DISSERTATION

THE EPIDEMIOLOGY AND ECOLOGY OF ANTIMICROBIAL USE AND RESISTANCE IN NORTH AMERICAN BEEF PRODUCTION SYSTEMS

Submitted by

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ABSTRACT

THE EPIDEMIOLOGY AND ECOLOGY OF ANTIMICROBIAL USE AND RESISTANCE IN NORTH AMERICAN BEEF PRODUCTION SYSTEMS

Antimicrobial resistance (AMR) is a critical public health issue (1), and analysis of historical *Escherichia coli* isolates reveals that AMR has been increasing steadily since the introduction of antimicrobial drugs (AMDs) (2). Meat production systems are thought to contribute to the problem by harboring a reservoir of AMR that interfaces with humans either through persistence in the food chain or dissemination of wastes into the environment (3–6). Antimicrobial use (AMU) in food producing animals is often cited as a driver of AMR in humans, but it is extremely challenging to design and execute studies that can be used to infer causality between the two. As a result, producers and policy makers alike have relatively little high-quality evidence on which to base informed and rational decisions with regard to AMU and other production management practices.

The four studies presented in this doctoral thesis attempt to overcome some of the obstacles that currently impede inferential analysis regarding AMU practices and AMR. The first two studies stem from a project in which detailed AMU and AMR data were collected throughout the feeding period for over 5,000 individual cattle across 300 pens. The unprecedented collection of prospective data from such a large number of uniquely identified commercial cattle enabled us to achieve a much more robust level of causal inference compared to many previous studies. The last two studies employed shotgun metagenomics to interrogate the entire AMR potential (the "resistome") of a given sample, enabling novel insight into the

longitudinal, microbe-level genetic ecology of AMR within beef production systems. Because AMR develops and is maintained within the genetic context a microbial population, the resistome-microbiome approach contributes a critical and long-lacking piece to the overall puzzle of AMR within beef production. Thus, while each study in this dissertation approaches the research question of AMR from a slightly different angle, all of them provide crucial and novel information to our scientific understanding of AMU and AMR in beef production.

The 4 studies also complement one another through investigation of different aspects of AMU and AMR across nearly the entire beef production system. The first study not only investigates AMU-AMR associations within *Mannheimia haemolytica*, but also examines how these associations affect respiratory-related morbidity and mortality outcomes in commercial cattle. As such, this study is focused on the animal health and economic dimensions of AMU and AMR in a critically important respiratory pathogen. The second study investigates within-feedlot AMU-AMR associations in non-type-specific *Escherichia coli*, a widely used "indicator" species for AMR, and compares different analytical methods for analyzing the types of data collected as part of ongoing surveillance of AMR in livestock production. Therefore, this study focuses on the public health and regulatory dimension of AMU-AMR in feedlot beef production. The third study tracks AMR in cattle production effluents such as feces, soil and water, thus encompassing the environmental dissemination routes that may play a role in the transmission of AMR from livestock to humans. And finally the fourth study tracks AMR in cattle and their environments from feedlot entry through slaughter and fabrication, thereby delving into the food supply dimension of beef production.

Importantly, all 4 studies were conducted in commercial beef feedlot operations, and samples are collected from commercial cattle and their environments. All 4 studies are strictly

observational; the participating operations did not alter their production practices and cattle were not managed differently for any of the studies. While this approach may add complexity to the interpretation of study findings, it has the distinct advantage of enabling insight into AMU-AMR dynamics on operations that constitute an integral part of the fabric of our society. The AMU and other production practices utilized on these operations were not contrived, and therefore the external validity of the study findings are more widely applicable than those gleaned from research animals and herds. The findings of the 4 studies in this dissertation are therefore novel, complementary and highly relevant to the societal, political and scientific debate surrounding AMU and AMR in beef production.

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PREFACE

Research in the life sciences is currently being transformed by the continued permeation of culture-independent approaches into diverse and disparate fields. While PCR and other culture-independent laboratory methods have been used for decades, the ability to easily and cheaply access thousands of microbes and their DNA (the "metagenome") from a single sample has only recently become available to the majority of life sciences researchers. I feel incredibly lucky that, through sheer coincidence of timing, I was able to conduct my doctoral training in the middle of this exciting transformation.

As a result, my doctoral dissertation is itself a very small microcosm of the much larger, inexorable change sweeping through life sciences research. The dissertation begins with a longitudinal epidemiological study that utilized culture-based approaches to investigate associations between antimicrobial use (AMU) in nearly 6,000 feedlot cattle and patterns of antimicrobial resistance (AMR) in Mannheimia haemolytica and Escherichia coli (E. coli) isolated from those same cattle. These culture-based studies in M. haemolytica and E. coli were the first to track AMU and AMR in a very large number of uniquely identified commercial beef feedlot cattle over the entire feeding period. The dissertation then shifts to two studies that utilized a metagenomics approach to investigate patterns of AMR across the beef production system. In these latter projects, the ability to sequence whole community DNA using next-generation sequencing technology allowed us to interrogate the entire genetic potential for resistance within collected samples, i.e., the "resistome". These culture-independent studies were the first to apply the metagenomics-based approach to beef production, and the first to describe the resistome of the beef production system. Despite the differing microbiological approaches,

the findings from each of these studies added uniquely to our collective knowledge of the epidemiology and ecology of AMR within beef production.

Beyond the subject-specific training and knowledge that I accrued in the process of developing this dissertation, I feel fortunate to have experienced "doing science" using a well-rooted, stolid, traditional approach, as well as a fledgling, mercurial, "cutting-edge" approach. The opportunity to work on the same topic using methodologies at such different stages of scientific development was both challenging and rewarding. As a result, I have deep respect for the former, and great optimism (plus a healthy dose of wariness) for the latter. Perhaps most importantly, working within the metagenomics arena has fostered the development of a profound sense of awe at just how much combined scientific effort is needed to move a novel research approach from infancy to adulthood. It has been a great privilege to be a very, very small part of this effort, and I am grateful to all of the amazing scientists who have helped me as I try to make my own, small contributions to the overall endeavor.

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CHAPTER 1: LITERATURE REVIEW

SOCIETAL CONTEXT OF AMU AND AMR IN LIVESTOCK PRODUCTION

Antimicrobial resistance (AMR) is a critical public health issue (1), and analysis of historical *Escherichia coli* isolates shows that AMR has been increasing steadily since the introduction of antimicrobial drugs (AMDs) (2). Infections with resistant versus susceptible pathogens are currently estimated to cause an additional 8 million hospitalization days per year in the U.S. (7). Methicillin-resistant *Staphylococcus aureus* (MRSA) infections alone caused 9,650 deaths in the US in 2011 (8). Meat production systems are thought to contribute to the problem by harboring a reservoir of AMR that interfaces with humans either through persistence in the food chain or dissemination of wastes into the environment (3–6). Antimicrobial use (AMU) in food producing animals is often cited as a driver of AMR in food production, but this blanket statement fails to recognize the extremely varied contexts in which such use occurs.

ANTIMICROBIAL USE IN BEEF PRODUCTION

Antimicrobial use in feedlots

In beef cattle production, antimicrobials are used to treat, control or prevent illness in individual or groups of animals, as well as historically to increase growth efficiency, although this latter use is being phased out of U.S. livestock production (9). Antimicrobials are administered either parenterally, or through the feed or drinking water of cattle. Detailed AMU data for beef production in Canada and the United States are not systematically collected on a national or state scale, but the National Animal Health Monitoring System conducted a nationally representative survey of AMU practices in U.S. feedlots in 2011 (10). Amongst all responding feedlots, these data indicate that 13.4% of cattle were treated at least once with

injectable antibiotics for respiratory disease, 21.3% were treated metaphylactically, and 18.4% and 71.2% received in-feed chlortetracycline and tylosin at some point during the feeding period, respectively (11). Cattle <700 lbs at feedlot placement were administered in-feed tylosin for an average of 168 days, while cattle >700 lbs at placement an average of 141 days (10). Of feedlots administering tylosin to cattle <700 lbs at placement, 28.3% reported using it for "growth promotion", compared to 32% of feedlots administering it to cattle >700 lbs at placement. Larger feedlots were more likely to use antimicrobials for all purposes, and cattle that were lighter-weight at feedlot placement were more likely to be administered antimicrobials than cattle that were heavier at placement. By far the most commonly used class of AMD, both in-feed and injectable, was macrolides (which include tylosin), followed by tetracyclines. Phenicols, betalactams, fluoroquinolones and sulfonamides were also used for treatment of respiratory disease and metaphylaxis, but less commonly (11). Temporal patterns of AMU vary within the feeding period, with a large proportion of parenteral AMU occurring in the early feeding period when cattle are transitioning to the feedlot environment and respiratory disease rates are highest. Overall, these data indicate that parenteral AMU is not uncommon in beef feedlots, in-feed AMU is very common, and macrolides and tetracyclines are the most widely used AMDs.

Antimicrobial use in cow-calf production

Patterns and rates of AMU on cow-calf operations differ greatly from those in feedlots. In the latest NAHMS study, 68% of operations reported using oral or injectable AMDs at least once, but only 7.2% of preweaned calves and 1.9% of cows were treated (12). The most commonly used injectable and orals AMDs were tetracyclines, followed by betalactams. Over 80% of cow-calf operations did not use any in-feed AMDs or ionophores for any purpose. These

data indicate that AMDs are used on the majority of cow-calf operations, but only a small percentage of animals on each operation are treated, primarily with tetracyclines and betalactams.

ANTIMICROBIAL RESISTANCE IN BEEF PRODUCTION

Antimicrobial resistance in beef feedlot cattle

Given the AMU patterns in beef cattle production, and the fact that feedlots are the primary users of AMDs, feedlots are considered the primary beef-related source from which AMR disseminates into the human population. However, very few countries and/or states undertake consistent and regular monitoring of resistance in healthy animals within livestock production facilities. Exceptions include the Canadian Integrated Program for Antimicrobial Resistance Surveillance (13), the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (14), and the Swedish Veterinary Antimicrobial Resistance Monitoring Program (15). In addition, the U.S. National Animal Health Monitoring System (NAHMS) includes pathogen isolation and susceptibility testing as part of its commodity studies; in 2011, NAHMS conducted a feedlot study in which *Salmonella spp*. were isolated from the feces of a nationally representative sample of feedlot cattle and tested for resistance to a panel of drugs (16). This study showed relatively low levels of resistance within *Salmonella spp*. (15.4%), with highest resistance to tetracycline (21.4%), sulfisoxazole (13.1%), chloramphenicol (8.8%) and streptomycin (8.6%) (16). Aside from this study, there are no nationally representative estimates of AMR in US and Canadian feedlots.

