Title

The Fecal Resistome of Beef Cattle from Conventional Grain-fed and Grass-fed Systems in the Western United States

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The Fecal Resistome of Beef Cattle from Conventional Grain-fed and Grass-fed Systems in the Western United States

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Abstract

Antibiotics have been used in the beef industry to improve animal health. However, this can potentially contribute to developing antimicrobial resistance (AMR) in the bacteria shedding from animal guts in feces. Antimicrobial resistance bacteria (ARB) and their AMR genes (ARGs) may be transmitted to humans via beef contaminated by animal feces during livestock production and slaughtering. However, whether various grass and grain beef feeding systems impact ARB or ARGs in cattle feces is currently unknown. Therefore, the objective of this study was to characterize and compare the fecal resistome of cattle raised in various grass and grain-feeding systems in the Western United States. Fecal samples were collected from individual cattle at 14 months of age as a baseline and collected again from the same animals two days before harvest. Groups included: 1) Conventional grain-fed (CON, n = 10), 2) Grass-fed for 20 months (20GF, n = 10), 3) Grass-fed and then grain-finished for 45 days (GR45, n = 10), 4) Grass-fed for 25 months (25GF, n = 10). Beef cattle raised in grass-fed systems did not receive any antibiotics, while some cattle from CON and GR45 received therapeutic antibiotics, and those finished in the feedlot received monensin in their feedlot rations. Total microbial DNA was extracted from samples and sequenced using the Illumina NovaSeq 6000 platform (250bp paired-end, Illumina, Inc., San Diego, CA, USA) for resistome analysis. In total 598 ARGs were identified, and 448 were kept after the filtration.

Regarding the Chao 1 diversity, the 25GF group had the smallest value compared to that of the other three groups ($P < 0.05$) at the harvest time. Shannon's diversity suggested that the richness and evenness of ARGs are greater than CON and GR45 compared with 20GF and 25GF ($P < 0.05$). The beta diversity for GR45 and CON differed concerning the abundance of ARGs and the predominant ARG classes, as compared to the samples collected from cattle within grass-

feeding systems at the harvest time and all the samples at the baseline (Stress = 0.07, $R = 0.2111$, $P = 0.018$), indicating the composition of fecal resistome were different between cattle under grain-finished groups and cattle under grass-feeding systems. The number of transferable ARGs, which can transfer from one environment to another by horizontal gene transfer, detected in CON, 20GF, GR45, and 25GF was 54, 29, 42, and 24, respectively. Among them, 14, 5, 2, and 2 unique transferable ARGs were identified, respectively. A total of 753 genes associated with resistance to biocides and metals (BMRGs) were identified, with 170 BMRGs found on plasmids and transposons, which were retained for further analysis after filtration. A smaller Shannon index of transferable BMRGs was observed for GR45 compared to 20GF and 25GF ($P < 0.05$). The results from the trial suggested that CON and GR45, which employed therapeutic and prophylactic antibiotics, enriched the diversity of ARGs, including transferable ARGs, in animals' feces. In grass-feeding systems where antibiotics were not administered, animals' feces exhibited greater diversity in transferable BMRGs, potentially creating selective pressure and promoting the development of ARGs. The enrichment in either ARG diversity or BMRG diversity of animal fecal resistome increases the spread of ARB in the production environment, which may eventually increase the risk of AMR in humans.

Keywords: Cattle, Resistome, Antimicrobial Resistance, Food Safety, Grass-fed

Table of Contents

Chapter 1: LITERATURE REVIEW

Introduction

Beef is a high-quality protein source for human consumption, and the global demand for beef continues to rise (Greenwood, 2021). In efforts to enhance feed efficiency and profitability within the beef industry, antibiotics were commonly employed in a prophylactic/metaphylactic and therapeutic manner (Sneeringer et al., 2015). However, studies have revealed that prolonged antibiotic use in animal can lead to the emergence of antimicrobial resistance (AMR), which can be transmitted to humans via consuming fecal-contaminated meat products (Guillemot et al., 2001; Gorji et al., 2022). The Antibiotic Resistance Threats in the U.S. report, published by the Centers for Disease Control and Prevention (2021), highlights that more than 2.8 million infections in the U.S. are attributed to AMR, resulting in approximately 35,000 deaths. Various factors can influence the development of AMR in animal fecal bacteria, including whether or not use antimicrobials, type of antibiotics, dosage of antibiotics, duration of antibiotics treatments ((Noyes et al., 2016; Vikram et al., 2017; Ma et al., 2021). The resistome is defined as the density and composition of antimicrobial-resistant genes (ARGs), representing the potential for AMR in a given sample (Noyes et al., 2016). Numerous studies have examined how different feeding systems and practices affect beef quality and safety. This literature review aims to describe: (1) the beef feeding systems employed in the U.S., (2) the utilization of antibiotics within these feeding systems, and (3) the fecal resistome of beef cattle, along with potential approaches to mitigating AMR in food-producing animals.

Beef Feeding Systems in the U.S.

Following World War II, a grain surplus prompted its incorporation into the diet of U.S. beef cattle as a high-energy source, resulting in a shortened beef production cycle and more

tender meat products (McCluskey et al., 2005; Mathews and Johnson, 2013). Subsequently, meat production has undergone increasing industrialization, with producers aiming for high stocking rates and breeding for rapid growth (Davis et al., 2022). Including a greater proportion of grain in animal diets has been shown to expedite weight gain, enabling animals to reach slaughter weight more quickly (Davis et al., 2022). As of 2021, the U.S. has produced 27.9 billion pounds of commercial beed and become the largest beef producer globally and ranks second in beef imports and exports (USDA FAS, 2022; USDA ERS, 2022). While the U.S. has emerged as the world's largest consumer of beef, the demand for high-quality grain-fed beef for exportation continues to rise, and the value of beef and beef products increased from 2020 to 2021 by $10.8 billion (USDA FAS, 2022; USDA ERS, 2022).

Approximately 80% of commercial beef products in the U.S. are grain-fed and consistently meet the requirements of more desirable USDA Grading categories (i.e., prime, choice, and select; Mathews and Johnson, 2013). After weaning, calves typically weigh about 700 pounds and are transferred from cow-calf operations to various programs (McCluskey et al., 2005). Cattle may enter a stocker program, where they graze for 3 to 4 months before moving to the feedlot, or a preconditioning program for 30 to 60 days that involves following animal health protocols (deworming, dehorning, vaccinating) (USDA ERS, 2022). Alternatively, they may undergo backgrounding in pens or lots, receiving a diet of dry forage, silage, and grain for 90 to 120 days (USDA ERS, 2022). Ultimately, cattle are moved to feedlots, where they typically spend 100 to 300 days before reaching the market weight of over 1000 pounds, usually achieved at 15 to 28 months of age (McCluskey et al., 2005; Drouillard, 2018). Producers always care about expenditures, which vary significantly due to various factors such as location, management of practice, and the scale of operation. Feed costs, which account for 60% or more of production

expenses, can be reduced by transitioning beef production to rangeland or grassland environments where grass serves as a sustainable and valuable source of nutrition for cattle (Greenwood, 2021). These lands typically are not suitable for growing crops for human consumption.

The production of 1 kg of meat in livestock production requires three to ten kg of grain as feed (Tilman et al., 2002). However, grain used in grain-based feeding systems is cultivated using substantial quantities of synthetic chemicals, water, and fossil fuels, leading to environmental concerns due to overuse (Conway and Pretty, 2013). In recent decades, consumer awareness regarding environmental impact and animal welfare has significantly increased (Razminowicz et al., 2006; Davis et al., 2022). A survey conducted by McCluskey et al. (2005) revealed that 55.4% of 509 beef consumers believe that grass-fed beef offers health benefits, contributing to the growing popularity of grass-based feeding systems. According to the USDA's definition of "grass-fed meat" (USDA, 2023), grass-fed cattle primarily consume grass or forage throughout their lifetime, except for milk consumption before weaning. However, providing cattle with high-quality grass or forage throughout their lives as their sole source of nutrients and energy can be challenging and costly due to seasonal growth patterns in the U.S. (Mathews and Johnson, 2013). Producers operating grass-fed feeding systems need greater control over the pricing of their products due to localized sales of grass-fed beef, as opposed to a more commodity like national market for grain-fed beef (Gwin, 2009).

Furthermore, several novel feeding systems exist that combine elements of both grain-based and grass-based approaches. Some early studies have explored raising cattle on pasture and finishing them on grain in feedlots to reduce the finishing time and enhance beef tenderness, and this feeding system has gradually become standard in the U.S. now (Griebenow et al., 1997; Mathews and Johnson, 2013). Grass-finished cattle, distinct from typical grass-fed cattle fed grass

for their whole life, are fed on grass or forage for only a financially feasible period before slaughter to reach market weight (Mathews and Johnson, 2013). Organic beef feeding systems exhibit variability in the utilization of grain or grass as feed sources, but organic systems must be devoid of chemical fertilizers, pesticides, antibiotics, feed additives, and growth hormones (Hafla et al., 2013; Mathews and Johnson, 2013). These restrictions may lead to some benefits as described by Hafla et al. (2013) who illustrated that due to the restrictions on synthetic fertilizer in organic systems, raising animals on pasture enables farmers to be able to recycle animal manure and compost, thereby maintaining or potentially increasing soil nitrogen levels. The Cornucopia Institute (2012) reported that 80% of organic beef is raised on pasture until slaughter, with 60% of the cattle being grass-fed and 20% receiving a small portion of grain in their pasture-based diet.

Use of Antibiotics in Beef Feeding Systems

Antibiotics had been extensively added in animals' diet in livestock production to promote animal growth, prevent diseases, and treat infections (Bretschneider et al., 2008). Many studies have demonstrated that the supplementation of antibiotics in feed effectively enhances feed efficiency and average daily weight gain in beef cattle (Murley et al., 1952; Bartley et al., 1953; Morris et al., 1990; Cusack, 2004; de Souza et al., 2018). However, the overuse of antibiotics has led to the development of drug-resistant bacteria, which can potentially enter the food chain (Trevisi et al., 2014). As a result, the use of antibiotics for production purposes in livestock production has gradually been restricted in many regions worldwide in recent years. In 2013, the U.S. Food and Drug Administration (FDA) issued guidance urging the voluntary phase-out of medically important antimicrobial drugs for production enhancement (FDA, 2018). In 2015, the FDA implemented the Veterinary Feed Directive (VFD) final rule, regulating the application of medically significant antimicrobial drugs in animal feed (FDA, 2023). By 2017, the use of

medically important antimicrobial drugs in food animals, administered through feed and water, had transitioned from over-the-counter to VFD or prescription-only use, effectively eliminating their use for production purposes including growth enhancement (FDA, 2023). In the U.S., the major non-medically important antimicrobial drugs used in food-producing animals include ionophores and pleuromutilins (FDA, 2022). At the same time, tetracyclines, penicillins, macrolides, aminoglycosides, sulfonamides, lincosamides, and amphenicols are the predominant medically important antimicrobial drugs (FDA, 2022).

