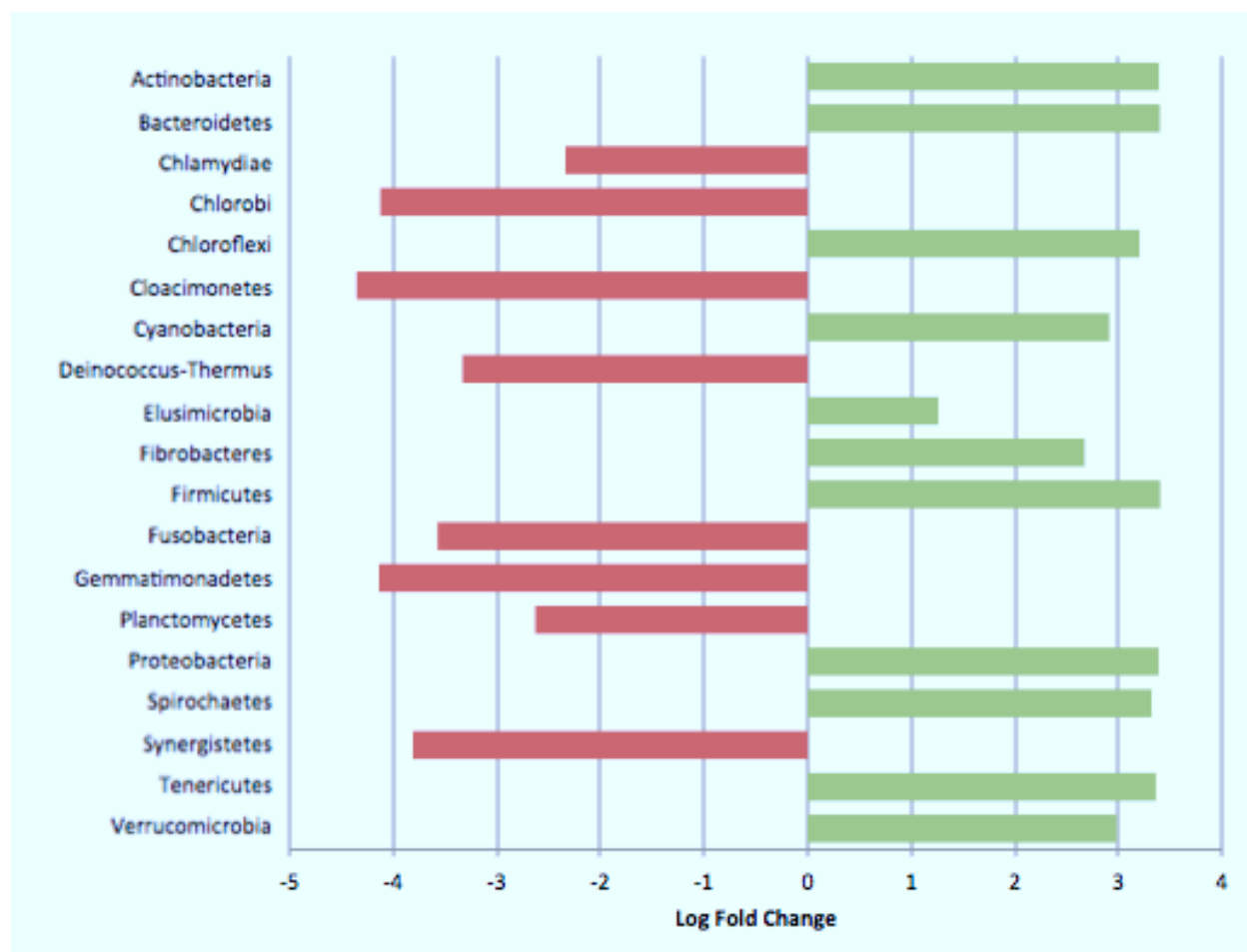
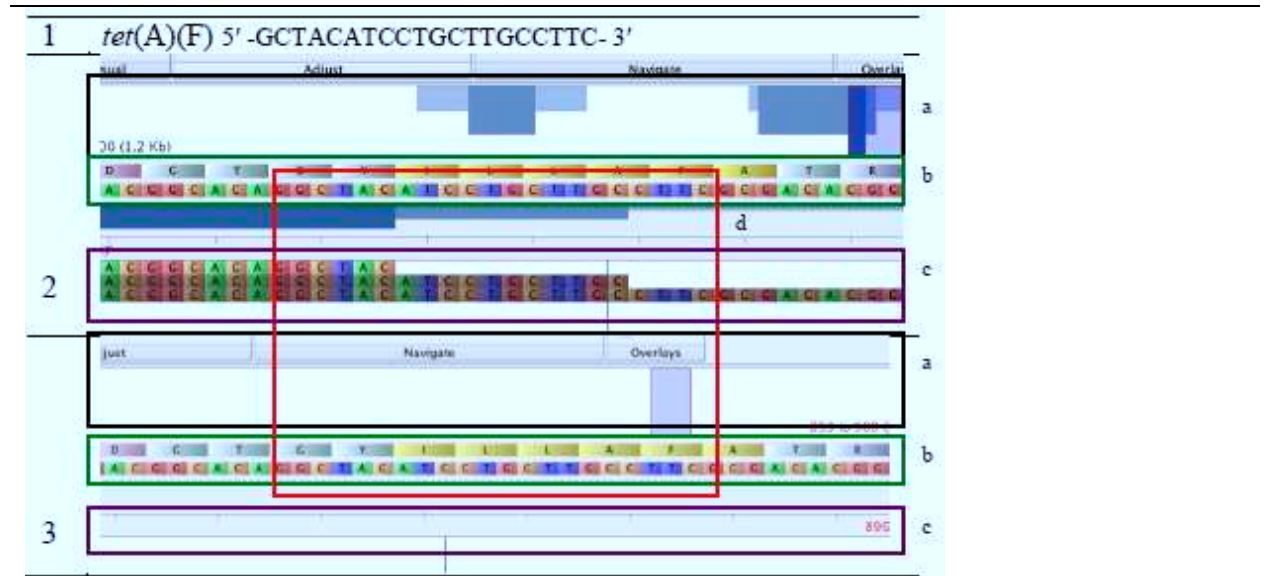


Figure 6: Significant ($P < 0.05$) log-fold changes in abundance of hits to taxa at the phylum level, from day 0 to day 26 sampling, pooled by treatment group. Positive values mean an increase in abundance from day 0 to day 26, while negative values mean a decrease in abundance from day 0 to day 26. All phyla identified were significantly different between day 0 to day 26 in relative abundance



- ^a Overall gene view, shows hits to entire region with blue box with shaded region denoting where on the gene zoom is applied
- ^b Reference gene sequence
- ^c If hits are present, where they are displayed
- ^d Primer sequence region at defined by box "1"

Figure 7: Visually alignment to hits to primer for *tet(A)* using Tablet. 1: the primer sequence for *tet(A)*F. 2: Sample with a hit to *tet(A)*. 3: Sample with no hit to *tet(A)*

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