In the absence of comprehensive resistance data, researchers must rely either on samples submitted by producers or veterinarians (for instance, samples tested at state or local diagnostic labs), or they must collect their own samples, typically from a select number of operations. Both

of these populations lack external validity and generally cannot be used to generate representative estimates of in-feedlot AMR prevalence. However, several consistent findings can be drawn from the collective body of scientific literature on AMR in beef feedlot cattle within North America. First, pan-susceptibility levels tend to vary widely even between studies conducted in large North American feedlots, i.e., 15.6% to 93% in generic *E. coli* (17–23), 0% to 95% in *Salmonella* (16,20,24,25) and 11% - 62% in *Campylobacter spp.* (20,26). Potential reasons for these discrepancies are numerous and include disparate sampling strategies, different study populations, and different susceptibility testing methods between studies. Resistance prevalence tends to be highest for tetracycline and streptomycin, and this holds true across varying geographic regions (20,27), feedlot management practices (21), and time points within the feeding period (22,23). This pattern also remains fairly consistent across commonly studied bacterial taxa, although *Salmonella spp*. isolates tend to exhibit higher prevalence of resistance to chloramphenicol and ampicillin than generic *E. coli* (24). Additionally, the few studies that have characterized AMR in cow-calf operations have reported lower prevalence of resistance overall compared to feedlot operations (17–19,28); given the multitude of differences between cow-calf and feedlot operations (including cattle genetics, cattle population characteristics, feed source, management practices, density of cattle stocking, and AMU patterns), it is difficult to tease apart what may be driving these differences. Taken as a whole, the literature also suggests that prevalence of resistance to most AMDs increases in the early stages of the feeding period, and then either levels off or declines (20–23,28). Some evidence suggests that this pattern is accompanied by a significant expansion of AMR gene diversity early in the feeding period, followed by a contraction as certain bacterial strains and/or plasmids gain a fitness advantage within the population (29).

Antimicrobial resistance in the food chain

The food chain has long been considered a primary route for transmission of resistant bacteria from livestock to humans, although attempts to quantify the amount of resistance being disseminated via this pathway are rarely described, especially for beef (for exceptions see 30–32). Due to regulatory, financial and public health concerns about the potential danger posed by contaminated food items, however, there are several well-established national programs that undertake regular sampling and susceptibility testing of in-plant and retail meat and poultry, including the National Antimicrobial Resistance Monitoring System in the U.S. Naturally, these programs focus sampling efforts on enteric foodborne pathogens of public health importance, including *E. coli* (33), *Salmonella enterica* (34,35) and *Campylobacter* (36). Outside of these national programs, resistance in other microbial species and groups have been described, including *Staphylococcus aureus* (37), extra-intestinal pathogenic *E. coli* (38) and *Enterobacteriaceae* (39).

Given the paramount importance to public health and national security of maintaining a safe food supply, the beef industry has instituted highly effective antibacterial interventions during the slaughter and fabrication process, including steam vacuuming, carcass washing, application of organic acid rinses and thermal pasteurization (40). These interventions have been shown to reduce bacterial loads by >5 log CFU/100cm^2 total plate count when comparing carcasses post-hide removal and at the end of the fabrication process (41). Despite the widespread use of these highly effective multiple-hurdle interventions in large beef harvest and fabrication facilities in the U.S. and Canada, susceptible and resistant foodborne and other pathogens continue to be isolated from both the harvest facilities themselves, as well as from retail beef products. Notably, however, prevalence of these recovered organisms from beef is

typically lower than for other livestock commodity products, including swine and poultry (33,36). *Campylobacter* prevalence is typically below 0.1% (36), and most large-scale studies of retail ground beef fail to recover a single *Salmonella* isolate (34,42–45). The U.S. Food Safety Inspection Service estimated the 2005-2007 prevalence of *Salmonella* in domestic beef manufacturing trimmings to be between 0.29% and 1.27% (46). However, when *Salmonella* and *Campylobacter* are recovered, typically >50% of the isolates exhibit phenotypic resistance, most frequently to multiple antimicrobials (35,45). Resistant *Salmonella spp.* isolates are also commonly resistant to tetracycline (35) while *Campylobacter* isolates exhibit relatively high prevalence of resistance to quinolones, nalidixic acid and ciprofloxacin, followed by tetracycline (45). Generic *E. coli* are more commonly recovered from both beef processing facilities and retail products than *Salmonella spp.* and *Campylobacter*, typically at >50% prevalence (33,47). However, only a minority of recovered isolates exhibit resistance (33), with highest prevalence for tetracycline, streptomycin, ampicillin and sulfamethoxazole-sulfisoxazole antimicrobials (33,45,47,48).

Antimicrobial resistance in the waste of beef production operations

In addition to being transmitted through food, resistant bacteria and resistance genes can potentially be transferred to humans through several livestock operation effluents that interface with aquatic, terrestrial and atmospheric ecosystems. Potential conduits include uncontained wastewater runoff, lagoon seepage, manure soil amendments, and airborne particulate matter (49–54). Historically, regulations concerning livestock production manure and wastewater focused on ensuring acceptable levels of organic nutrients, e.g., phosphorous and nitrogen, within ground and surface waters and amended soils. However, there is increasing concern that livestock production effluents may contribute to AMR within the human population via

transmittance of both AMD residues and AMR genes to surface and ground waters as well as to crops produced for human consumption (55). Some of this concern is likely fueled by recent evidence that foodborne illnesses associated with vegetables may originate from animal manure (56–59).

Concerns about these environmental routes of AMR dissemination are relatively new, and no formal, representative monitoring programs exist to document AMR prevalence and patterns. Furthermore, little is known about the normal or baseline AMR profile of soils and water not impacted by agricultural wastes (60). Recent studies suggest that even pristine environmental samples contain high levels of both phenotypic and genotypic AMR, even to synthetic AMDs (61–66). The resistance potential of soil is especially high, and an extremely high proportion of soil bacteria are multi-drug resistant (61,67). Phenotypic resistance has been shown to be uncorrelated with anthropogenic impacts on soils, likely due to the presence of multi-purpose efflux mechanisms within environmental bacteria (61,68). On the other hand, culture-independent studies suggest that the abundance of AMR genes in soil and feces is correlated with level of anthropogenic activity (69), but this evidence is still tenuous due to a reliance on incomplete databases of known resistance genes (60,68,69). A recent study reports higher levels of resistance in active versus ancient layers of permafrost, although bacteria from the ancient layers exhibited resistance to semi-synthetic AMDs (66). In addition, while high levels of phenotypic and genetic resistance have been reported in so-called pristine environments, other studies suggest that many of these ecosystems may in fact be substantially impacted by human activity (70).

Given the abundance and diversity of resistance across almost all environmental landscapes sampled to-date, it is extremely challenging to establish an accurate estimate of the prevalence of agriculture-related resistance in terrestrial and aquatic ecosystems.

ASSOCIATIONS BETWEEN ANTIMICROBIAL USE AND ANTIMICROBIAL RESISTANCE

Associations between antimicrobial use in livestock and antimicrobial resistance in livestock

If the food chain and feedlot environmental effluents are considered the primary conduits of AMR originating in beef production, then the cattle in the feedlot are considered the primary generators. Under this hypothesis, cattle are exposed to AMDs and then either excrete a resistance-enriched population of microbes into the environment, or carry it into the food chain. While this seems like a fairly straightforward hypothesis to test, the realities of beef production render it extremely challenging to establish causal inference, especially if the goal is to understand AMU-AMR associations within "real-world" commercial feedlot operations. Challenges include uniquely identifying and tracking animals over time, maintaining and accessing detailed health records for individual animals, and obtaining biological samples longitudinally from a sufficient number of these uniquely identified animals.

Due to these challenges, there are no identified randomized, controlled trials of AMU-AMR associations in the reviewed literature that follow commercial cattle from placement through shipment and/or slaughter. Case-control studies are also extremely uncommon due to the challenge of retroactively collecting AMU data on individual animals. Cohort studies are also not reported in the literature, likely due to the fact that cattle enter feedlots already colonized with AMR bacteria; in other words, it is difficult to identify "outcome-free" populations. For purposes of establishing causality, we are therefore left with longitudinal studies of commercial

beef cattle, of which there are two identified in the literature (20,21). Both of these studies found statistically significant differences in prevalence of resistance to tetracycline, sulfamethoxazole and streptomycin based on AMD exposures. In one study, pens with no antimicrobial exposures were significantly less likely to harbor generic *E. coli* with resistance to tetracycline, sulfamethoxazole and streptomycin than pens with low or high exposures (20). In the other study, pens with no in-feed AMD exposures were significantly less likely to harbor generic *E. coli* with resistance to tetracycline, sulfamethoxazole, streptomycin and chloramphenicol, compared to pens with in-feed AMD exposures (21). In the former study, all three resistance phenotypes were significantly associated with exposure specifically to in-feed and injectable tetracycline AMDs. The latter study did not identify any significant associations between AMD exposures and AMR, but the study feedlots did not use tetracycline AMDs, and therefore this relationship could not be examined.

There are also several longitudinal studies that describe AMU-AMR relationships in large groups of beef cattle raised in research herds. In general, these studies find that parenteral and in-feed AMD exposures for purposes of prophylaxis do not significantly influence resistance prevalence in generic *E. coli* from fecal samples collected immediately prior to slaughter (22,23). However, in-feed AMD exposures for purposes of growth promotion, and specifically chlortetracycline, were associated with a much higher likelihood of recovering tetracycline, sulfamethoxazole, chloramphenicol and ampicillin resistant generic *E. coli* as well as tetracycline and erythromycin resistant *Campylobacter spp*. in feces of pre-slaughter cattle, compared to cattle without this exposure or with exposure to other in-feed antimicrobials (71–73). This effect was not observed for feed supplemented with tylosin (72), however in-feed tylosin both alone

and with monensin and copper has been reported to significantly increase prevalence of macrolide-resistant fecal enterococci (74,75).

Another set of studies investigated shorter-term AMU-AMR dynamics for specific AMD-AMR combinations within small groups of cattle purchased and managed specifically for research purposes. These studies report a significant but transient increase in extended-spectrum betalactam (ESBL) and tetracycline resistance subsequent to single-dose ceftiofur exposures (76–78), however persistently high levels of ESBL resistance can be maintained if ceftiofur is given in combination with in-feed tetracycline exposures (79). In-feed (tylosin) and injectable (tulathromycin) macrolides have been associated with greatly increased levels of erythromycin resistance in *Enterococci* spp. in the short term (80). In-feed tetracycline with and without sulfamethazine has been shown to increase levels of tetracycline, sulfamethoxazole, chloramphenicol and ampicillin resistant *E. coli* (29,81), although some of these associations are strictly transient (82).

In contrast to the dairy literature, there are very few studies that compare resistance in the feces of organically- and conventionally- raised cattle. Those that do are cross-sectional and do not account for the myriad of potential confounders that exist when comparing organic and conventional production. These studies document the presence of multi-drug resistant *E. coli* and tetracycline resistance genes in the feces of both conventionally and organically/non-intensively raised beef cattle, but find significantly higher levels in the conventional operations (28,83).

In addition to animal- and herd-level studies, several studies describe positive associations between AMU and AMR in livestock at the national level (84). However, these studies are ecological in nature and do not contribute significantly to establishing causality.

Taken as a whole, the literature on associations between use and resistance in beef cattle is best described as patchy and ambiguous. Most of the studies have been conducted in non-commercial settings, and this limits the external validity of the findings. Several studies document associations between in-feed tetracycline and macrolide exposures and increased tetracycline and macrolide resistance, respectively, in generic *E. coli* (20,21,29,71–73,80,82,85,86). Additionally, exposure to injectable ceftiofur consistently produces increased resistance to several classes of drugs, but these increases are short-lived unless in-feed drugs are also administered to exposed cattle (77–79). The extent to which such associations significantly impact public health is not known, although quantitative risk assessments suggest that the negative consequences of macrolide use are extremely low (30,31).