Currently, non-medically important antimicrobial drugs are widely employed in animal operations (FDA, 2023). Ranchers tend to prefer ionophores over non-ionophores due to their cost-effectiveness (Samuelson et al., 2016; Maciel et al., 2019). Ionophores can alter ruminal metabolism by selectively targeting gram-positive bacteria, leading to increased ruminal propionate concentration (the only glucogenic short-chain fatty acid), which improves feed efficiency and cattle performance (Ogunade et al., 2018; Marques and Cooke, 2021). Additionally, ionophores can reduce ruminal proteolysis and ammonia synthesis, thereby increasing the protein bypass to the small intestine (Ogunade et al., 2018; Marques and Cooke, 2021). Ionophores may also decrease mortality rates in ruminant herds by reducing ingestion, metabolic stress, bloat, and enterotoxemia (Novilla, 2018). The high-grain diet provided to cattle to enhance productivity poses a risk of metabolic diseases such as liver abscesses (Nagaraja and Lechtenberg, 2007). Still, using ionophores helps significantly decrease the risk of metabolic disease and liver abscesses in beef cattle. According to a survey conducted by Samuelson (2016), 92.3% of 24 consulting nutritionists reported that their clients included ionophores in the diet of feedlot beef cattle, while 97.3% of their clients applied ionophores in the finishing diet. This survey also revealed that the most commonly used ionophore in feed rations, accounting for 77.3% of ionophore use, was

Monensin, used by all clients who indicated they use ionophores in the finishing diets (Samuelson et al., 2016).

Currently, livestock production antibiotics are primarily employed for prophylaxis/prevention and metaphylaxis. Prophylaxis/prevention and metaphylaxis involve administering antimicrobial drugs to healthy animals, with prophylaxis being based on a risk assessment of disease consequences and metaphylaxis being implemented when clinical cases within a group reach a certain threshold (Lees and Shojaee Aliabadi, 2002; Baptiste and Kyvsgaard, 2017). Prophylactic administration of antimicrobial drugs is a common and sensible practice, as it improves animal welfare and decreases morbidity. One study showed that calves receiving prophylactic chlortetracycline treatment were 28 times less likely to become sick compared to calves not receiving treatment (Agga et al., 2016); and another study demonstrated that providing florfenicol to newly arrived heifers decreased baseline morbidity resulting from bovine respiratory disease (Keyser et al., 2007). Furthermore, prophylactic approaches reduce the necessity for administering VFD medications, which are more similar to human medicines, thereby decreasing the potential for developing and transmitting AMR, potentially more harmful to humans (Cox and Ricci, 2008; Miller et al., 2018). Importantly, studies have demonstrated that prophylactic and metaphylactic administration does not have long-term effects on AMR (Zaheer et al., 2013; Agga et al., 2016; Miller et al., 2018). In 2021, 65% of medically important antimicrobial drugs administered to food-producing animals belonged to the tetracycline class, with 43% of tetracycline use intended for cattle (FDA, 2022). Among the tetracycline class, oxytetracycline, tetracycline, doxycycline, and chlortetracycline are widely used for the treatment of infected animals due to their broad activity spectrum and low cost (Granados-Chinchilla and Rodríguez, 2017). Agga et al. (2016) found that tetracycline-resistant *Escherichia coli* increased after five

days of administering chlortetracycline prophylaxis in cattle feed. However, the resistance levels became consistent after 27 days of treatment and remained stable during the last sampling occasion (117 days of treatment), compared to cattle that did not receive chlortetracycline.

The administration approaches of antimicrobials differ according to the feeding system. Antimicrobials are typically added to cattle feed for disease control in feedlots, while it is challenging to distribute antimicrobials on grasslands evenly. Therefore, in grass-based feeding systems, antimicrobials are usually administered therapeutically to diseased individuals (Santamaría, 2011; Rovira, 2023). Although no regulatory mandates compel producers to follow this practice, animals raised in grass-based feeding systems typically do not receive antibiotics and hormones throughout their lives (Gwin, 2009).

Beef Cattle Fecal Resistome

The overuse of antibiotics contributes to the development of AMR, particularly in livestock production, where more antibiotics are used compared to human medicine, making animals a reservoir of antimicrobial-resistant bacteria (ARB; van den Bogaard, 2000; Davies and Davies, 2010; WHO, 2021). Resistome, the density and composition of ARGs, is always used to study the potential for AMR (Noelle et al., 2016). When ARGs' density, diversity, and richness are more significant, the likelihood of AMR development at the phenotypic level increases. Animals and humans share similar antimicrobial drugs, and ARGs in commensal bacteria in animals can be transmitted to pathogenic bacteria in animals and eventually transferred to humans through various routes (Guillemot et al., 2001; WHO, 2021). Fecal contamination is one of the major pathways for AMR transmission from food-producing animals to humans via consumption of ARB contaminated meat (Gorji et al., 2022). The potential for AMR transmission poses a significant

threat to human health as the effectiveness of antimicrobials for treating common infections and major surgeries would be dramatically reduced (Laxminarayan et al., 2013).

A recent study summarizing previous studies on metagenomics data of rumen content and fecal resistome demonstrated that the dominant classes of drugs that bacteria have resistance for in the rumen and feces are tetracyclines, macrolides-lincosamides-streptogramins (MLS), aminoglycosides, and beta-lactams (Haley and Van Kessel, 2022), which may be the result of corresponding antibiotics use. Tylosin are usually added in feed for feedlot cattle to reduce liver abcesses, and the application of tylosin increases the proportion of macrolide-resistant enterococci in cattle's gastrointestinal tract (Cazer et al., 2020). Ciprofloxacin and ceftriaxone are the common antibiotics that are injected to cattle to control bovine respiratory disease, and the study showed that either ciprofloxacin or ceftriaxone increase the proportion of ciprofloxacin-resistant *E. coli* in the feces (Pereira et al., 2020). Besides, antibiotic usage, diet and age also play crucial roles in shaping the resistome of beef cattle (Edrington et al., 2012; Liu et al., 2019; Gaire et al., 2021). Diet indirectly influences the resistome by altering microbiome assembly, as diet significantly impacts the shift in microbiome composition in ruminants, and different microbiota may carry different sets of ARGs (Henderson et al., 2015; Auffret et al., 2017). Regarding age effects, studies have shown that younger cattle feces exhibit higher levels of AMR in both the phenotypical and genotypical dynamics of *Escherichia coli* (Gaire et al., 2021). Liu et al. (2019) suggested that colostrum may serve as a carrier for transmitting ARGs to newborn calves, initiating the development of AMR in gut microbiota, thus highlighting the importance of early intervention to prevent ARG colonization (Haley and Van Kessel, 2022). Age-dependent AMR dynamics have also been observed in humans, where the prevalence of bacteria resistant to trimethoprim, trimethoprim-sulfamethoxazole, and cephalexin increases with age, while the prevalence of

bacteria resistant to extended-spectrum beta-lactamase is high in the very young or elderly population (Robey et al., 2017).

Different beef cattle feeding systems differ in diet and the administration of antibiotics, which can lead to variations in the resistome between other systems. Historically, it has been impractical to directly add antibiotics to feed beef cattle in grass-based feeding systems due to grazing practices. In contrast, preventive antibiotics are commonly administered in the feed for cattle in conventional grain-based feeding systems, which may result in a richer and more extensive resistome in the animals under these systems. However, only some studies have comprehensively compared the resistome across different feeding systems. Some studies have focused on specific bacterial resistances or compared the resistome of cattle raised under the same systems with or without antibiotics, but the results could be more consistent. Zhang et al. (2010) demonstrated that the prevalence of antibiotic-resistant *E. coli*, coliforms, and *Salmonella* does not differ between conventionally raised beef and grass-fed labeled beef (mixed with solid cuts and ground beef). Another study investigated the prevalence of fecal *E. coli* strains resistant to streptomycin, sulfadiazine, and tetracycline, revealing a correlation with supplement of vitamin D independent of oxytetracycline usage on dairy farms (Khachatryan et al., 2006). Nevertheless, research has shown more antibiotic-resistant bacteria in air samples near conventional beef operations than in organic operations (Sancheza et al., 2016). Certain tetracycline and MLS genes are abundant in beef cattle raised in conventional feeding systems with antibiotic use (Vikram et al., 2017).

The inherent nature of different feeding systems may also contribute to the composition of the resistome. Cattle in grass-based feeding systems primarily graze on pasture, while cattle in grain-based feeding systems are more intensively managed and live more closely together,

increasing the potential for transmission of ARGs and ARB in grain-based systems (Barlow et al., 2009). The transmission rate of bacteria among animals is also influenced by minerals usage and stress factors, including pH changes, heat shock, and starvation (Noyes et al., 2016; Huemer et al., 2020).

Minerals are commonly added as supplements to calf or cattle feed to ensure high productivity and increased feed efficiency (Peel, 2003; KyselkovÃ et al., 2015; Sukhanova et al., 2019). Based on a survey conducted in the U.S., the majority of surveyed nutritionists recommend major minerals such as calcium, phosphorus, magnesium, potassium, and salt in the receiving and finishing diet of beef cattle, as well as trace minerals including copper, cobalt, iodine, iron, manganese, selenium, and zinc (Samuelson et al., 2016). However, studies have shown that minerals, as sources of heavy metals, are co-selected with ARGs in animals, especially zinc, copper, chromium, arsenic, cadmium, and lead (Zhang et al., 2012; Haley and Van Kessel, 2022). Bednorz et al. (2013) demonstrated that zinc feed supplementation significantly increased the prevalence of multi-resistant *E. coli* in piglets' gut. Co-selection can occur through co-resistance, where ARGs and metal-resistant genes are physically linked on the same genetic element, or through cross-resistance, where resistance to metals accompanies antibiotic resistance when the resistance mechanisms are shared (Baker-Austin et al., 2006). Copper, cobalt, zinc, cadmium, nickel, and arsenic share resistance mechanisms with tetracyclines, chloramphenicol, and β-lactam antibiotics (Baker-Austin et al., 2006). Liu et al. (2019) conducted a correlation analysis between biocide and metal-resistant genes and ARGs, revealing significant correlations between copper-, cadmium-, silver-, and tellurium-resistant genes and aminoglycoside-resistant ARGs, as well as one tetracycline-resistant ARG.