Associations between antimicrobial use in livestock and antimicrobial resistance in the food chain

There are currently no published studies that report tracking AMD exposures and resistance outcomes in individual or pens of cattle from feedlot entry through slaughter and retail distribution. Therefore, it is impossible to draw conclusions about causal associations between AMU within the feedlot setting, and AMR in the beef supply chain. However, several studies have undertaken descriptive analysis of resistance patterns on carcasses and retail products from conventionally versus organically raised cattle. Carcasses of conventionally raised cattle have been found to harbor a significantly higher proportion of *E. coli* resistant to ampicillin, ciprofloxacin, doxycycline, gentamycin and sulfisoxazole, although this finding was not reflected within isolates of *Listeria monocytogenes* (43). However, a similar study of *E. coli* O157:H7 isolated from carcasses found that the MIC's of isolates from organically raised cattle were significantly higher than those for conventionally raised cattle for cefoxitin, ceftriaxone,

nalidixic acid, rifampin and tetracycline, and lower for gentamycin, neomycin and streptomycin (87).

Within the retail environment, results are also mixed. One study of conventionally versus organically labeled beef products identified significant increases in prevalence of *E. coli* resistant to ceftiofur and chloramphenicol among conventionally labeled beef products (88). Another study that found that generic *E. coli* isolated from beef purchased at natural-foods stores had significantly lower likelihood of testing resistant than *E. coli* from beef purchased from other grocery stores (89).

Associations between AMU in livestock and AMR in the environment

Given our nascent understanding of resistance within the soil and water microbiome, it is difficult to establish causal links between AMU practices in beef production and AMR that may be found in ecosystems that interface with beef production operations. However, there seems to be mounting evidence to suggest that the combination of AMU within feedlots, resultant AMD residues in lagoons and manures, and poor waste management systems together produce especially high levels of resistance in surrounding locations (90).

The presence of intact AMD residues within feces and wastewater (91,92) is an additional consideration when investigating the potential for AMR transmittance via environmental routes. While AMD residues are strictly controlled and monitored in the beef supply chain, most regulations pertaining to management of livestock wastes were not formulated to control AMR residues in waters and soils. Recent studies have documented a synergistic relationship between AMD residues and AMR gene abundance within contaminated water, composting manure and amended soils (91,93–97). Evidence also suggests that some AMD-AMR dynamics within these systems can be significantly altered by compounds such as

organic acids and ammonia (98,99), which are readily available in beef production settings. However, the ability of AMDs to remain chemically active within different matrices and in the presence of different organic and inorganic compounds varies considerably by AMD (92,97,100), and therefore further research is needed.

Most studies of associations between AMU practices and AMR levels in manure, soils and wastewater have been conducted in swine production, primarily in China (101). As a body of work, these studies indicate that AMU practices significantly impact levels of AMR within waste effluents, although the applicability of these findings to beef production in the U.S. is not clear, especially given substantial differences in both AMU practices and waste management systems between North America and China, as well as between swine and beef production. Among studies conducted in North American cattle production settings, one found significantly higher levels of 6 tetracycline resistance genes in the lagoons of conventional versus organic feedlots (102). Another study that investigated AMR in composting feces from cattle administered in-feed tetracycline and/or tylosin versus unexposed cattle documented increased abundance of tetracycline, sulfonamide and erythromycin resistance genes in the treated versus the control cattle feces, but overall AMR levels in nearly all groups returned to baseline levels by the end of the study (85). AMD residues and/or exposures are not necessary for significant ARD increases in soils after cattle manure application (103), and some studies show that ARD increases in manure-amended soil only occur when application rates are high or manure storage is not properly managed (90).

The potential public health impact of AMU-engendered AMR in soils and wastewater is unknown. At a very broad level, soil bacteria and human pathogens have been shown to exchange resistance genes (104), but other evidence suggests that such exchange is rare (105),

and whether livestock AMU influences this exchange is not known. Interrogation of resistance levels in meticulously archived historical soils since the 1940s shows increasing levels of many resistance classes, but especially tetracyclines since the 1970s (106), which could be explained by increasing use of tetracyclines in agriculture beginning in the 1970s, although many other factors could also explain this finding. Resistant *E. coli* isolated from field lettuce was found to most closely resemble resistant *E. coli* recovered from cattle, but similarly the influence of AMU practices on the presence of the resistant *E. coli* was not explored (59).

Associations between antimicrobial use in livestock and antimicrobial resistance in the general population

Evidence that livestock AMU may increase levels of AMR in food or the environment is not necessarily evidence that AMU in livestock is a significant risk to public health. To make this claim, the chain of causality needs to be extended from food, water systems and/or soils to the places that people live, work and eat, and ultimately to healthcare centers where people with AMR infections are diagnosed and treated (107). Following AMR prospectively through this part of the transmittance chain is nearly impossible. MRSA, for example, is diagnosed at an incidence rate of 24/100,000 U.S. inhabitants per year (108). Cohort, longitudinal and controlled studies of such rare outcomes are cost-prohibitive, leaving aside the logistical difficulties. A case-control approach would enable more targeted enrollment of people with AMR infections, but it is then impossible to retroactively obtain AMD exposure data for the specific meat and poultry products that each study participant consumed, even if participants could recall with specificity what they ate in the past. Therefore, we are left with "natural experiments" and cross-sectional studies.

Natural experiments include instances in which AMR data for a large population are available before and after livestock production AMU practices undergo a drastic change, whether through regulatory changes or introduction of a new AMD onto the market. The widespread adoption and subsequent ban of avoparcin in livestock production in Europe is one example of the former, and many studies documented a substantial decrease in vancomycin-resistant *Enterococci* (VRE) in both animals and humans after the ban went into effect (109,110). Much of the reporting out of Denmark also utilizes this type of correlative analysis, with reports of decreases in various types of AMR infections in humans after widespread livestock AMU restrictions went into effect (14). However, these studies fail to account for numerous other factors that could potentially explain differences between AMR levels before and after alteration of AMU practices, including coinciding changes to livestock management practices, population demographics, and human medical AMU prescribing practices. Therefore, while temporality can be established, causality cannot.

Cross-sectional studies do not establish temporality between exposures and outcomes and therefore also do not support causal interpretations, even when confounders can be taken into account. Despite this, much of the literature cited as evidence of causal associations between livestock AMU and AMR in humans is, in fact, cross-sectional. This includes studies that identify sequence similarity between bacterial isolates from AMR outbreaks in humans and isolates recovered from food animals. For instance, researchers have described substantial genetic similarities between retail beef and outbreak strains of extraintestinal *E. coli* from humans, although beef samples were less likely to exhibit these similarities than poultry or swine (111,112). However, more comprehensive studies have shown that genetically similar outbreak strains can arise within animal and human populations separately (113). Furthermore, evidence

of AMR transmission from livestock to humans is not in itself evidence that AMU practices created the AMR; organic livestock operations and so-called pristine environments harbor a diverse resistance profile, indicating that AMU is not the only determinant of AMR presence.

The inability to definitively support or dispute links between livestock AMU and AMR in humans is not for lack of trying. Given the nature of livestock production and its integral role in numerous complex human ecosystems (i.e., food, water, air and soil), it is simply impossible to conduct sufficiently evidentiary studies to infer causality between AMU in livestock and AMR in humans. Therefore, instead of attempting to apply traditional epidemiological study design methods to this seemingly intractable problem, it may be more fruitful to frame the problem in terms of systems and ecosystems, while keeping issues such as bias and validity at the forefront of study design and interpretation. For instance, one potential approach would be to undertake a comprehensive risk analysis that applies risk probabilities around key components of the livestock production ecosystem and then models the system to quantify the overall risk to human and public health. This approach allows not only quantification of risk, but comparison of different production practices and interventions and their effects on the overall risk. This is not a new approach even within beef production (30,31), however it has yet to be applied to transmission routes other than the beef supply chain or to organisms other than major foodborne pathogens. Given recent evidence that livestock effluents may carry significant amounts of AMR determinants and bacteria, it is critical to include these transmission routes in a comprehensive risk analysis. Furthermore, such an analysis will not be comprehensive unless the entire AMR potential of a given microbial population is included in the assessment, as non-pathogenic and pathogenic bacteria can exchange ARDs and various management practices can co-select for ARDs of unrelated antibiotic classes in a networked fashion (79,104,114). Studies

have shown that bacteria increase rates of horizontal gene transfer of ARDs under stress conditions, including antibiotic exposure, and these dynamics must be included in the assessment of risk under different management practices (115,116).

Until recently, this type of analysis was not feasible given the limitations of culture- and PCR-based methods. However, with the advent of next-generation sequencers and subsequent dramatic decline in cost, it is now possible to sequence the DNA of an entire sample (the "metagenome"), and therefore interrogate all its genetic resistance potential, i.e. the "resistome". This has been done in several large-scale studies in humans (117,118). Only two such studies have been conducted in cattle, both in feces, one of which analyzed 5 samples from 4 cows and the other 1 sample composited from 6 cows (119,120). Significant variability within the human fecal resistome has been documented, and it is highly unlikely that these studies provide a representative description of the cattle fecal resistome. Diet, AMU, co-location, and other factors have all been shown to significantly influence resistome composition, and further descriptive studies in cattle are needed to document how these and other management factors change the resistome of beef cattle production. This baseline knowledge will constitute an integral and previously lacking component of a comprehensive risk analysis of AMU in livestock production.

CONCLUSION

Research involving AMU and AMR in the context of livestock production has been, and will continue to be, extremely challenging. The challenges stem not only from practical, logistical and financial considerations, but also political, societal, and economic ones. Increasing societal pressure is creating incentive for both politicians and industry leaders to address this topic. The scientific community now has new tools to respond in kind. However, with any new

technology comes peril and promise. The promise of a shotgun metagenomics approach to AMU-AMR research is both readily apparent and continually evolving. The ability to describe, quantify and modulate the resistome holds immense potential for ensuring that livestock production practices are optimized towards the goal of protecting both the efficacy of AMDs and the public health. However, this potential can only be realized if the metagenomics approach is executed in the context of sound science. The peril of the metagenomics approach is that the shininess of the new technology will blind us to its limitations and caveats, and that we will fail to utilize the technology in the pursuit of actionable, applied scientific results. Ultimately, a combination of sound epidemiological study design, ecosystems thinking, detailed metagenomic data, and phenotypic susceptibility testing will likely work in concert to significantly advance our understanding of AMU and AMR in food production.

CHAPTER 2: MANNHEIMIA HAEMOLYTICA IN FEEDLOT CATTLE: PREVALENCE OF RECOVERY AND ASSOCIATIONS WITH ANTIMICROBIAL USE, RESISTANCE AND HEALTH OUTCOMES.

SUMMARY

Background

Mannheimia haemolytica is an important etiological agent in bovine respiratory disease.

Objectives

Explore risk factors for recovery of susceptible and resistant M. haemolytica in feedlot cattle and explore associations with health outcomes.

Animals

Cattle (n=5,498) from 4 feedlots were sampled at arrival and later in the feeding period.

Methods

Susceptibility of M. haemolytica isolates was tested for 21 antimicrobials. Records of antimicrobial use (AMU) and health events were analyzed using multivariable regression.

Results

M. haemolytica was recovered from 29% of cattle (1,596/5,498), 13.1% at arrival (95% CI, 12.3% - 14.1%) and 19.8% at second sampling (95% CI, 18.7% - 20.9%). Nearly half of study cattle received antimicrobial drugs (AMDs) parenterally, mostly as metaphylactic therapy at arrival. Individual parenteral AMD exposures were associated with decreased recovery of M. haemolytica (OR, 0.2; 95% CI, 0.02 – 1.2), whereas exposure in penmates was associated with increased recovery (OR, 1.5; 95% CI, 1.05 – 2.2). Most isolates were pan-susceptible (87.8%; 95% CI, 87.0%–89.4%). AMD exposures were not associated with resistance to any single drug. Multiply-resistant isolates were rare (5.9%; 95% CI, 5.1% - 6.9%), but AMD exposures in pen

mates were associated with increased odds of recovering multiply-resistant M. haemolytica (OR, 23.9; 95% CI, 8.4 – 68.3). Cattle positive for M. haemolytica on arrival were more likely to become ill within 10 days (OR, 1.7; 95% CI, 1.1 – 2.4).

Conclusions

Resistance generally was rare in M. haemolytica. Antimicrobial drug exposures in penmates increased the risk of isolating susceptible and multiply-resistant M. haemolytica, a finding that could be explained by contagious spread.

INTRODUCTION

Bovine respiratory disease (BRD) is a major economic burden to feedlot operators. It is estimated that BRD-associated morbidity and mortality result in annual loss of one billion USD for North American feedlots (121). BRD-related costs can account for 7% of total production costs, and per-calf revenue losses associated with treatment of BRD are estimated at up to $292 USD for animals requiring 3 antimicrobial treatments (122,123).

Although the etiology of BRD is multifactorial, *M. haemolytica* is arguably the most important associated bacterial pathogen, primarily because of virulence factors that induce severe morbidity. *M. haemolytica* is typically the most common agent isolated from post-mortem samples of cattle with BRD (124,125).

Treatment of BRD in large commercial feedlots is focused on antimicrobial therapy in clinically ill animals and antimicrobial metaphylactic treatment of high-risk animals. Sick animals that fail to respond to initial treatment typically are re-treated with a different antimicrobial (personal communication: Calvin Booker). Recently, BRD treatment strategies have come under scrutiny because of a perception of antimicrobial resistance (AMR) in *M. haemolytica* isolates recovered from feedlot cattle, including multiply-resistant isolates (126).

Despite the putative importance of BRD and *M. haemolytica* for feedlot economics and animal health, ambiguity persists regarding colonization dynamics of *M. haemolytica* and associations with clinical disease.

Primary objectives of this study were to describe the prevalence of *M. haemolytica* in isolates obtained from commercial feedlot beef cattle, to describe resistance prevalence and patterns in isolates, and to investigate associations between antimicrobial use (AMU) and resistant isolates. A secondary objective was to investigate associations between *M. haemolytica* isolation and morbidity and mortality outcomes.

MATERIALS AND METHODS

Study Overview

Isolates evaluated in this study were collected as part of a project to develop and evaluate surveillance methods of AMR in feedlots (127). The study population, sampling methods and laboratory procedures, and interpretive criteria for antimicrobial susceptibility have been described (127). Briefly, 5,968 individual cattle were enrolled using a 2-stage random sampling as they entered 4 feedlots in Alberta, Canada. Morbidity, mortality and antimicrobial treatment events were tracked throughout the study. Deep nasopharyngeal swabs were collected at arrival to the feedlot ("arrival sample") and again later in the feeding period ("second sample") and cultured for M. haemolytica. Isolates with morphologic characteristics of M. haemolytica were confirmed using biochemical tests and PCR (127,128). Confirmed isolates were evaluated for resistance to antimicrobial drugs (AMDs; Table 2.1) using broth microdilution (Supplemental Table 2.1), disk diffusion (Supplemental Table 2.2) or both. Prevalence of and risk factors for isolation of M. haemolytica were described, and multivariable logistic regression was used to

investigate associations between AMU and AMR in M. haemolytica isolates and between M. haemolytica isolation and health outcomes.

Study Population

Four feedlots in Alberta, Canada with one-time holding capacities of between 15,000 and 20,000 cattle were purposively selected based on their ability to track AMU and other health data as well as their willingness to participate. Production conditions were typical for North American commercial cattle feedlots, and veterinary care was managed by Feedlot Health Management Services (FHMS). Cattle handling and sampling procedures were approved by the Animal Care Committee of the University of Calgary (Protocol Number M07031).

Cattle were sourced from across Canada through auction markets, and entered the feedlots at a range of weights (225 – 400 kg), ages, frame sizes and sexes. Based upon these factors and historical patterns of illness in similar cattle, arriving groups were assigned an ordinal category of perceived risk for developing BRD (low risk to very high risk), which was used to employ prevention and treatment protocols. All cattle received a growth implant, vaccines against selected pathogens, and topical anthelmintic upon arrival. Very high risk cattle received *M. haemolytica* anti-leukotoxin vaccine, and high risk cattle received AMDs as metaphylaxis for respiratory disease, whereas lower risk, non-clinical cattle did not (Table 2.1). Cattle in higher-risk categories received drugs shown to have greater efficacy for prevention and treatment of respiratory disease (129). Cattle were fed a diet that met or exceeded the National Research Council requirements for beef cattle until reaching a body weight of 550-650 kg, at which time they were sent to slaughter, typically 120-250 days after arrival in the feedlot (130).

Trained feedlot personnel evaluated cattle for signs of illness at arrival and daily thereafter. Animals exhibiting systemic illness (e.g., dyspnea, lack of response to stimulation,

reluctance to move, abnormal carriage or posture of the head, or some combination of these signs) were assigned a diagnosis of "undifferentiated systemic illness" with or without fever based on a body temperature of higher or lower than 40.5°C, respectively, and treated using antimicrobial protocols formulated specifically for their diagnosis and risk status. All cattle that died underwent necropsy by a FHMS veterinarian, who used clinical history and physical findings to classify the cause of death as either BRD, bovine viral diarrhea-associated disease, disease caused by *Histophilus somni*, diseases of the appendicular skeleton, metabolic disease, and miscellaneous heath events (e.g., trauma).

Animal and Pen Record Management

A computerized data collection system[a] was used to track the date each animal arrived at the feedlot, the number of cattle in the pen, the BRD risk status of each animal, and all health events, including treatments (date, drug administered, dose, and route of administration) and clinical and necropsy diagnoses. Only in-feedlot AMD exposures were included in this study due to a lack of information on management of cattle prior to arrival in the feedlot. Most cattle's pen assignments did not change after arrival, exceptions being pens that were split or mixed for the purposes of marketing homogenous groups of cattle; such pens were excluded from analysis due to an inability to accurately characterize AMU for penmates. Nasopharyngeal sample collection dates, culture results, *M. haemolytica* isolate identification numbers, and resistance testing results were compiled and linked using the unique animal identification assigned to each animal upon arrival.

Data Analysis

Descriptive analyses and data distributions were explored graphically and through numerical summaries. Adjusted CI for binomial proportions (adding 2 successes and 2 failures)

were estimated (131). Crude prevalence of susceptible and resistant *M. haemolytica* at arrival and second sampling was calculated and compared. When drugs were tested by both broth microdilution and disk diffusion (e.g., ampicillin, ceftiofur), isolates were classified as resistant if either test result indicated resistance. "Pan-susceptibility" was defined as phenotypic susceptibility to all drugs tested. "Multiple resistance" was defined as phenotypic resistance to ≥2 antimicrobials, regardless of drug class and whether results were obtained from broth microdilution or disk diffusion, because the drugs included in these panels differed. McNemar's test was used to detect significant differences in isolation of *M. haemolytica* within individual cattle between the 2 sampling points. Least-square means estimates from generalized estimating equations (GEE) were used to determine significant changes in the overall prevalence of *M. haemolytica* between the 2 sampling points, with cattle ID specified as a repeated measure.

Inferential analyses were performed with commercial software[b] using logistic regression with GEE to control for clustering within pens, specifying an exchangeable correlation structure. Feedlot was included in all models as a fixed effect. The distributions of AMU were strongly right-skewed and zero-inflated, and therefore were modeled dichotomously (i.e., no exposure vs. any exposure). Each antimicrobial class (tetracycline, macrolide, beta-lactam, phenicol, sulfonamide and quinolone) and route of exposure (in-feed vs. parenteral) was modeled separately. Parenteral drugs were grouped into a single variable if exposures were too sparse for model convergence.

The primary study outcome was isolation of *M. haemolytica* (yes or no) in the second sample. Primary exposure variables of interest were previous exposure to parenteral antimicrobials of any type, and in-feed macrolides and tetracyclines. Exposures were classified as direct (i.e., administered directly to the enrolled individual) or indirect (i.e., administered to

penmates of the enrolled individual). Furthermore, exposures were dichotomized as occurring >7 or ≤ 7 days from sample collection. Arrival sample *M. haemolytica* status (positive or negative) and cattle risk level also were risk factors of interest. Pen size was added as a potential confounder.

Secondary outcomes were resistance in second sample *M. haemolytica* isolates, both to each of the 21 drugs tested in the 2 panels, as well as to ≥2 drugs, that is multiply-resistant (i.e., multiply-resistant vs. singly-resistant or susceptible). Isolates tested by broth microdilution and disk diffusion were considered multiply-resistant if either method showed multiple resistance. Primary exposure variables included individual and penmate parenteral exposure to betalactams, sulfonamides, phenicols, quinolones, macrolides, and tetracyclines and in-feed macrolides and tetracyclines at any point before sample collection. For the outcome of multiply-resistant *M. haemolytica,* all parenterally administered drugs were grouped together into a single exposure variable. *M. haemolytica* status of cattle at arrival was the primary risk factor of interest for resistance outcomes. Secondary risk factors included the season of feedlot arrival and sample collection (Jan-March, Apr-June, July-Sept, Oct-Dec), the risk status assigned to each animal (low, medium, high, or very high), and the number of cattle in the pen (<100, 101-200, 201-300, 301-400, or >400). The number of days cattle had been in the feedlot at sample collection was forced into all models as a potential confounder. To account for repeated measures on samples from testing of multiple isolates, we specified "sample" as a subcluster with a "1-(nested log odds ratio)" structure using GEE with alternating logistic regression (ALR) (132). Some isolates were tested by both broth microdilution and disk diffusion, and therefore "test type" was added as a fixed effect.

To model isolation of susceptible and resistant *M. haemolytica,* each AMU variable was first modeled individually. Variables exhibiting a *P* value of ≤ 0.20 were included in multivariable modeling, which proceeded in a backwards stepwise fashion with a critical alpha for retention of 0.05. Variables with a relatively large effect size and biological relevance also were retained in the final model. Confounding (defined as parameter estimate change of ≥20%) was assessed for all excluded variables. Collinearity was evaluated using the variance inflation factor (VIF) and Chi-square test for continuous and categorical variables, respectively (133).