Slowing down the development of AMR and mitigating its transmission between food-producing animals and humans is a global goal. The first strategy is to reduce the pathogen on live animal by vaccination and improving waste management systems in animal operations (da Costa et al., 2023). According to the global action plan on AMR published by the World Health Organization (WHO), vaccines can prevent infectious diseases that would otherwise require antibiotic treatment. They can also minimize primary viral infections and prevent secondary conditions that would necessitate antibiotic treatment (WHO, 2015). Studies have shown that ARGs remain present in high concentrations in the soil of animal operations even two years after cattle removal (Agga et al., 2019). Therefore, farmers and ranchers should enhance their manure management practices to reduce the aggregation of ARGs in soil and transmission from soil to newly arrived cattle. The highest concentration of bacteria has been found around feeders and water foundations in feedlots (Agga et al., 2019), highlighting the need for frequent antibacterial actions to prevent further transmission from communal feeding areas to individual animals. The second strategy is to prevent fecal contamination during slaughtering, and during processing, and in the event that it occurs, effective interventions such as steam vacuuming, the use of organic acids and hot water for spraying whole carcasses, and ionizing radiation can be employed to eliminate bacteria carrying ARGs (Koohmaraie et al., 2005; Han et al., 2020). The third strategy is to minimize the long-term use of the same antibiotics in food animals by discovering new antibiotics, exploring alternative options to antibiotics, and developing vaccines to prevent the prevalence of diseases that would require antibiotic treatment. New antibiotics should target bacteria that have already acquired ARGs and multidrug-resistant genes, and collaboration among stakeholders and policymakers is necessary to prevent the overuse of new antibiotics (Bassetti et al., 2013). However, the innovation of new antibiotics faces challenges due to limited commercial

attractiveness and a lack of public support. As a result, many large pharmaceutical companies divert their attention from this area, while small and medium-sized enterprises struggle to financially support their research (Årdal et al., 2020). Some studies have suggested that bacteriophages, lysins, antimicrobial peptides, probiotics, prebiotics, and symbiotics can serve as preventive and therapeutic alternatives to antibiotics (Cheng et al., 2014; Ghosh et al., 2019). However, thus far, no other opetions are as effective as antibiotics while meeting the requirements for antibiotic substitution (Cheng et al., 2014; Ghosh et al., 2019).

Conclusion

The use of antibiotics in animal production has been shown to improve animal health and welfare by reducing prevalence of diseases and also enhance feed efficiency and animal production effectively, but it also contributes to the emergence of AMR. Previous research has primarily focused on investigating individual factors, such as the presence or absence of antibiotics, while neglecting the comprehensive consideration of all influencing factors, including age, diet, antibiotic exposure, and the nature of animals' living environment. Examining the resistome in the context of different production systems is crucial, as these systems can represent the multiple factors that exert influence, and thus by comparing the resistome across various production systems, a more comprehensive understanding of AMR development and transmission can be obtained.

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Chapter 2: FECAL RESISTOME

Introduction

The Centers for Disease Control and Prevention (CDC; 2013) reported that AMR is responsible for over 2 million infections and 23,000 deaths annually. Furthermore, the Organization for Economic Co-operation and Development has predicted that between 2015 and 2050, AMR will cause 30,000 deaths in the United States. Consequently, it is crucial to address the development of AMR and antimicrobial-resistant genes (ARGs) to mitigate their impact on public health. The widespread use of antibiotics in livestock industries for disease control and enhancing feed efficiency has been a long-standing practice (Sneeringer et al., 2015). However, the latter purpose was prohibited by the U.S. Food and Drug Administration (FDA) in 2017 (FDA, 2023). In the United States, the largest beef-producing nation, antibiotics such as fluoroquinolones, macrolides, and third-generation cephalosporins are commonly utilized in the beef industry to prevent or treat diseases (Greenwood, 2021; Noyes et al., 2016; Lim and Page, 2022). Nonetheless, research has demonstrated that the routine administration of antibiotics in livestock may contribute to the selective pressure of bacteria, thereby fostering antimicrobial resistance (AMR) that could be transmitted to humans through the consumption of fecal-contaminated food (Economou and Gousia, 2015; Gorji et al., 2022; Van Boeckel et al., 2015), which may increase the risk of AMR in bacteria that cause human infection.

Recently, consumer awareness regarding food safety has significantly increased, leading to a greater willingness to invest in food products perceived as safer by the public (Lim et al., 2013; Alimi and Workneh, 2016; Riccioli et al., 2020). Beef constitutes a widely consumed source of protein for humans, with global demand for it exhibiting a steady growth (Greenwood, 2021). The United States accounts for the highest consumption of beef, particularly grain-fed beef, worldwide

(Lim and Page, 2022). However, recent data indicates that approximately 40% of consumers perceive grass-fed beef (cattle fed 100% grass from weaning to harvest, USDA FSIS, 2019) as safer than conventional grain-fed beef (Beef that has finished in a feedyard for over 100 days). Factors such as animal welfare, environmental sustainability, and health considerations contribute to an increasing preference for grass-fed beef, with some consumers demonstrating a willingness to pay more for it (Lim and Page, 2022; Van Elswyk and McNeill, 2014). Grass-feeding systems strictly prohibit the utilization of grains and grain byproducts (USDA-FSIS, 2019), and although not explicitly specified by the USDA, many who market grass-fed beef avoid using antibiotics or hormones (Gwin, 2009).

Conventional grain-based feeding systems, known as the feedlot, and grass-feeding systems exhibit differences in diet and in antibiotic usage. However, it remains uncertain whether different feeding systems employed in beef cattle production led to an influence on the resistome, which is defined as the density and composition of ARGs representing the AMR potential (Noyes et al., 2016). Previous research has yielded varying results concerning the differences in resistant bacteria or resistance genes arising from distinct cattle operations (Zhang et al., 2010; Edrington et al., 2012: Sancheza et al., 2016; Vikram et al., 2017). However, the early research primarily focused on analysis of the prevalence data (Zhang et al., 2010) or the specific bacteria (Edrington et al., 2012) that exhibited the expressed ARGs at phenotypical level. These approaches cannot capture the overall picture of the antibiotic resistance landscape, as they ignore non-expressed genes that may be activated under certain conditions. Thus, metagenomics, which involves sequencing the whole DNA content from a given sample recently has become a better approach to be introduced to analyze the resistome from ecological perspective (Weinroth et al., 2018; Huebner et al., 2019).

In the Western United States, particularly in California, the number of beef cattle grew, with a recorded population of 670,000 in 2021 and a 2.2% increase from the previous year (USDA, 2021), which inferred that demand for grass-fed beef in this region had also risen. Consequently, it was crucial to examine how beef cattle feeding systems impacted meat safety in this area. Previous studies had primarily focused on characterizing the profile of AMR in cattle raised with or without antibiotics within the same feeding system. However, some studies show that antimicrobial use is not directly linked to changes in microbial resistance, and thus other (environmental) factors must be taken into consideration (Khachatryan et al., 2006; Barlow et al., 2008; Zhang et al., 2010; Thomas et al., 2017; Vikram et al., 2017). Studying the systems enables the identification of complex relationships and interactions by considering factors such as age, animal intensity, and environmental conditions. Thus, this study was conducted as the first to investigate AMR in both grain and grass-feeding systems currently utilized by ranchers in California. As our study aimed to characterize and compare the fecal resistome of cattle raised in different grass- and grain-feeding systems within the Western United States.

Material and Methods

Sample Collection

This project was approved by the Institutional Animal Care and Committee at the University of California Davis (UCD; protocol #20560). Fecal samples were collected from animals used by Klopatek et al. (2021). In June 2018, forty Angus and Angus-Herford cross beef steers were fenceline weaned at University of California Sierra Foothill Research and Extension Center (Browns Valley, CA). In total, 68 cattle (average initial weight of 284 kg ± 27.57 kg) were kept in an animal trial done by Klopatek et al. (2021), and 10 steers were randomly selected from each group (4 groups) for the present study to characterize the fecal resistome using metagenomic

sequencing. The four groups were: 1) Conventional grain-fed (CON), 2) Grass-fed for 20 months (20GF,), 3) Grass-fed and then grain-finished for 45 days (GR45,), and 4) Grass-fed for 25 months (25GF). All steers grazed on pasture in Maxwell, CA from June to November 2018. Afterward, CON steers were moved to the UCD feedlot and fed a starter ration for 14 days, followed by an intermediate ration for 14 days, and finally finished on a high-energy corn-based ration for 100 days. Meanwhile, 20GF and 25GF steers were transported to the Sierra Field Research Station in Browns Valley, CA, where they were provided a mixture of grasses.

The 20GF steers were harvested at the end of the winter-spring grazing season, while all GR45 cattle were those diagnosed with pinkeye infection and treated with antibiotics and then at the end of the winter-spring grazing season moved to the UCD feedlot and fed a starter ration for 7 days, followed by an intermediate ration for 10 days, and finally finished on a high-energy corn-based ration for 45 days. Also, at the end of the winter-spring grazing season, the 25GF steers were transported to the UCD flood-irrigated pasture in Davis, CA, where they were given a mixture of perennial grasses. Cattle in the 20GF and 25GF groups were never given antibiotics or an ionophore. Monensin was included in the ration for CON and GR45 steers. In addition, Oxytetracycline and florfenicol was administered to CON (6 of 10) and GR45 (10 out of 10) steers who presented the symptoms for pinkeye, liver abscess, or pneumonia. Minerals were added to the animals' feed at the University of California Sierra Foothill Research and Extension Center and the UCD feedlot. The CON and GR45 steers were harvested at a large-scale commercial processing plant in Fresno, CA, while the 20GF and 25GF steers were harvested at a natural/organic beef packing plant in Merced, CA.

Rectal fecal samples were collected from each steer as a baseline before they were assigned to a group, and again one week before harvest. Approximately 50g of fecal samples were collected

per sampling time from each individual and stored in separate sterile 24 oz Whirl-Pak sampling bags (Whirl-Pak, Madison, WI). The samples were then transported to UCD within two hours and stored at -80°C for further analysis.

Sample Processing and DNA Extraction

The fecal samples that were stored at -80°C were moved to 4°C to be thawed overnight. Total microbial DNA was extracted from the fecal samples using the DNeasy PowerSoil Pro Kit (Qiagen, Valencia, CA), following the manufacturer's protocol. The concentration of the extracted DNA was determined by measuring the absorbance at 260 nm using the Invitrogen Qubit Fluorometer (Thermo Fisher Scientific, Inc., Pittsburgh, PA). The purity of the extracted DNA was assessed using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Pittsburgh, PA). For an accepted concentration, the 260/280 and 260/230 ratios were >= 20ng/μL, 1.8-2, and >2, respectively. For the samples with low concentration, QIAquick PCR Purification Kit was used (Qiagen, Valencia, CA) following the manufacturer's protocol to increase the concentration of the samples.

Library preparation and sequencing

The extracted DNA samples were sent to UC Davis Genomic Center for library preparation and metagenomic sequencing. The sample libraries were built by using the Illumina TruSeq DNA library kit (Illumina, Inc., San Diego, CA). Library sequencing (paired-end, 2 ×150 bp) was performed on the Illumina NovaSeq 6000 in the Genome Center at University of California, Davis.