A third set of outcomes included BRD-associated mortality and morbidity (diagnosis of systemic illness with fever at arrival, at any time during the study period, and within 10 days after sample collection). For these 4 outcomes, the primary risk factor of interest was arrival sample *M. haemolytica* status. Secondary *a priori* risk factors included in all models were BRD risk status, number of cattle in the pen, and the season of arrival.

RESULTS

Samples

A total of 5,968 cattle from 288 pens housing 56,080 cattle were enrolled in the study. During the study period, 71 cattle died and were not sampled a second time. Approximately 7.9% (470/5,968) of arrival samples and 15.6% (918/5,897) of second samples were excluded from analysis, resulting in 10,477 samples available for analyses (5,498 arrival samples and 4,979 second samples). The majority of exclusions occurred as a result of split/mixed pens, but 1.4% (20/1,388) were excluded because of missing sample numbers and laboratory results.

Second samples were collected throughout the feeding period: 14.5% (721/4,979) were obtained between 30 and 60 DOF; 49.0% (2,441/4,979) between 61 and 90 DOF; 10.1%

(502/4,979) between 91 and 120 DOF; 17.5% (873/4,979) between 121 and 150 DOF; 6.0% (300/4,979) between 151 and 180 DOF; and 2.9% (142/4,979) at >180 DOF.

Study Population

The 5,498 cattle represented a diversity of BRD risk categories (Table 2.2). Most cattle were housed with 101 - 300 animals (59.4%, 3,267/5,498), and entered the feedlot in the summer and fall (68.6%, 3,771/5,498).

Prevalence of Mannheimia haemolytica Recovery

A total of 10,477 nasopharyngeal samples were obtained, and *M. haemolytica* was isolated from 16.6% (1,744/10,477; 95% CI, 15.9% - 17.4%). Overall, 29% of cattle (1,596/5,498) were culture-positive for *M. haemolytica* at least once, and there was significant discordance in recovery likelihood between arrival and second samples (McNemar's $P<0.001$), that is, a majority of positive cattle (90.7%; 1,448/1,596) were culture-positive only once. There was a significant increase ($P < 0.001$) in the likelihood of recovery from arrival to second sample (13.1%; 95% CI, 12.3% - 14.1% and 19.8%; 95% CI, 18.7% - 20.9, respectively).

Antimicrobial Use

All enrolled cattle that were sampled twice received tetracycline and 9.6% (477/4,979) received macrolides in-feed for liver abscess control before second sampling (Table 2.3). Parenteral drugs were given to 47.5% (2,611/5,498) of all enrolled cattle for which treatment records were available, most commonly during initial processing as metaphylaxis for respiratory disease. Tetracyclines and macrolides were the most common parenterally administered antimicrobials, with 31% (1,563/4,979) and 23% (1,158/4,979) of enrolled cattle exposed during

the study, respectively. Other parenterally administered AMDs were each given to <2% of study cattle.

Risk Factors for Recovery of of M. haemolytica

Odds of isolating *M. haemolytica* in second samples from cattle that received any parenterally administered drug ≤ 7 days preceding sample collection were about 5 times lower than for cattle that did not receive parenterally administered drugs in this same timeframe (OR, 0.2; 95% CI, 0.02 – 1.2; *P*=0.006; Table 2.4). Non-treated enrolled cattle housed in a pen with cattle that received injections >7 days before sample collection were about 1.5-times more likely to be colonized with *M. haemolytica* than study cattle that did not have treated penmates (OR, 1.5; 95% CI, 1.05 – 2.2; *P*=0.02; Table 2.4). Arrival sample *M. haemolytica* status was not significantly associated with second sample *M. haemolytica* status. BRD risk status was collinear with AMD exposure and could not be modeled.

Antimicrobial Resistance

Susceptibility testing was performed on 2,989 isolates taken from 1,744 culture-positive nasopharyngeal samples. A total of 1,200 isolates were tested with only broth microdilution, 215 with only disk diffusion, and 1,574 with both methods. Over eighty-seven percent of isolates (2,623/2,989; 95% CI, 87.0% – 89.4%) were pan-susceptible. Most single-drug phenotypes exhibited crude prevalence ≤2.0%, with insufficient occurrence to support logistic regression modeling (Table 2.5). The relatively low prevalence of resistance across all drugs is also reflected in the distributions of minimum inhibitory concentration (MIC) and zone diameter data (Supplementary Tables 2.3 and 2.4). Spectinomycin, which was included only on the disk diffusion testing panel, exhibited the highest resistance prevalence at 4.5% (81/1,789), followed by tetracycline (which was tested for on both disk diffusion and broth microdilution panels)

(4.4%, 204/4,622), streptomycin (4.3%, 119/2,833), and kanamycin (3.8%, 108/2,833; Table 2.5), both of which were included only on broth microdilution panels. No AMD exposures were significantly associated with resistance to any of these 4 drugs.

A subset of the 2,989 isolates tested for susceptibility (8.6%, 415/2,989) was excluded from inferential analyses of resistant *M. haemolytica* because of missing AMD exposure information when pens were split or mixed before sampling. A small proportion of remaining isolates was multiply-resistant (5.9%;152/2,574; 95% CI, 5.1% - 6.9%), comprising 3.8% of arrival isolates (47/1,225; 95% CI, 2.9% - 5.1%) and 7.8% of second sample isolates (105/1,348; 95% CI, 6.5% - 9.4%). Combined kanamycin and streptomycin resistance was the most common multiple-resistant phenotype among all susceptibility test results at 47.1% (80/170; 95% CI, 39.7% - 54.5%; Table 2.6). Although multiple resistance was rare, odds of recovery was much more likely when penmates of sampled individuals received parenterally administered AMDs (OR, 23.9; 95% CI, 8.4 – 68.3; $P<0.001$; Table 2.7). The wide CI for this estimate indicates a predictable lack of precision given the relatively rare occurrence of multiple resistance. Parenteral AMU in sampled cattle also was associated with increased odds of recovering multiply-resistant *M. haemolytica* from second samples, but was collinear with parenteral exposure in penmates; individual exposures were removed from the model because of a weaker magnitude of effect on recovery of multiply-resistant *M. haemolytica*. Multiple-resistance status was not associated with BRD risk status, pen size, or diagnosis of systemic illness or fever.

Morbidity and Mortality

Among cattle for which treatment records were available, 7% (401/5,498) were diagnosed with systemic illness requiring treatment, 19% of which were febrile at arrival

(75/401), 50% of which became systemically ill and febrile while in the feedlot (200/401), and 31% of which were identified as ill at some point during the feeding period but were not febrile (126/401). Of the 401 sick cattle, 41% (164/401) were diagnosed as ill <10 days after sample collection, the majority (95.7%; 157/164) <10 days after arrival sampling.

Among cattle for which treatment records were available, approximately 1.3% died during the study (71/5,498), with 21% (15/71) attributed to metabolic disease, 21% (15/71) to *Histophilus somni*, 13% (9/71) to lameness, 11% (8/71) to BRD, and 3% (2/71) to mucosal disease caused by bovine viral diarrhea virus. The remaining 31% (22/71) succumbed to miscellaneous causes.

M. haemolytica Isolation as a Risk Factor for Respiratory Morbidity and Mortality

Isolation of *M. haemolytica* at arrival was not a significant predictor of mortality, arrival diagnosis with systemic illness and fever, or diagnosis of systemic illness and fever later in the feeding period (Table 2.8). However, cattle that were culture-positive for *M. haemolytica* on arrival had almost twice the likelihood of being identified as systemically ill and febrile <10 days after arrival as compared to culture-negative cattle (OR, 1.7; 95% CI, 1.1 – 2.4; P=0.07).

DISCUSSION

M. haemolytica prevalence in this study was similar to that of previous reports (123,134), but recovery was significantly lower at arrival than later in the feeding period. Almost 90% of isolates were susceptible to all AMDs evaluated (21 AMDs from 9 different drug classes), and only approximately 6% were multiply-resistant. The likelihood of recovery was decreased in cattle that received AMDs parenterally, but increased in untreated cattle whose penmates received antimicrobials parenterally, which may be an indicator of contagious transmission

within pens. Additionally, parenteral AMD exposures in penmates greatly increased the odds of recovering multiply-resistant *M. haemolytica* in study subjects.

These findings are especially relevant to producers and their veterinarians because they stem from a longitudinal study conducted in commercial cattle under typical feedlot conditions. Deep nasopharyngeal sampling was done on live feedlot cattle regardless of clinical signs, as opposed to sampling post-mortem lung tissues, and therefore provides a potentially more relevant picture of *M. haemolytica* transmission dynamics. Although live animal sampling is unique, it must be noted that cattle were sampled only twice and outcomes therefore represent only a snapshot of *M. haemolytica* feedlot dynamics.

All cattle were exposed to in-feed AMDs and 50% to parenterally administered AMDs, primarily for BRD metaphylaxis. Most AMDs used in this population were macrolides and tetracyclines, while other classes were relatively infrequently used, including antimicrobials germane to AMR in *M. haemolytica* (e.g., ceftiofur). Although these AMD use patterns reflect real world practices, they can also hamper analytic analysis because of sparse data distributions for AMD exposure measures, as well as rare resistance outcomes. Given low parenteral AMU rates, randomized controlled trials may be necessary to evaluate specific hypotheses regarding the impact of use on *M. haemolytica* recovery and AMR, particularly for specific resistances that pose substantial human, animal or economic health risk. However, we believe that the observational nature of this study better reflects real world ecological impact of AMU on *M. haemolytica* recovery and AMR.

A striking finding of this study is that parenteral AMU in penmates not only modestly increased the odds of isolating any *M. haemolytica*, but also dramatically increased the likelihood of recovering rare multiply-resistant isolates (Table 2.4 and 2.7). Parenteral treatment

is a marker of disease occurrence under the management strategy used in this population, and therefore this finding could suggest that contagious spread is predicted by disease occurrence in penmates. If this is true, the use of arrival metaphylaxis in high-risk populations may be effective in controlling disease in clinically ill cattle, as well as preventing colonization of healthy penmates (129,135,136). However, it might also suggest that treatment selects for more resistant bacterial populations, which spread among penmates. Indeed, this theory is supported by the large effect of parenteral treatment on increasing the likelihood of isolating multiply-resistant *M. haemolytica*. Together, these findings suggest that metaphylaxis treatment protocols may be striking a delicate balance between the competing interests of animal health and antimicrobial resistance. This ecological impact warrants further investigation given the importance of *M. haemolytica* in feedlot cattle.

We also found that feedlot-of-origin exhibited a strong and consistent association with the 4 single-resistance outcomes that could be modeled (i.e., spectinomycin, tetracycline, kanamycin and streptomycin). If *M. haemolytica* undergoes contagious spread, we would expect resistance patterns to be strongly associated with geographic location (i.e., feedlot). Indeed, the contagious nature of *M. haemolytica* previously has been suggested based on evidence of BRD clustering within transport trucks and pens (137).

This finding also suggests that *M. haemolytica* subpopulations may undergo clonal expansion, and that resistant strains are maintained at low levels within a feedlot. Previous studies have shown a link between resistance patterns and *M. haemolytica* subtype (138). Furthermore, the significant increase in prevalence of *M. haemolytica* from arrival to second samples (13% to 20%) could suggest that phenotypic characteristics (e.g., virulence) of *M. haemolytica* might influence treatment decisions and thus transmission dynamics. For

example, arriving cattle with clinical signs might be colonized with a particularly virulent strain of *M. haemolytica*. Treatment of these cattle could then increase the likelihood that persistent *M. haemolytica* is resistant, and this resistant strain could then spread to untreated penmates, who subsequently exhibit a higher likelihood of colonization even in the absence of clinical illness. *M. haemolytica* serotypes have been shown to differ in virulence, and cattle exhibiting clinical BRD signs are more likely to be colonized with more virulent serotypes (139). Future studies should include isolate typing to gain a clearer understanding of transmission dynamics.

Published rates of multiply-resistant *M. haemolytica* range from 0% to 50%, but it is difficult to compare the prevalence found in this study, because the only identified studies used small numbers of young animals in non-commercial settings (sample sizes ranging from 4 to 27) (140–143). One recent, larger study examined samples from over 350 cattle and found an increase in multiply-resistant *M. haemolytica* from 5% to 35% between 2009 and 2011, but evaluated only isolates from cattle with terminal respiratory disease (126). As stated above, information regarding *M. haemolytica* susceptibility obtained from our study is particularly relevant to veterinarians and producers because isolates were obtained from randomly selected live cattle without considering treatment history or disease status.

Several lines of evidence indicate that isolates obtained in this study were representative of a highly susceptible bacterial population. The large majority of *M. haemolytica* isolates (88%) were pan-susceptible, there was low resistance prevalence to all drugs, and distributions of susceptibility information (MICs and zones of inhibition) were highly suggestive of a largely susceptible population. The prevalence of multiply-resistant *M. haemolytica* isolates also was much lower than indicated by other recent research (126).

In addition to a largely susceptible bacterial population, it should be noted that no associations were found between AMD exposures among enrolled cattle or their penmates, and resistance to single drugs. Furthermore, the most prevalent resistance phenotypes observed were for AMDs not used in the study population (e.g., kanamycin, streptomycin and spectinomycin). This finding is consistent with a recent study that showed no correlation between antemortem treatment regimens and resistance patterns in *M. haemolytica* recovered from necropsy lung samples (144). Together, these findings highlight the complexity of AMR and suggest that AMU practices do not necessarily impact development of AMR in a predictable manner. Furthermore, these results support the contention that decreased efficacy of BRD treatment stems from chronic and repeatedly treated BRD cases, rather than from AMU practices. The association between treatment with parenterally administered antimicrobials and recovery of multiply-resistant *M. haemolytica* deserves closer study to determine whether this relationship affects BRD control or treatment efficacy in feedlot populations.

Recovery of *M. haemolytica* from 20% of cattle after arrival was higher than expected, as was the significant increase in prevalence over time. However, recovery of *M. haemolytica* in the second sample was not associated with increased morbidity or mortality, suggesting that post-arrival colonization is more likely to be subclinical and may not be as great a concern for feedlot operators. In contrast, isolation of *M. haemolytica* on arrival was associated with a short-term, significant increase in risk of clinical illness. Thus, although prevalence of *M. haemolytica* was lower at arrival, the clinical and economic relevance of such colonization was greater. Additionally, our results showed that cattle receiving parenterally administered drugs were at decreased risk of *M. haemolytica* colonization in the short-term, a finding that supports AMU in high-risk individuals during a defined period of stress, such as arrival in the

feedlot. These findings highlights the complexity of colonization, treatment and clinical illness, and support the belief that aggravating factors such as transport and handling stress are critical for causing cattle to develop overt disease.

ENDNOTE

[a]*i*FHMS, FHMS, Okotoks, AB

[b]SAS 9.3 (SAS Institute Inc., Cary, NC).

Table 2.1. Antimicrobial Drugs used in this study population.

Antimicrobial Drug and Dosage	Primary Reason for Use	Class
Parenteral		
Ceftiofur sodium 1 mg/kg BW	BRD Treatment	Beta lactam
Ceftiofur crystalline free acid 6.6 mg/kg BW	BRD Treatment	Beta lactam
Ceftiofur hydrochloride 1.1 mg/kg BW	BRD Treatment	Beta lactam
Enrofloxacin 7.7 mg/kg BW	Relapse BRD Treatment	Quinolone
Florfenicol 40 mg/kg BW	BRD Treatment	Phenicol
Florfenicol 40 mg/kg BW & Flunixin meglumine 2.2 mg/kg BW	BRD Treatment	Phenicol
Oxytetracycline		
10 mg/kg BW	BRD Prevention/Treatment	Tetracycline
20 mg/kg BW	BRD Prevention/Treatment	Tetracycline
30 mg/kg BW	BRD Prevention/Treatment	Tetracycline
Tilmicosin 10 mg/kg BW	BRD Prevention/Treatment	Macrolide
Trimethoprim and sulfadoxine 16 mg/kg BW	BRD Treatment	Sulfonamide
Tulathromycin 2.5 mg/kg BW	BRD Prevention/Treatment	Macrolide
Tylosin tartrate 29 mg	Implant Site Abscess Prevention	Macrolide
In-Feed		
Chlortetracycline @		
35 mg/kg diet dry matter	Liver Abscess Prevention	Tetracycline
1 g/head/day	Histophilosis Prevention/Treatment	Tetracycline
3 g/head/day	Histophilosis Prevention/Treatment	Tetracycline
6 g/head/day	Histophilosis Prevention/Treatment	Tetracycline
Tylosin phosphate @ 11 mg/kg diet dry matter	Liver Abscess Prevention	Macrolide

Table 2.2. Demographics of study population.

	No. of cattle	*% of cattle*
Risk status of cattle		
Low risk	2,420	*44.0*
Medium risk	832	*15.1*
High risk	1,356	*24.7*
Very high risk	890	*16.2*
Arrival season of cattle		
Winter (Jan-Mar)	876	*15.9*
Spring (Apr-June)	851	*15.5*
Summer (July-Sept)	1,623	*29.5*
Fall (Oct-Dec)	2,148	*39.1*
Pen Size		
<101	459	*8.4*
101-200	1,858	*33.8*
201-300	1,409	*25.6*
301-400	1,173	*21.3*
>400	599	*10.9*

Table 2.3. Drug use before the time of second sampling, by class.

Drug Class	Total ADD's	*% of ADD's*	*No. of Cattle Exposed*	*% of Cattle Exposed*
Parenteral Betalactam	211	*0.4*	73	*1.5*
Parenteral Quinolone	57	*0.1*	19	*0.4*
Parenteral Phenicol	81	*0.2*	27	*0.5*
Parenteral Macrolide	3,166	*5.7*	1,158	*23.3*
Parenteral Sulfonamide	51	*0.1*	17	*0.3*
Parenteral Tetracycline	4,540	*8.2*	1,563	*31.4*
In-feed Tetracycline	47,178	*85.2*	4,979	*100.0*
In-feed Macrolide	63	*0.1*	477	*9.6*
Total	55,346			

[a]ADD=Animal Daily Dose, defined as the number of days that a single treatment remains in the target tissue(s) at therapeutic concentrations.

Table 2.4. Risk factors associated with the isolation of *M. haemolytica* in second samples.

Predictor	Level	Odds Ratio	95% Confidence Interval	p-value
Parenteral drugs given to sampled individual within 7 days of sample collection	any exposure	0.16	(0.02 – 1.23)	0.006
	no exposure	Reference	Reference	Reference
Parenteral drugs given to penmates of sampled individual at least 7 days prior to sample collection	any exposure	1.52	(1.05 – 2.19)	0.023
	no exposure	Reference	Reference	Reference
Pen Size	*confounded*	*confounded*	*confounded*	*confounded*

Table 2.5. Crude prevalence of resistance of *M. haemolytica* isolates (n=2,989).[a]

Resistance Phenotype	No. of isolates	*% (95%CI)*[d]
Pan-Susceptible	2,623	*87.8 (87.0-89.4)*
Amikacin[b]	3	*0.1 (0.0 – 0.3)*
Amoxicillin-clavulanate[c]	34	*0.7 (0.5 – 1.0)*
Ampicillin[c]	70	*1.5 (1.2 – 1.9)*
Cefoxitin[b]	5	*0.2 (0.1 – 0.4)*
Ceftiofur[c]	2	*0.0 (0.0 – 0.2)*
Ceftriaxone[b]	1	*0.0 (0.0 – 0.2)*
Chloramphenicol[b]	0	*0.0 (0.0 – 0.1)*
Ciprofloxacin[b]	0	*0.0 (0.0 – 0.2)*
Enrofloxacin[d]	1	*0.0 (0.0 – 0.3)*
Florfenicol[d]	2	*0.1 (0.0 – 0.4)*
Gentamicin[c]	0	*0.0 (0.0 – 0.1)*
Kanamycin[b]	108	*3.8 (3.2 – 4.6)*
Nalidix acid[b]	4	*0.1 (0.0 – 0.3)*
Streptomycin[b]	119	*4.2 (3.5 – 5.0)*
Sulfonamide[b]	12	*0.4 (0.2 – 0.8)*
Spectinomycin[d]	81	*4.5 (3.7 – 5.6)*
Danofloxacin[d]	35	*2.0 (1.4 – 2.7)*
Tilmicosin[d]	5	*0.3 (0.1 – 0.7)*
Tulathromycin[d]	2	*0.1 (0.0 – 0.4)*
Tetracycline[c]	204	*4.4 (3.9 – 5.1)*
Trimethoprim-sulfadiazine[c]	9	*0.2 (0.1 – 0.4)*

[a]Isolates can be listed more than once if they were multiply resistant; 1,574 isolates were tested by both broth microdilution and disk diffusion, 1,200 isolates were tested by only broth microdilution, and 215 isolates tested only by disk diffusion, for a total of 2,833 test results from broth microdilution and 1,789 from disk diffusion (4,622 total test results).
[b]Tested by broth microdilution only.
[c]Tested by both broth microdilution and disk diffusion.
[d]Tested by disk diffusion only.
[d]Adjusted CI for binomial proportions (adding 2 successes and 2 failures) were estimated as previously described (131)

Table 2.6. Most common phenotypes among multiply-resistant isolates (n=152).

Frequency of Resistance Phenotype[a]	*% (95% CI)*[b]	Phenotype
80	*47.1 (39.7 – 54.5)*	Kanamycin, Streptomycin
11	*6.5 (3.6 – 11.4)*	Ampicillin, Amoxicillin-Clavulanate
8	*4.7 (2.3 – 9.2)*	Kanamycin, Streptomycin, Tetracycline
8	*4.7 (2.3 – 9.2)*	Ampicillin-Clavulanate, Tetracycline
7	*4.1 (1.9 – 8.5)*	Spectinomycin, Danofloxacin
7	*4.1 (1.9 – 8.5)*	Spectinomycin, Danofloxacin, Tetracycline
6	*3.5 (1.5 – 7.7)*	Kanamycin, Streptomycin, Ampicillin-Clavulanate
25	*16.4 (10.5 – 22.4)*	25 other multiply-resistant phenotypes

[a]From a total of 32 multiply-resistance phenotypes; the phenotypes listed had a frequency of >2% among
multiply-resistant *M. haemolytica* isolates.
[b]Adjusted CI for binomial proportions (adding 2 successes and 2 failures) were estimated as previously described.[9]

Table 2.7. Final multivariable model for risk factors associated with recovery of multiply-resistant *M. haemolytica* in second sample (multiply-resistant vs. singly-resistant or susceptible).