Bioinformatics

The sequencing reads were processed by first trimming and merging them using HTStream (version 1.3.3) (https://s4hts.github.io/HTStream/), and then the steps and the results were visualized by MultiQC (version 1.14) (Ewels et al., 2016). BWA (version 0.7.12) (Li and Durbin,

2009) was used to filter out host DNAs (Bos Taurus, UMD3.1). The filtered reads were then matched to the MEGARes (version 2.0) (Doster et al., 2020), which classifies the antimicrobial drug, biocide, and metal resistance factors in metagenomic sequencing data, using BWA with the default settings, and ARGs were identified at gene, mechanism, and class levels, using ResistomeAnalyzer (https://github.com/cdeanj/resistomeanalyzer). Next, the identified ARG reads were assembled into contigs using MEGAHIT (version 1.2.9) (Li et al., 2015), and BWA-MEM (Li and Durbin, 2009) was used to align the contigs that contained ARG reads for identifying bacterial origin of these contigs. The unassembled reads that were identified as ARGs were also classified to a customized database including RUG2 (Stewart et al., 2019) FASTA sequences, which contains the extensive amount of assembled rumen genomes to identify the potential bacteria origin of the ARGs at species level, and RefSeq (O'Leary et al., 2016) FASTA sequences, which contains sequences from more than 55000 organisms for better taxonomic representation, using Kaiju (version 1.9) (Menzel et al., 2016). The contigs were compared with the reference genome in Kaiju database for getting the number of reads that were assigned to each taxon. The merged reads were aligned to Resfinder (version 4.0) (Florensa et al., 2022) with a 90% identity threshold and 60% minimum length match for identifying transferable ARGs. Additionally, the host-removed, non-merged reads were aligned to the BacMet database with experimentally confirmed resistant genes (version 2.0) (Pal et al., 2014) using DIAMOND (version 2.0.15) (Buchfink et al., 2021) with an E-value cutoff of $\leq 10^{-10}$ to identify the biocide and metal resistant genes (BMRGs).

Statistical Analysis

The resistome was analyzed based on the relative abundance of ARGs and BMRGs, which was calculated using the number of reads of each gene/class/mechanism divided by the total reads

present in this sample. To normalize the relative abundance, the cumulative sum scaling (CSS) method was used using metagenomeSeq R package (*v1.42.0*; Paulson et al., 2013) with default setting. Shannon index (representing richness and evenness) and Chao1 index (representing richness) of ARGs and BMRGs were calculated using the vegan package (*v2.6-4*; Oksanen et al., 2013). and then were utilized to analyze alpha diversity. Beta diversity of ARGs and BMRGs was represented by Non-Metric Multidimensional Scaling (NMDS) using Bray–Curtis dissimilarity calculated through the vegan package. Multivariate homogeneity of groups variances following PERMANOVA with 999 permutations was examined using the vegan package. To identify changes in relative abundance from baseline to harvest within each feeding system, the mean relative abundance of each ARG was calculated for all groups. To ensure valid data, a value of one was added to each data point in the data frame., one was added to every datapoint in the data frame, and log2 fold change was applied to the modified mean of normalized relative abundance between harvest and baseline for each group at the class level.

$$Mean_{class1}\ at\ harvest\ of\ trt1 = \frac{sum\ of\ relative\ abundance\ of\ ARGs\ in\ class1\ of\ trt1\ at\ harvest}{10}$$

$$Mean_{class1}\ at\ baseline\ of\ trt1 = \frac{sum\ of\ relative\ abundance\ of\ ARGs\ in\ class1\ of\ trt1\ at\ baseline}{10}$$

$$log2\ fold\ change\ of\ class1\ of\ trt1 = \frac{log_2(Mean_{class1}\ at\ harvest\ of\ trt1 + 1)}{log_2(Mean_{class1}\ at\ baseline\ of\ trt1 + 1)}$$

The completely randomized design was used to examine the feeding effects and age effects on the resistome. Feeding effects were defined as the difference between grass-feeding system and grain-feeding system at harvest, while age effects were defined as the difference between baseline and harvest for each feeding system. Accordingly, a two-way analysis of variance (ANOVA) with

Tukey test were applied to explore the feeding effects, age effects, and corresponding interaction effects on the alpha diversity. Pairwise comparisons were done on the top 10 classes of ARGs between groups using t-test. The P-values obtained from multiple comparisons were adjusted by the Bonferroni method. In addition, the feeding effects on the microbiome were tested by one-way ANOVA. All statistical analyses and visualization were conducted in R statistical software (version 4.1.2), with an alpha level of 0.05 applied to test statistical significance.

The visualizations of alpha diversity, predominant classes, and comparisons of classes by time for each feeding system were programmed through the ggplot2 package (*v3.4.2*; Wickham, 2016). The NMDS ordination plots were visualized using base R. Heatmaps for relative abundance of ARGs was created using the ComplexHeatmap package (*v2.16.0*; Gu, 2022). The normalized relative abundance of ARGs was further normalized by row using the Euclidean method through the wordspace package (*v0.2-8*; Evert, 2014) for better visualization. The transferable ARGs of each feeding systems, regardless of age, were visualized using the VennDiagram package (*v1.7.3*; Chen and Boutros, 2011).

Results

Fecal resistome differs between feeding systems.

Over 41 million reads were kept after filtration, and these reads were aligned to 448 ARGs categorized into 40 classes of resistance and 89 mechanisms, with 341 ARGs identified across all groups. The average number of ARGs varied among groups, with the highest number identified in the CON (203, range from 74 to 440), followed by GR45 (185, range from 90 to 412), 20GF (158, range from 72 to 377), and 25GF (119, range from 66 to 373). A total of 132 out of 448 ARGs (29.5%) were identified across all groups at baseline. At harvest, among 448 ARGs, 233 ARGs

(52%) were identified in CON samples, 222 ARGs (49.6%) in GR45 samples, 164 (36.6%) ARGs in 20GF samples, and 88 ARGs (19.7%) in 25GF samples (Fig 1).

At the gene level, the baseline alpha diversity, as determined by the Shannon and Chao1 indices, did not (P = 1.00) exhibit a difference across all groups. The Shannon index for grain-finished groups was higher ($P \leq 0.001$) than for grass-feeding groups at the time of harvest (Fig 2a). However, CON and GR45 had similar Shannon indexes (P = 1.00), and 20GF and 25GF also showed similar Shannon indexes (P = 0.49) (Fig 2a). Extensive grass-feeding resulted in a lower Chao1 index for 25GF compared with CON (P = 0.0004) and GR45 (P = 0.0014), while 20GF, CON, and GR45 had similar ($P \geq 0.45$) Chao1 indexes (Fig 2b). The findings indicate that fecal bacteria from the 25GF cattle had a lower number of ARGs, and these ARGs were less unevenly distributed among the samples in this group. Furthermore, cattle feces from 20GF exhibited a fewer ARGs, but these ARGs were evenly distributed across the group samples.

The of resistome in cattle feces differed (Stress = 0.07, R = 0.211, P = 0.018) between CON and GR45 cattle feces and grass-fed cattle feces, regardless of time of sample collection (Fig 3a). When considering harvest samples, the difference lead by feeding effects was more evident, showing a difference (Stress = 0.06, R = 0.403, P = 0.001) in beta diversity of 20GF and 25GF compared to CON and GR45 (Fig 3b). Specially, the resistome of 25GF was different (P = 0.006) from the resistome of CON and GR45, while the resistome of 20GF, CON and GR45 did not ($P \geq$ 0.054) show differences. The results demonstrate that different feeding systems give rise to distinct resistome. However, it is noteworthy that the duration of grazing serves as a critical factor in shaping the resistome within various grass-feeding systems. Cattle from the similar feeding systems had a similar composition of ARGs in their feces, but the variance may vary based on animal age. Taken the alpha diversity into the consideration, the grain-finished groups enrich the

diversity of the ARGs in cattle feces, whereas beef cattle raised under grass-feeding systems may harbor a low diversity of resistome.

The top 3 most abundant classes of antibiotics for alignment encoding resistance were macrolide-lincosamide-streptogramin (MLS), aminoglycosides, and oxazolidinone. Cattle raised under similar feeding systems had a similar composition and distribution of antibiotic classes in their feces. The relative abundance of tetracycline-associated ARGs in the CON (15.2%) and GR45 (15.8%) at harvest was higher ($P < 0.0001$) compared to both the baseline (8.66%) and grass-fed groups at harvest (6.72% for 20GF and 6.84% for 25GF). Fusidic acid, which was the 10th abundant class at the baseline, disappeared from the top ten classes for CON and GR45 cattle but became the 9th abundant class for 20GF and 25GF cattle, and a new class – betalactams – appeared in the top ten classes in CON and GR45 (Fig 4).

Fecal resistome differs as cattle grow older.

Notably, no age difference in alpha diversity was observed between baseline to harvest for 20GF ($P = 0.60$ for Shannon index; $P = 1.00$ for Chao1 index) and 25GF ($P = 1.00$ for Shannon index and Chao1 index). However, at the time of harvest, an increase ($P < 0.001$) in alpha diversity was observed in the cattle feces of CON and GR45. Both the Shannon index ($P < 0.001$) and Chao1 index ($P = 0.017$) exhibited an increase as the cattle under GR45 group advanced in age (Fig 2a & 2b). Conversely, only the Shannon index displayed an increase ($P < 0.001$) over time for cattle under the CON group (Fig 2a). These findings imply that with increasing age in cattle under the GR45 group, more ARGs were developed, and these ARGs exhibited an even distribution across the groups. On the other hand, in some of the CON cattle, an increase in the number of ARGs was observed over time.

For CON, GR45, and 20GF, the overall trend of the relative abundance of all the classes inclined at harvest compared with the relative abundance at baseline. However, it was reduced for 25GF from baseline to harvest and ARGs of 17 classes disappeared at harvest while they presented in the feces at baseline (Fig 5). Mercury, nucleosides, phenicol, and tellurium resistance occurred at harvest only for CON; mercury and phenicol resistance occurred at harvest only for 20GF; bacitracin, mupirocin, nucleosides, and tellurium resistance occurred at harvest only for GR45; and aluminum and mercury resistance occurred at only harvest for 25GF (Fig 5).

Microbiome shedding of ARGs is consistent between feeding systems.

Overall, 19.5% of the reads were able to be classified into bacteria that shed the ARGs using RefSeq and RUG2 databases. *Pseudomonas*, *Escherichia*, *Klebsiella*, *Salmonella*, and *Synechocystis* were identified as the five most dominant genera for four groups, but the ranking was different in different groups (Table 1). No difference ($P \geq 0.8$) was observed in the relative abundance of these genera across the different feeding systems. *Pseudomonas* and *Escherichia*, as the top two most abundant genera across all groups, collectively constituted more than 11.6% of classable reads. The third most abundant genus was *Klebsiella* for both CON and GR45 groups, while *Synechocystis* occupied the same position for 20GF and 25GF groups. Notably, three out of the five most dominant genera belonged to the family *Enterobacteriaceae*, encompassing 10.3% of classable reads. The top five abundant bacteria species that shed ARGs were *Escherichia coli*, *Pseudomonas monteilii*, *Synechocystis sp. PCC 6803*, *Salmonella enterica*, and *Klebsiella pneumoniae*, ranked by order of abundance for 20GF, GR45, and 25GF, while the place of *Synechocystis sp. PCC 6803* and *Salmonella enterica* were switched for CON (Table 2). The relative abundance of either the same genus or the same species showed consistency ($P \geq 0.13$) among different groups. Overall, the findings indicate that the relative abundance of specific

bacteria shedding ARGs remained consistent at both the genus and species levels across different feeding systems. However, it is notable that the feeding systems might exert a slight influence on the composition of the predominant genus and species within each respective feeding system.

Feeding systems lead to different numbers of transferable ARGs.

The number of transferable ARGs identified for CON, 20GF, GR45, and 25GF were 54, 29, 42, and 24, respectively, while the number of unique transferable ARGs identified was 14, 5, 2, and 2, respectively. Eighteen common transferable ARGs were presented in feces from cattle in all groups (Fig. 6). Among the common transferable ARGs, eight of them exhibited resistance to tetracycline, two were resistant to beta-lactam, two were resistant to lincosamide, and two showed resistances to chloramphenicol-florfenicol.

Biocide and metal resistome are the same between feeding systems.

Out of the total 753 BMRGs identified, 170 of them were confirmed to be located on plasmid and transposon (PTBMGRs), which were selected for further analysis. Regardless of feeding system, no difference ($P = 0.45$) was detected in Chao index. However, a lower Shannon index (2.82) was found for GR45 cattle comparing with those for 20GF (2.92; $P = 0.0093$) and 25GF (2.96; $P = 0.013$)) cattle (Fig 7). Beta diversity of PTBMGRs was the same (Stress = 0.14, $R = 0.20$, $P = 0.16$) among all the groups (Fig 8), indicating that the investigated feeding systems in present study didn't influence the biocide and metal resistome in cattle feces

Discussion

The study demonstrated that cattle raised in grass-feeding systems exhibited lower richness and evenness of ARGs in their feces than cattle raised on conventional grain-finished feeding systems which incorporated antibiotics in the feed, as indicated by the Shannon and Chao1 indices. The long-term use of monensin, a well-known antibacterial ionophore, in grain-feeding systems

may contribute to developing resistance in fecal bacteria. Previous research has suggested that monensin exerts selective pressure on gram-positive cells, leading to alterations in the prevalence of *Enterococcus spp*. in the rumen and feces, which can impact the growth of *Enterococcus faecium* and *Enterococcus faecalis*, potentially influencing resistance to ARGs (Nisbet et al., 2008; Congilosi and Aga, 2021; Callaway et al., 2003). *Enterococcus* has been detected across all the groups in this study; however, it explained only 0.19% of the ARG reads in the fecal samples of cattle treated with monensin. Therefore, Enterococcus may not significantly contribute to the observed AMR patterns in this investigation. Similar findings were reported in another study conducted by Rovira et al. (2019), where it was observed that the number of ARGs identified in the conventional cattle feeding system (beef and dairy) was 51% higher than in feeding systems without antibiotic administration after excluding ARGs found in wastewater. Similar to this experiment, Rovira et al. (2019) considered antibiotic administration and assessed environmental factors such as water sources when evaluating AMR in cattle from different feeding systems. Unlike our study, where baseline samples were collected prior to assigning cattle to groups, Rovira et al. (2019) collected fecal samples during the early feeding period (13 ± 11 days) and late feeding period (243 ± 48 days) in the feedlot. Nevertheless, both studies yield similar results that the fecal resistome remained consistent among groups during the early period in their study and at baseline in this study, indicating that resistome transforms after a few days of switching feeding systems. Our study showed that the composition of the top ten ARG classes of GR45 was closer to that of CON than that of 20GF. The quick change of the fecal resistome of GR45 cattle towards the cattle fecal resistome in the conventional feeding system can be attributed to the 45-day finishing period in the feedlot. Compared to the 20GF resistome, the composition of ARG classes in GR45 is much closer to that of CON, which indicates that the conventional feedlot, which usually supplement

antibiotics in feed ration, has a powerful potential to restructure the resistome within a short-term period. Animal feces can contaminate the environment by contaminate the water and feed and promote the AMR spread by horizontal gene transfer of ARGs among bacteria (Agga et al., 2019). Feedlots, which are used to enhance the efficiency of beef production, are known for their dense conditions where cattle typically congregate around feed and water areas, and a notable presence of ARGs has been detected around feed and water sources in the feedlot (Wang et al., 2021). A study conducted by Agga et al. (2019) demonstrated that the concentration of ARGs specifically related to sulfonamide is the highest in the proximity of waterers and feeders, while it is lowest in grazing areas located farthest from the feeding area. Therefore, the different fecal resistome between cattle under different feeding systems can be attributed to the higher population densities of cattle in conventional systems, where they are often confined to close living quarters. It is possible that beef cattle from CON and GR45 gained more ARGs from their living environment, such as soil, and then ARGs were further transmitted to others via close interaction, including sharing feeder and waterer. In contrast, grass-feeding systems allow cattle to roam and graze on pastures, which may limit the transmission and acquisition of ARGs. This theory can also explain the higher detection of transferable ARGs in CON, followed by GR45, 20GF, and 25GF. The number of transferable ARGs could increase dramatically within 45 days for GR45 in the feedlot, as the intensive population in feedlots creates an ideal environment for ARG transfer.

The most abundant class of ARGs in the fecal samples was MLS, followed by aminoglycosides, oxazolidinone, or tetracyclines, regardless of the groups and age. Similar results have been shown in the earlier studies where the resistance of MLS, aminoglycosides, and tetracyclines were all observed in greater than 50% of the samples from cattle (Noyes et al., 2016; Huebner et al., 2019; Lim et al., 2020). In present study, more than 70% of ARGs belong to these

three classes regardless of groups, and greater than 80% of ARGs are categorized in these three classes for baseline and grain-feeding groups. Previous studies have shown that the therapeutic use of oxytetracycline could increase the minimal inhibitory concentration of tetracycline by encoding *tet*(A) and *tet*(B) in *E. coli*, and the subcutaneous injection of oxytetracycline can traverse the biliary route and exert selection pressure on intestinal bacteria (Agwuh, 2006; Alexander et al., 2013; O'Connor et al.). In present study, two cattle from CON received oxytetracycline injections either in May or June 2018. Meanwhile, seven cattle from GR45 received it between June and July 2018, and one cattle under GR45 received it in October. The fecal samples were collected in November. Therefore, the increase in tetracycline-associated ARGs in cattle feces within grain-feeding systems might be attributed to the therapeutic use of oxytetracycline.

Environmental factors could also have played a role in shifting the resistome. Long et al. (2022) and Shawver (2021) revealed that soil samples exposed to manure from cattle, including both antibiotic-free and antibiotic-treated cattle, exhibited a high abundance of ARGs belonging to tetracycline, aminoglycoside, and sulfonamide classes. According to the annual summary report on antimicrobials sold or distributed for use in food-producing animals by the FDA (2022), 45% of sulfonamides, 52% of aminoglycosides, and 43% of tetracyclines were intended for medical use in cattle in 2021. A significant proportion of administered antimicrobial substances, ranging from 20% to 80%, is excreted in the form of metabolites through urine and feces, which can persist in the soil even after the animals have been removed (Andersson and Hughes, 2014; Agga et al., 2019). Therefore, when antibiotics were previously administered to cattle within the same feedlot, residual traces of these antibiotics could be excreted by the cattle into the soil. The dispersion of antibiotics, accompanied by flying dust, could potentially lead to the contamination of feed or water, facilitated by cattle movement. The hypothesis that ARGs present in the animals' living

environment can colonize in the animals even without direct exposure is further supported by other studies (Thomas et al., 2017; Mir et al., 2018; Ma et al., 2019).

In this study, the results revealed that age could contribute to shifting resistome abundance. For 25GF, 42.5% (17 classes) of ARG classes showed reductions at harvest, and 42.5% (17 classes) of ARG classes were present at baseline but disappeared at harvest, while 7.5% (3 classes) increased in prevalence from baseline to harvest. The cattle in 20GF, which received the grass diet but were harvested earlier, did not exhibit a significant decrease in the prevalence of ARGs from the baseline to the harvest stage. This lack of reduction could be attributed to the additional five months of grazing on irrigated pasture in 25GF or to the oldest harvest age for 25GF cattle. This idea is supported by previous research that the prevalence of ARGs in fecal samples decreased in aged beef cattle, dairy cattle, and swine (Gaire et al., 2021). In the present study, the 25GF cattle exhibited the greatest ARG prevalence reduction in their feces at harvest. However, this does not imply that older animals always harbor fewer ARGs, as there may be a significant increase of AMR development after reaching a certain age. According to Robey et al. (2017), in humans, resistance to amoxicillin, cefalexin, ciprofloxacin, co-amoxiclav, and extended-spectrum beta-lactamases (ESBL)-producing *Escherichia coli* decreases (amoxicillin, ciprofloxacin, and co-amoxiclav) or remains stable (cefalexin) during early ages, and then increases as individuals grow older. In humans, specific change points for amoxicillin, cefalexin, ciprofloxacin, and co-amoxiclav are observed at 12 years, 54 years, two years, and 33 years, respectively (Robey et al., 2017). These findings, along with previous studies, suggested that the age-dependent dynamics of ARG fluctuations vary across animal species and types/classes of antibiotics. Further research is necessary to determine the age at which cattle harbor the lowest abundance of ARGs to minimize the transmission of AMR.

Nevertheless, in this study, age and feeding systems could potentially serve as confounding factors. The cattle in the 25GF group are not only the oldest among the groups but also have not been directly exposed to antibiotics for a longer period. This lack of direct antibiotic exposure could also be a contributing factor leading to the lower abundance of ARGs observed in this particular group.

In contrast to another study (Noyes et al., 2016), our research identified a significant occurrence of oxazolidinone resistance across all groups. It was important to note that while oxazolidinones were approved for clinical use in humans and companion animals, their use was never approved for food-producing animals in the USA (Tyson et al., 2018). A similar situation was reported that 30% of the poultry carried fluoroquinolone-resistant *E.coli* in Australia where fluoroquinolone is prohibited in food-producing animals (Ingram et al., 2013). Therefore, the presence of oxazolidinone resistance may have originated from the workers involved in cattle production. Another potential source could be companion animals with access to the production systems, which may excrete feces with ARGs. This could facilitate horizontal gene transfer, where bacteria shedding resistance genes are transferred from companion animals to the soil and subsequently to cattle. Among the 18 common transferable ARGs investigated, 16 of them demonstrated resistance to tetracycline, beta-lactam, or lincosamide antibiotics, which are widely utilized in both veterinary and human medicine (Chopra and Roberts, 2001; Chen et al., 2010; Lima et al., 2020). On the other hand, two of the ARGs showed resistance to chloramphenicol-florfenicol. Notably, florfenicol is restricted in human medicine due to its severe side effects but remains used for veterinary purposes concerning pets and food-producing animals (Schwarz, 2004; Picco, 2001). Consequently, the concern about florfenicol-resistant genes alone in cattle feces might not be overly significant for human; however, co-selection mechanisms between florfenicol-

resistant genes and other classes of resistant genes could be a potential concern. Of interest, chloramphenicol is approved for treating infections in pets but is considered illegal for use in cattle and pigs and is only used for serious human diseases (FDA, 2006). Thus, the presence of chloramphenicol resistant genes across all groups could be explained by close contact between farm workers and possible pets.