Predictor	Level	Odds Ratio	95% Confidence Interval	p-value
Parenteral drugs given to penmates of sampled individual at any time prior to sample collection	any exposure	23.9	(8.4 – 68.3)	<0.0001
	no exposure	Reference	Reference	
Arrival Season	Fall (Oct-Dec)	1.2	(0.5 – 3.1)	0.07
	Summer (July-Sept)	0.6	(0.2 – 1.9)	
	Spring (Apr-June)	0.2	(0.1 – 1.0)	
	Winter	Reference	Reference	

Table 2.8. Odds ratio, 95% confidence interval[a], and p-value for *a priori* risk factors of respiratory morbidity and mortality.

Risk Factor	Outcome							
	Mortality at any time during the feeding period		Diagnosis of fever[b] on arrival		Diagnosis of fever[b] after arrival, at any time in feeding period		Diagnosis of fever[b] after arrival, within 10 days after sample collection	
M. haemolytica status of arrival sample								
Negative	Reference		Reference		Reference		Reference	
Positive	1.1 *(0.3 – 2.9)*	0.93	1.4 *(0.7 – 1.7)*	0.38	1.2 *(1.0 – 1.6)*	0.24	1.7 *(1.1 – 2.4)*	0.07
Risk status of cattle								
Low risk	Reference	0.03	Reference	0.008	Reference	<0.001	Reference	<0.001
Medium risk	3.5 *(0.4 – 90.2)*		2.9 *(2.1 – 7.0)*		1.7 *(1.7 – 3.2)*		2.1 *(1.5 – 4.0)*	
High risk	13.7 *(2.2 – 285.7)*		2.7 *(1.4 – 4.3)*		5.6 *(4.1 – 7.8)*		2.5 *(1.5 – 4.1)*	
Very high risk	3.8 *(0.4 – 111.7)*		0.5 *(0.2 – 0.9)*		2.7 *(1.8 – 3.8)*		0.3 *(0.1 – 0.6)*	
Arrival season of cattle								
Winter (Jan-Mar)	Reference	0.21	Reference	0.09	Reference	0.002	Reference	0.002
Spring (Apr-June)	0.3 *(0.01 – 2.7)*		5.2 *(2.2 – 11.4)*		1.3 *(0.6 – 1.7)*		1.2 *(0.5 – 2.5)*	
Summer (July-Sept)	0.3 *(0.03 – 2.0)*		2.3 *(0.9 – 4.7)*		2.5 *(1.4 – 3.0)*		1.2 *(0.5 – 1.0)*	
Fall (Oct-Dec)	1.3 *(0.3 – 5.2)*		2.7 *(1.1 – 5.2)*		3.9 *(2.5 – 5.1)*		3.1 *(1.6 – 5.1)*	
Pen Size								
<101	Reference	0.23	Reference	0.38	Reference	0.24	Reference	0.97
101-200	3.1 *(0.54– 56.6)*		1.2 *(0.6 – 2.7)*		1.7 *(1.2 – 2.8)*		1.0 *(0.5 – 2.0)*	
201-300	1.6 *(0.1 – 37.0)*		0.4 *(0.1 – 1.5)*		1.6 *(1.0 – 2.8)*		1.0 *(0.5 – 2.4)*	
301-400	0.4 *(0.02 – 15.4)*		1.8 *(0.9 – 6.1)*		1.6 *(1.0 – 2.9)*		0.8 *(0.4 – 2.2)*	
>400	1.6 *(0.2 – 92.6)*		0.6 *(0.3 – 2.5)*		2.8 *(1.6 – 5.2)*		0.9 *(0.3 – 2.7)*	

[a]95% CI represent likelihood ratio-based confidence interval
[b]Systemic illness with fever (bovine respiratory disease)

Supplemental Table 2.1. Interpretive criteria (minimum inhibitory concentrations [ug/ml]) for *Mannheimia haemolytica* susceptibility testing using broth microdilution

Antimicrobial	Susceptible	Intermediate	Resistant	Reference
Amikacin	≤16	32	≥64	*CLSI M100-S22, 2012
Ampicillin	≤0.5	-	-	CLSI M45-A2, 2010
Amoxicillin-Clavulanic acid	≤0.5/0.25	-	-	CLSI M45-A2, 2010
Cefoxitin	≤8	16	≥32	*CLSI M100-S22, 2012
Ceftiofur	≤2	4	≥8	CSLI M31-A4, 2013
Ceftriaxone	≤1	2	≥4	*CLSI M100-S22, 2012
Chloramphenicol	≤8	16	≥32	*CLSI M100-S22, 2012
Ciprofloxacin	≤1	2	≥4	*CLSI M100-S21, 2011
Gentamicin	≤4	8	≥16	*CLSI M100-S22, 2012
Kanamycin	≤16	32	≥64	*CLSI M100-S22, 2012
Nalidixic Acid	≤16	-	≥32	*CLSI M100-S22, 2012
Streptomycin	≤32	-	≥64	NARMS Executive Report 2009
Sulfisoxazole	≤256	-	≥512	*CLSI M100-S22, 2012
Tetracycline	≤2	4	≥8	CLSI M31-A4, 2013
Trimethoprim-Sulfamethoxazole	≤0.5/9.5	-	-	CLSI M45-A2, 2010

* Interpretive criteria for *E. coli* used in lieu of *M. haemolytica* since CLSI does not define breakpoints for *M. haemolytica* and these antimicrobial drugs. CLSI = Clinical and Laboratory Standards Institute.

Supplemental Table 2.2. Interpretive criteria (inhibition zone diameters [mm]) for *Mannheimia haemolytica* susceptibility testing using disk diffusion

Antimicrobial	**[Disk] (µg)**	**Susceptible**	**Intermediate**	**Resistant**	**Reference**
Ampicillin	10	≥27	-	-	CLSI M45-A2, 2010
Amoxicillin-Clavulanic acid	20/10	≥27	-	-	CLSI M45-A2, 2010
Ceftiofur	30	≥21	18-20	≤17	CLSI M31-A3, 2008
Danofloxacin	5	≥22	-	-	CLSI M31-A3, 2008
Enrofloxacin	5	≥21	17-20	≤16	CLSI M31-A4, 2013
Florfenicol	30	≥19	15-18	≤14	CLSI M31-A4, 2013
Gentamicin	10	≥15	-	≤12	Catry et al., 2007
Spectinomycin	100	≥14	11-13	≤10	CLSI M31-A3, 2008
Sulfisoxazole	300	≥17	13-16	≤12	CLSI M31-A3, 2008
Tetracycline	30	≥23	-	-	CLSI M45-A2, 2010
Tilmicosin	15	≥14	11-13	≤10	CLSI M31-A4, 2013
Trimethoprim-Sulfamethoxazole	1.25/23.75	≥24	-	-	CLSI M45-A2, 2010
Tulathromycin	30	≥18	15-17	≤14	CLSI M31-A4, 2013

CLSI = Clinical and Laboratory Standards Institute.

Supplemental Table 2.3. Minimum inhibitory concentrations for *Mannheimia haemolytica* isolates recovered from deep nasopharyngeal swabs obtained from feedlot cattle (n=2,833 isolates)

MIC[a] (µg/ml)	Amikacin	[b]Amoxicillin-Clavulanate	Ampicillin	Cefoxitin	Ceftiofur	Ceftriaxone	Chloramphenicol	Ciprofloxacin	Gentamicin	Kanamycin	Nalidixic Acid	Streptomycin	Sulfonamide	Tetracycline	[c]Trimethoprim-Sulfamethoxazole
0.016								2804 (99.0%)							
0.031								23 (0.8%)							
0.063								3 (0.1%)							
0.125					2781 (98.2%)			0 (0%)							2820 (99.5%)
0.25					21 (0.7%)	2805 (99.0%)		2 (0.1%)	30 (1.1%)						1 (0.04%)
0.5	30 (1.1%)			2493 (88%)	11 (0.4%)	16 (0.6%)		1 (0.04%)	6 (0.2%)		105 (3.7%)				4 (0.1%)
1	2 (0.1%)	2824 (99.7%)	2800 (98.8%)	318 (11.2%)	8 (0.3%)	9 (0.3%)		0 (0%)	216 (7.6%)		1600 (56.5%)				3 (0.1%)
2	5 (0.2%)	6 (0.2%)	6 (0.2%)	7 (0.2%)	6 (0.2%)	2 (0.1%)	2823 (99.6%)		2449 (86.4%)		1113 (39.3%)				0 (0%)
4	32 (1.1%)	0 (0%)	1 (0.04%)	9 (0.3%)	4 (0.1%)	0 (0%)	9 (0.3%)		132 (4.7%)		10 (0.4%)			2755 (97.2%)	5 (0.2%)
8	1753 (61.9%)	0 (0%)	3 (0.1%)	1 (0.04%)	1 (0.04%)	0 (0%)	1 (0.04%)		0 (0%)	2535 (89.5%)	0 (0%)			16 (0.6%)	0 (0%)
16	995 (35.1%)	0 (0%)	5 (0.2%)	0 (0%)	1 (0.04%)	0 (0%)	0 (0%)			189 (6.7%)	1 (0%)		1771 (62.5%)	13 (0.5%)	
32	13 (0.5%)	0 (0%)	13 (0.5%)	3 (0.1%)		0 (0%)				1 (0.04%)	1 (0%)	2714 (95.8%)	451 (15.9%)	47 (1.7%)	
64	3 (0.1%)	3 (0.1%)	5 (0.2%)	2 (0.1%)		1 (0.04%)				108 (3.8%)	3 (0.1%)	12 (0.4%)	423 (14.9%)	2 (0.1%)	
128	0 (0%)					0 (0%)						107 (3.8%)	88 (3.1%)		
256													88 (3.1%)		
512													12 (0.4%)		

[a] Minimum inhibitory concentration obtained from broth microdilution assays; smaller values represent greater susceptibility. Lowest MIC values represent the lowest concentration tested and actual MIC values could be at or below (≤) this value. Highest MIC values represent MIC results that were > than the 2nd highest value, and actual MIC values could be any value greater than the 2nd highest value.

[b] Values for amoxicillin–clavulanate refer to amoxicillin concentrations (clavulanate was included in wells at half of the amoxicillin concentration).

[c] Values for trimethoprim–sulfamethoxazole represent trimethoprim concentrations (sulfamethoxazole was included in wells at 19 times the concentration of trimethoprim).