In our study, *Enterobacteriaceae*, including *Escherichia*, *Klebsiella*, and *Salmonella*, emerges as the predominant family that shed ARGs across all groups. *Enterobacteriaceae* has been exhibited to produce 16S rRNA methyltransferase, which is a high-level resistance mechanism against aminoglycosides (Wachino and Arakawa, 2012), which leads to the natural development of aminoglycoside resistance in *Enterobacteriaceae*. *Escherichia* and *Klebsiella*, the two predominant bacterial genera that shed ARGs in this study, are known to be primary hosts for multidrug-resistant genes (Xiong et al., 2018), which might be the result of the naturally occurred resistant mechanisms conferring cross-resistance to multiple antibiotic classes. Coque et al. (2008) demonstrated that ESBL-producing *Enterobacteriaceae* develop resistance to beta-lactam antibiotics and co-select ARGs for fluoroquinolones or aminoglycosides. In our study, ESBL-producing *Enterobacteriaceae*, including *Escherichia coli* and *Klebsiella pneumoniae*, emerged as the most and fifth abundant species responsible for shedding ARGs across all groups. The inherent capability of producing ESBL renders these bacteria resistant to beta-lactam antibiotics, and the high abundance of aminoglycoside and fluoroquinolone resistant genes may be attributed to co-selection mechanisms. Of significance, the relative abundance of the predominant genera and classes of bacteria shedding ARGs did not show significant variation between groups. This finding suggests that feeding systems may not influence certain bacterial ability to shed ARGs. Furthermore, studying the AMR patterns of a few individual bacterial species may inadequately

capture the comprehensive AMR landscape of the entire system. The employment of metagenomics to investigate the resistome holds the potential to enhance the understanding of resistant genes and mechanisms, extending its efficacy to contain even those bacterial species that are not culturable (De Abreu et al., 2021).

The exposure of cattle feces to biocides and heavy metals has been shown to induce the development of BMRGs (Chapman, 2003; Roosa et al., 2014). Interestingly, in this study, no significant differences in the biocide and metal resistome were detected across the different groups. This lack of variation could be attributed to the fact that all cattle involved in the study had received mineral supplementation at Sierra before being assigned to their respective groups. However, a previous study yielded different results, indicating that the total abundance of BMRGs in grazing cattle feces was notably lower than in intensive feeding cattle feces (Fan et al., 2023). The disparity might arise from the fact that the grazing cattle in that study did not have direct exposure to any antimicrobials, biocides, or heavy metals. Furthermore, all the feedlot cattle were provided with mineral supplementation in their feed ration in our study, which implies that the duration of exposure to heavy metals might not exert an influence on the metal and biocide resistome as long as direct exposure has occurred. Nonetheless, this hypothesis requires confirmation through future research. Notably, the diversity of BMRGs in the CON cattle feces was lower than in grass-feeding systems. A previous study has demonstrated that fertilization with chloride increases the concentration of chloride in the grass (Pehrson et al., 1999). Therefore, in this study, it is likely that fertilizers might have been used to elevate the mineral concentration in the grass, which, in turn, could have induced the development of BMRGs through the consumption of high mineral content grass.

Conclusion

The findings of our study revealed a significant difference in the fecal resistome of cattle raised under conventional and grass-feeding systems. Overall, conventional feedlot cattle and grain-finished feedlot cattle exhibited a greater diversity of ARGs in their feces compared to 100% grass-fed beef. The similarity in diversity and composition of ARGs between CON and GR45 suggests that even a short period of antibiotic administration can shift the fecal resistome of cattle. In conclusion, the predominant factors contributing to the shift of resistome in conventional feedlots are likely the use of antibiotics for disease prevention and treatment, in addition to other potential factors such as the living environment and age of the cattle. Further studies should be conducted to fully comprehend the independent effects of living environment and slaughtering age on the resistome shift in different feeding systems.

Additionally, the extended grazing period appears to be a critical factor in reducing AMR in cattle feces, which was shown by the lowest abundance of ARGs in 25 GF cattle. While, the duration of feeding cattle in the CON, 20GF, GR45, and 25GF system were about 18 months, 20 months, 21-22 months, and 25 months, respectively, further studies are required to determine the optimal feeding duration associated with the minimized AMR risk.

Figure 1. Heatmap of ARGs identified in fecal samples collected at baseline combining four groups (n=40) and harvest for CON (n=10), 20GF (n=10), GR45 (n=10), and 25GF (n=10).

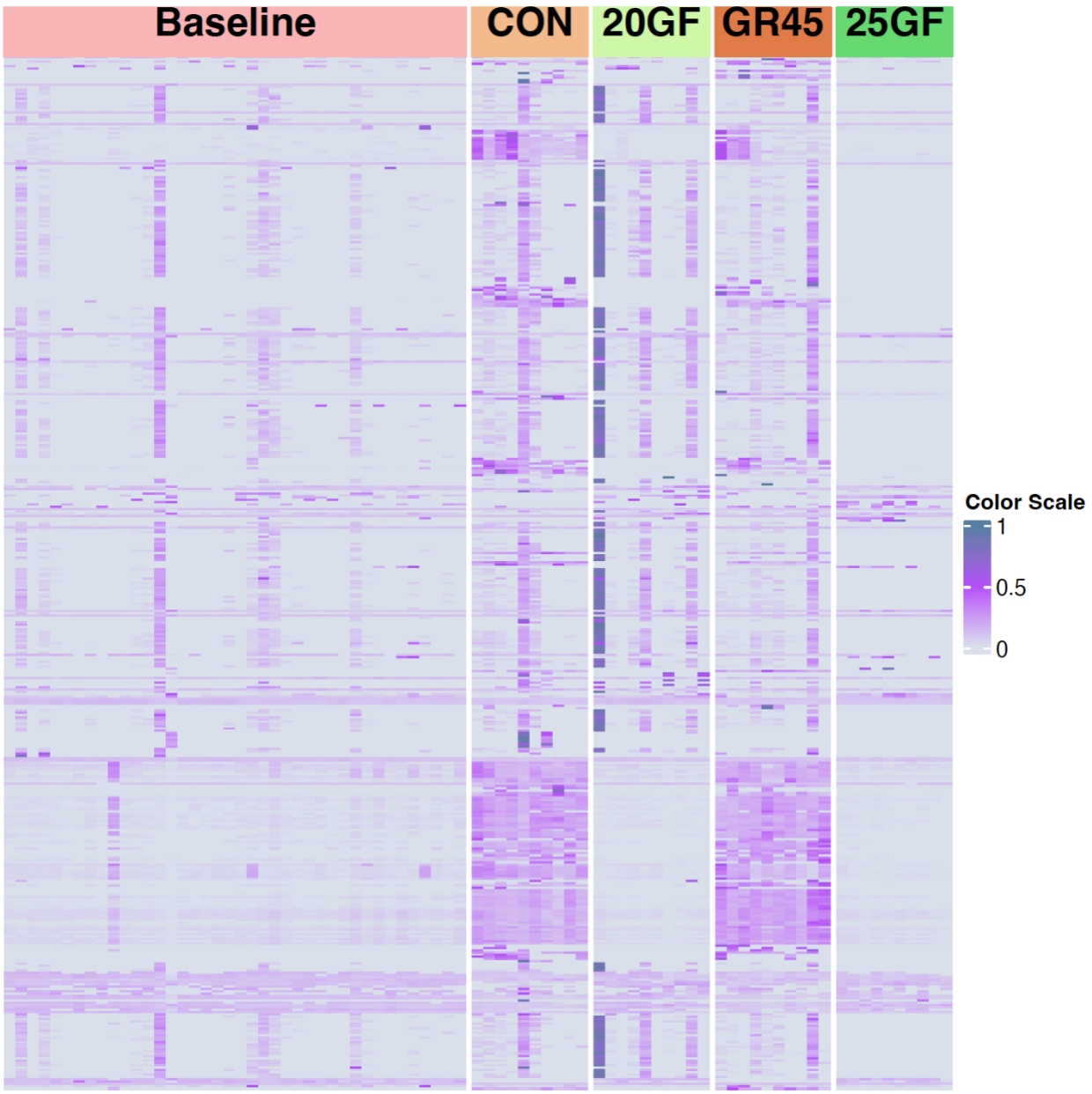


Figure 2. The comparisons of alpha diversity of ARGs at gene level. Lower cases (a and b) show the feeding effects between conventional grass-fed cattle (CON), 20 months grass-fed cattle (20GF), 20 months grass-fed and 45 days grain-finished cattle (GR45), and 25 months grass-fed cattle (25GF) at the same age. Upper cases (A and B) show the age effect between baseline and harvest of the same group. (a). Shannon diversity. (b). Chao1 diversity.

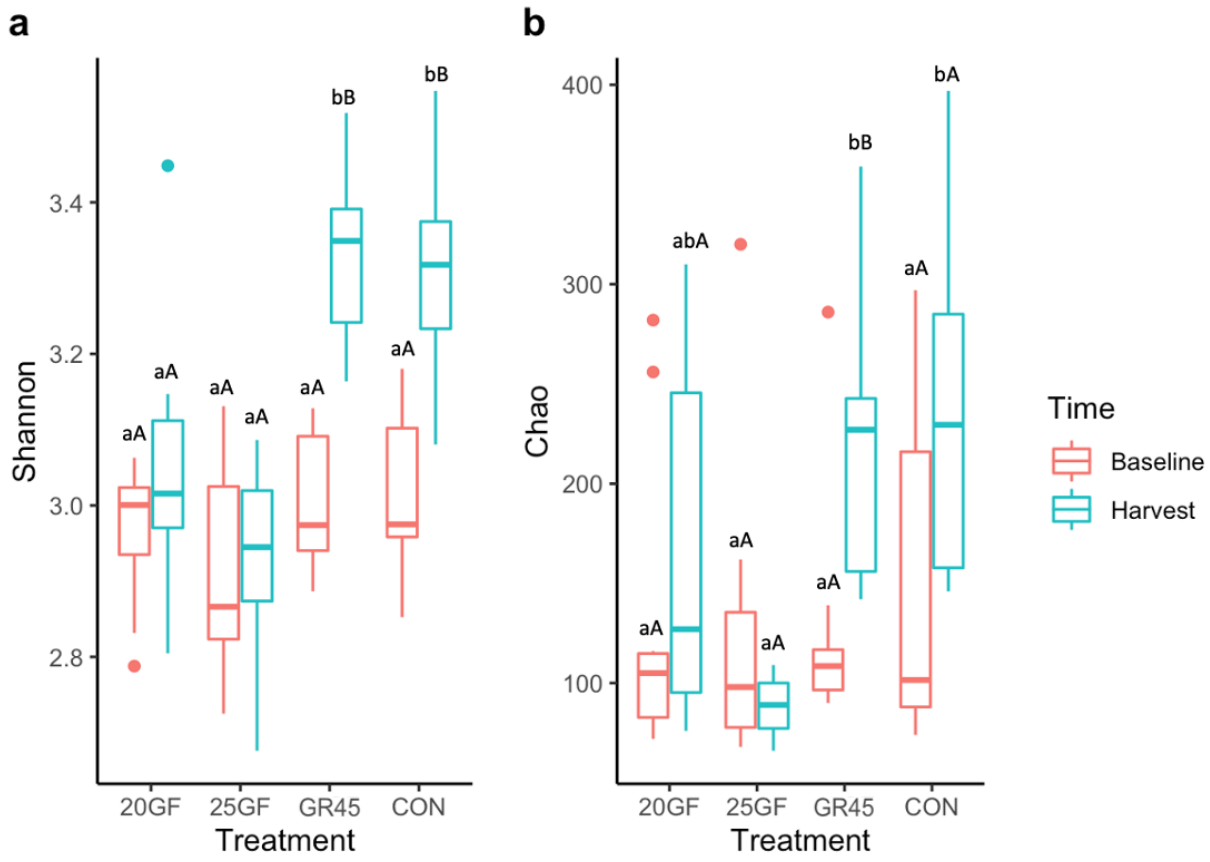


Figure 3. A comparison of fecal samples revealing by NMDS ordination of resistome at gene level from (a). four groups at two sampling time. B represents samples collected at baseline and H represents samples collected at harvest. (Stress = 0.07, R = 0.211, P = 0.018). (b). four groups at harvest. (Stress = 0.06, R = 0.403, P = 0.001).

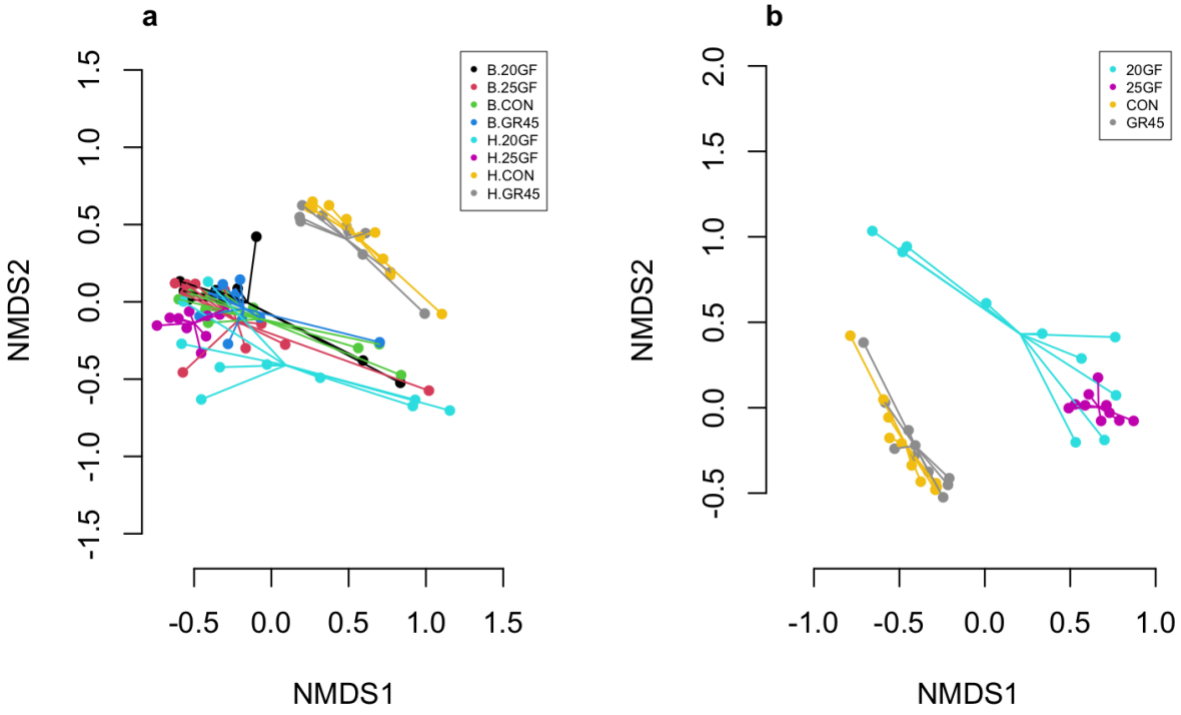


Figure 4. The relative abundance of top 10 ARGs classes at baseline (n=40) and at harvest specifically for CON (n=10), 20GF (n=10), GR45 (n=10), and 25GF (n=10).

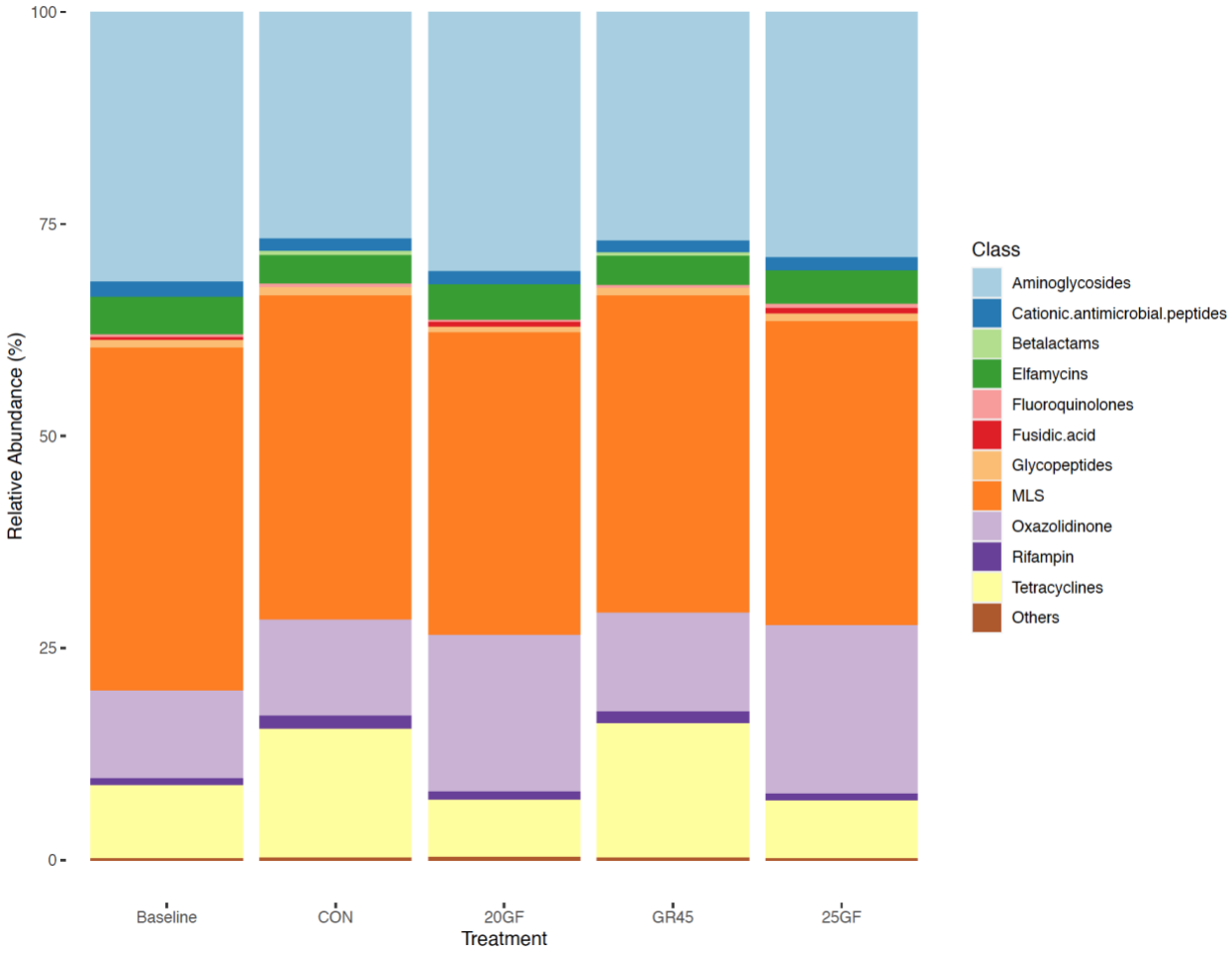


Figure 5. The comparisons of relative abundance of ARGs between baseline and harvest at class level. Red bar represents the reduction from baseline to harvest, while green bar represents the increment from baseline to harvest.

Figure 6. Transferable ARGs identified in conventional grass-fed cattle (CON), 20 months grass-fed cattle (20GF), 20 months grass-fed and 45 days grain-finished cattle (GR45), and 25 months grass-fed cattle (25GF) regardless of age.

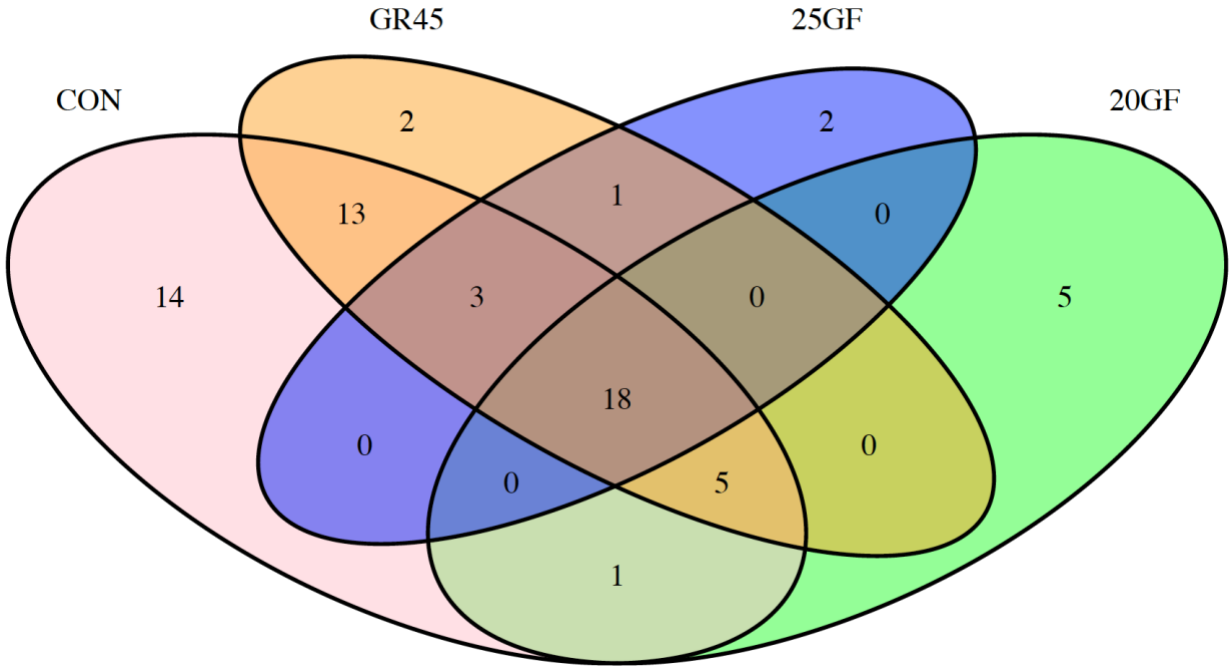


Table 1. The relative abundance of five predominant genera of bacteria in fecal samples

	Groups[1]				
Genus	CON	20GF	GR45	25GF	*p-Value*
Pseudomonas	1.81%	1.92%	1.84%	1.91%	0.99
Escherichia	1.61%	1.47%	1.61%	1.43%	0.98
Klebsiella	0.83%	0.69%	0.82%	0.69%	0.88
Salmonella	0.75%	0.67%	0.74%	0.65%	0.97
Synechocystis	0.72%	0.89%	0.74%	0.85%	0.80

[1]Groups: conventional grain-fed beef (CON), 20 months grass-fed beef (20GF), 20 months grass-fed and 45 days grain-finished feed (GR45), 25 months grass-fed beef (25GF).

Table 2. The relative abundance of Five predominant species of bacteria in fecal samples

	Groups[1]				
Species	CON	20GF	GR45	25GF	*p-Value*
Escherichia coli	1.58%	1.44%	1.40%	1.58%	0.26
Pseudomonas monteilii	0.87%	1.07%	1.04%	0.91%	0.21
Salmonella enterica	0.73%	0.66%	0.65%	0.73%	0.21
Synechocystis sp. PCC 6803	0.71%	0.89%	0.84%	0.74%	0.16
Klebsiella pneumoniae	0.69%	0.58%	0.59%	0.69%	0.13

[1]Groups: conventional grain-fed beef (CON), 20 months grass-fed beef (20GF), 20 months grass-fed and 45 days grain-finished feed (GR45), 25 months grass-fed beef (25GF).

Figure 7. The comparisons of diversity of BMRGs at gene level. Lower cases (a and b) show the feeding effects between conventional grass-fed cattle (CON), 20 months grass-fed cattle (20GF), 20 months grass-fed and 45 days grain-finished cattle (GR45), and 25 months grass-fed cattle (25GF) at the same age. Upper cases (A and B) show the age effect between baseline and harvest of the same group. (a). Shannon diversity. (b). Chao1 diversity.

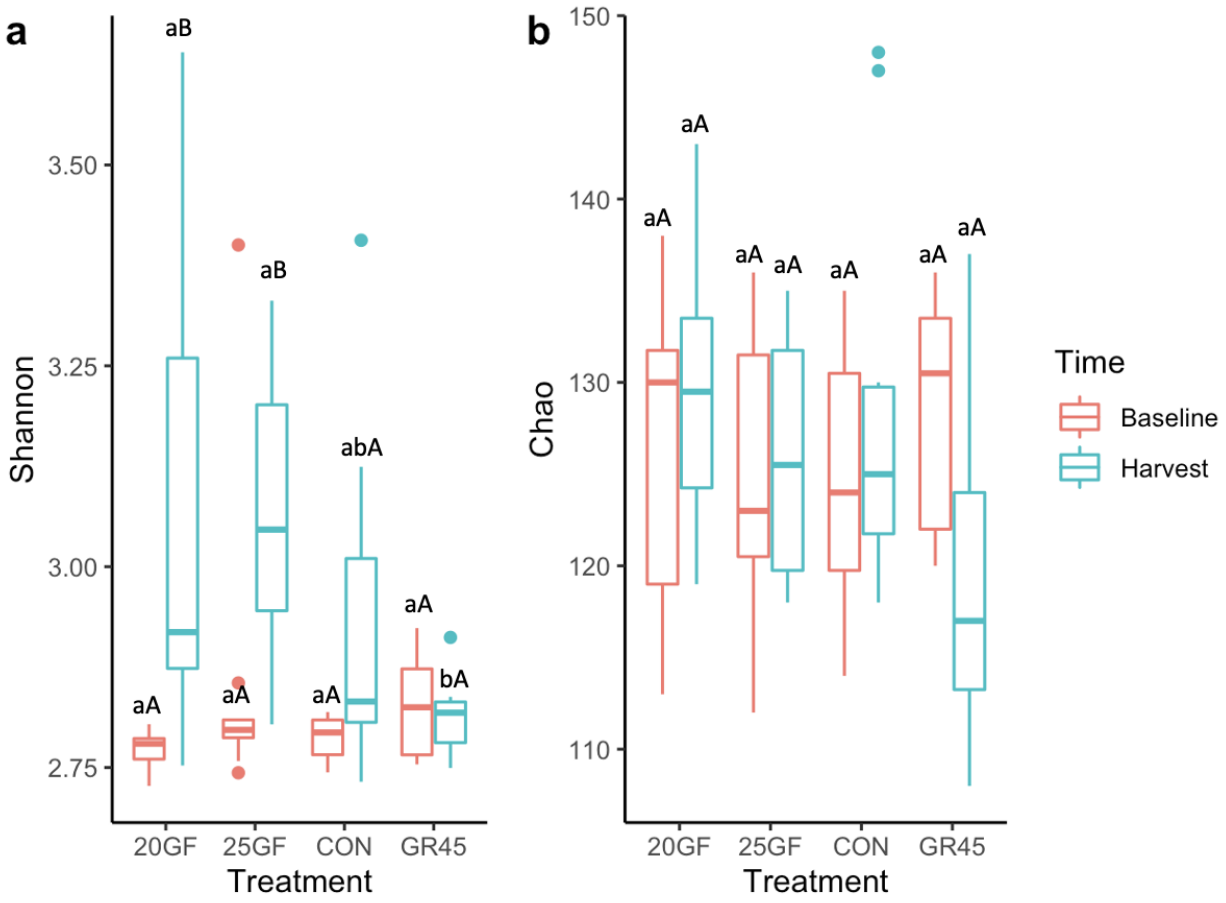
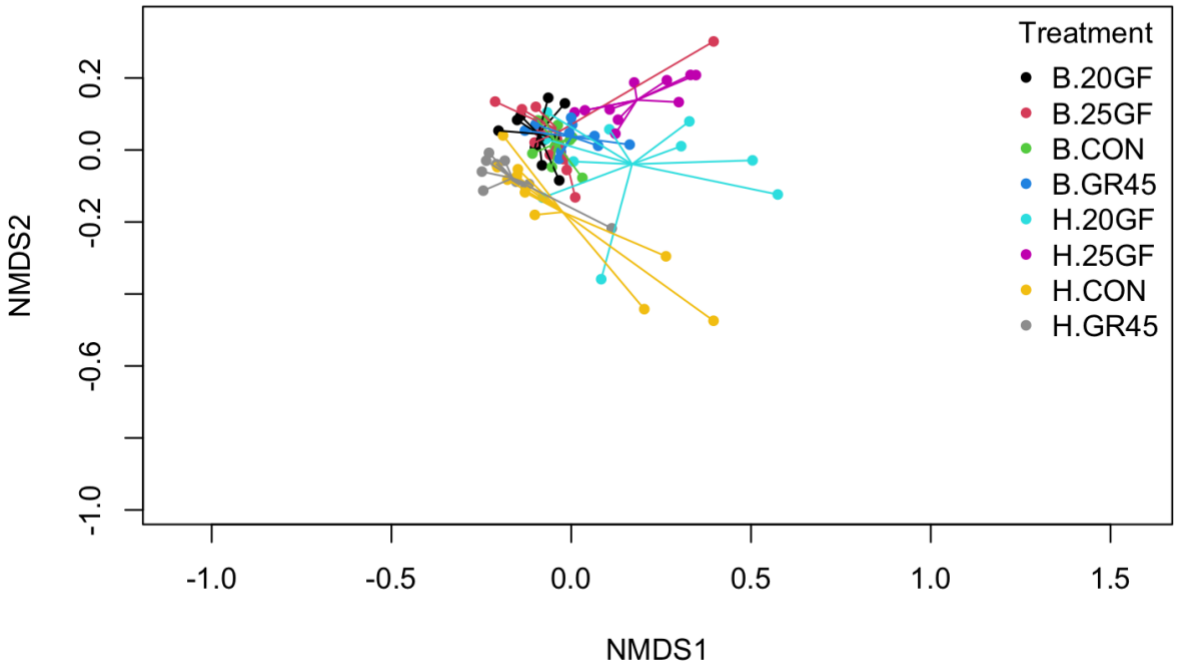


Figure 8. A comparison of fecal samples revealing by NMDS ordination of biocide and metal resistome at gene level from four groups at two sampling time (Stress = 0.14, $R = 0.20$, $P = 0.16$). B represents samples collected at baseline and H represents samples collected at harvest.



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Chapter 3: SUMMARY

Grain-feeding systems and grass-feeding systems result in distinct diversities of ARGs in cattle fecal bacteria and lead to variations in the fecal resistome. Specifically, grain-feeding systems, often associated with feedlots, exhibit more ARGs, including more transferable ARGs, and shift resistome in the animal feces. This shift in the fecal resistome may be attributed to incorporating antibiotics into animal feed and the therapeutic administration of antibiotics. Notably, similar trends in ARG class composition shifts were observed when comparing cattle raised post-weaning on a grain-feeding system to those finished on the grain-feeding system. This suggests that even a short-period exposure to antibiotics in conventional feedlots can significantly alter the resistome and enrich the diversity of ARGs. Extended grazing durations in the grass-feeding system may reduce AMR in cattle fecal bacteria. However, subsequent research is required to confirm the ideal grazing duration to mitigate the emergence of AMR development resulting from the inherent dynamics specific to grass-fed cattle.

There was no observed difference in beef cattle's fecal biocide and metal resistome between grain and grass-feeding systems. Nonetheless, a reduction ($P < 0.05$) in Shannon diversity of transferable BMRGs was noted in GR45 cattle compared to those raised exclusively on grass-feeding systems. Given the nature of grass-feeding systems, cattle may experience added selective pressure for BMRGs from environmental factors and diet, possibly due to mineral contamination. This hypothesis warrants further investigation. Overall, further studies with a more complex experimental design should be conducted on the soil and feedstuffs from each feeding system, considering the feeding duration, to optimize profitability and minimize the risk of AMR.