Supplemental Table 2.4. Results of disk diffusion susceptibility testing of *Mannheimia haemolytica* isolates recovered from deep nasopharyngeal swabs obtained from feedlot cattle (n=1,789 isolates)

Diameter of Inhibition Zone (mm)[a]	Ampicillin	Amoxicillin-Clavulanate	Ceftiofur	Danofloxacin	Enrofloxacin	Florfenicol	Gentamicin	Oxytetracycline	Spectinomycin	Tilmicosin	Trimethoprim-Sulfamethoxazole	Tulathramycin
50 to 55	0 (0%)	0 (0%)	5 (0.3%)	0 (0%)	1 (0.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
45 to 50	3 (0.2%)	0 (0%)	44 (2.5%)	4 (0.2%)	6 (0.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
40 to 45	67 (3.7%)	40 (2.2%)	366 (20.5%)	48 (2.7%)	52 (2.9%)	3 (0.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	26 (1.5%)	0 (0%)
35 to 40	524 (29.3%)	552 (30.9%)	777 (43.4%)	275 (15.4%)	351 (19.6%)	80 (4.5%)	0 (0%)	8 (0.4%)	0 (0%)	0 (0%)	520 (29.1%)	1 (0.1%)
30 to 35	897 (50.1%)	903 (50.5%)	568 (31.7%)	775 (43.3%)	907 (50.7%)	557 (31.1%)	0 (0%)	311 (17.4%)	35 (2%)	2 (0.1%)	1016 (56.8%)	66 (3.7%)
25 to 30	285 (15.9%)	292 (16.3%)	28 (1.6%)	292 (16.3%)	469 (26.2%)	1022 (57.1%)	150 (8.4%)	1301 (72.7%)	663 (37.1%)	32 (1.8%)	218 (12.2%)	881 (49.2%)
20 to 25	1 (0.1%)	2 (0.1%)	1 (0.1%)	88 (4.9%)	2 (0.1%)	125 (7%)	1407 (78.6%)	29 (1.6%)	944 (52.8%)	1100 (61.5%)	6 (0.3%)	830 (46.4%)
15 to 20	2 (0.1%)	0 (0%)	0 (0%)	104 (5.8%)	0 (0%)	0 (0%)	232 (13%)	22 (1.2%)	32 (1.8%)	641 (35.8%)	1 (0.1%)	9 (0.5%)
10 to 15	5 (0.3%)	0 (0%)	0 (0%)	135 (7.5%)	0 (0%)	0 (0%)	0 (0%)	64 (3.6%)	29 (1.6%)	10 (0.6%)	1 (0.1%)	2 (0.1%)
6 to 10[b]	5 (0.3%)	0 (0%)	0 (0%)	68 (3.8%)	1 (0.1%)	2 (0.1%)	0 (0%)	54 (3%)	86 (4.8%)	4 (0.2%)	1 (0.1%)	0 (0%)

[a] Diameters of zones of inhibition for susceptibility measured with disk diffusion assays; larger values represent greater susceptibility.
[b] Disks impregnated with antimicrobial drugs were 6 mm in diameter, which is therefore the minimum possible size of inhibition

CHAPTER 3: MODELING CONSIDERATIONS IN THE ANALYSIS OF ASSOCIATIONS BETWEEN ANTIMICROBIAL USE AND RESISTANCE IN BEEF FEEDLOT CATTLE

SUMMARY

A number of sophisticated modeling approaches are available to investigate potential associations between antimicrobial use (AMU) and resistance (AMR) in animal health settings. All have their advantages and disadvantages, making it unclear as to which model is most appropriate. We used advanced regression modeling to investigate AMU-AMR associations in faecal generic *Escherichia coli* (NTSEC) isolates recovered from 275 pens of feedlot cattle. Ten modeling strategies were employed to investigate AMU associations with resistance to chloramphenicol, ampicillin, sulfisoxazole, tetracycline and streptomycin. Goodness-of-fit statistics did not show a consistent advantage for any one model type. Three AMU-AMR associations were significant in all models. Recent parenteral tetracycline use increased the odds of finding tetracycline-resistant NTSEC (odds ratios [OR] ranged from 1.1 to 3.2); recent parenteral sulfonamide use increased the odds of finding sulfisoxazole-resistant NTSEC (ORs ranged from 1.4 to 2.5); and recent parenteral macrolide use decreased the odds of recovering ampicillin-resistant NTSEC (ORs ranged from 0.03 to 0.2). Other results varied dramatically depending on the modeling approach, emphasizing the importance of exploring and reporting multiple modeling methods based on a balanced consideration of important factors such as study design, mathematical appropriateness, research question and target audience.

INTRODUCTION

Antimicrobial resistance (AMR) is a leading public health concern, causing significant morbidity and mortality, and increased healthcare-related costs (145). Antimicrobial use (AMU) in food animals has been posited as a driver of AMR by national and international

governing agencies (146), however studies in this area have produced ambiguous results (18–22). The significance of AMU in animals on AMR outcomes in humans is difficult to quantify (30–32,147,148). Despite best efforts to address this knowledge gap, substantial logistical challenges remain with respect to designing and conducting commercial field studies to quantify associations between AMU and AMR in food animals. Such challenges include uniquely identifying and tracking animals over time, maintaining and accessing detailed health records for individual animals, and obtaining biological samples longitudinally from a sufficient number of these uniquely identified animals.

Even studies that have overcome these challenges have produced inconsistent results (18,19,22), including differences in detecting associations between tetracycline use and resistance in beef cattle feedlots (20,21), and differences in the association between tetracycline resistance and use of chlortetracycline and oxytetracycline in swine (149). One reason for such differential results may stem from differences in laboratory and analytical methods. Potential bench-side differences include the use of multiple resistance testing methods that may produce different results (e.g., disk diffusion vs. broth microdilution) (150), a lack of host- and agent-specific breakpoints for interpretation of resistance results, and debate as to which indicator bacterial species best represent resistance dynamics in food animals. Analytically, the datasets resulting from large representative studies of AMU-AMR in commercial settings present several challenges including accounting for multiple levels of non-independence (i.e., clustering), accounting for time of sampling, validating quantification of antimicrobial drug (AMD) exposures, and classifying resistance outcomes when there are many isolates within a sample, and one can use multiple testing methods for resistance characterization.

This report is part of a larger study that aimed to overcome many of these obstacles with the goal of developing a longitudinal AMR surveillance system for use in beef feedlot cattle (127). The purpose of this investigation was to estimate resistance prevalence in isolates of non-type-specific *E. coli* (NTSEC) recovered from faeces of beef feedlot cattle, to model associations between AMU and AMR in these isolates using various modeling strategies, and to compare these strategies in order to understand how they may differentially impact measures of association between AMU and AMR.

MATERIALS AND METHODS

Study Overview

Pens of cattle (N=300) in four feedlots in Alberta, Canada were enrolled in the study and composite pen-floor faecal samples were collected at the beginning of the feeding period, and at least once again during the remainder of the feeding period. Samples were cultured for NTSEC and isolates were susceptibility tested using broth microdilution (BM) and disk diffusion (DD). Antimicrobial exposures for all cattle in enrolled pens were tracked throughout the study period. The prevalence of recovery of AMR NTSEC was estimated, various methods of multivariable logistic regression were used to investigate associations between AMU and AMR in NTSEC isolates, and model results were compared.

Sample Collection

Bacterial isolates were collected as part of a project intended to develop and evaluate methods for surveillance of AMR in beef feedlot cattle, the details of which have been reported elsewhere (127). Sample collection ran from September 2007 to January 2010, during which time feedlot collaborators enrolled 30% of all arriving pens using a randomization table. Pens were sampled when filled to capacity, and at least once again later during the feeding period. Twenty

fresh tablespoon-size faecal samples from each pen floor were collected in a standard spatial pattern and then mixed together for 1 minute, and 10 g was removed for further processing. All cattle handling and sampling procedures were approved by the Animal Care Committee of Feedlot Health Management Services Ltd. (FHMS) and the Institutional Animal Care and Use Committee of Colorado State University.

Susceptibility Testing

Faecal samples were cultured for NTSEC, and up to five isolates from each sample were selected for susceptibility testing, which was performed using both automated DD (BioMIC®) and BM (Sensititre® panel type: CMV1AGNF). The antimicrobial panels differed between the two test methods, as they were selected independently for surveillance purposes. An automated visual imaging system recorded zone diameter from DD tests, while laboratory personnel recorded the minimum inhibitory concentration (MIC) from BM tests. All susceptibility testing was conducted in accordance with standards established by the Clinical Laboratory Standards Institute (151–155). Details about quality control assessments and interpretive criteria have been previously described in detail (127).

Antimicrobial Use Data

Feedlot personnel used a chute-side, customized health information system (iFHMS© software) to record all parenteral and in-feed AMD treatments for all cattle in enrolled pens from the day of arrival until the day that the last sample was collected [Table 3.1]. Records were subsequently exported for analyses, and included individual animal and pen identification, AMD type, dosage, and route and date(s) of administration. For analysis, antimicrobial doses were converted to units of animal defined daily dose (ADD), a metric that defines the number of days that a single dose remains in the recipient's target tissue(s) at therapeutic concentrations (156).

This standardization enables comparison of AMD exposures across varying sizes of cattle with varying antimicrobial dosages. To aggregate AMD exposures at the group (pen) level, each treatment was converted to ADDs, multiplied by both the number of animals exposed and the duration of treatment (in days), and then summed by AMD class (betalactams, phenicols, quinolones, sulfonamides, tetracyclines and macrolides). The AMD categories were further subdivided into the route of administration as being in-feed or parenteral.

Multivariable modeling to estimate adjusted prevalence of resistance

Adjusted prevalence of resistance among individual isolates was estimated from least-square means obtained from Poisson regression modeling. Estimates were stratified by the number of days that cattle had been in the feedlot when faecal samples were collected: 0-3 days-on-feed (DOF), 4-70, 71-120, 121-180, and >180 DOF. This enabled us to adjust resistance prevalence relative to DOF, and to determine if DOF was a statistically significant predictor of AMR levels. Cut-offs for DOF categorization were chosen based on the likelihood of disease occurrence and therefore AMU at different phases of the feeding period, as well as the distribution of collected samples. Generalized estimating equations (GEE) with an exchangeable correlation structure were used to control for clustering of isolates within samples. For isolates that were susceptibility tested using both BM and DD (repeated measures), we included only BM results in order to meet the assumption of independence between observations. Pen size and feedlot could potentially confound resistance prevalence, and thus were included as fixed effects.

Multivariable modeling to test for associations between use and resistance

A variety of multivariable modeling techniques were used to analyze potential associations between AMU and AMR in NTSEC isolates. The primary outcome of interest in

all models was the antimicrobial susceptibility status of NTSEC isolates, defined dichotomously as either resistant or non-resistant, the latter of which included intermediate and susceptible classifications. In order to maintain temporal logic for associations between AMU and AMR, only AMDs given prior to sample collection were included in these analyses, and therefore only isolates recovered from non-arrival samples were interrogated. Resistance status for each of the 19 AMDs included on the test panels was evaluated in parallel in separate models.

The primary independent variables of interest were exposures to AMDs, which were categorized and summed as described above. Despite the inherently continuous nature of pen-level AMD data, class-specific distributions for AMDs were strongly right-skewed and zero-inflated. We attempted to assess linearity using quadratic terms for AMD exposures, as well as by modeling deciles and quintiles of AMD exposures in order to compare parameter estimates with the logit of the resistance outcome. However, these models either would not converge or the Hessian matrix was not positive definite, and linearity could not be formally assessed. Therefore, AMD exposures were modeled as both continuous and categorical variables and model results were compared [Table 3.3].

. For categorization, parenteral AMD exposures were dichotomized ("no exposure" vs. "any exposure"), while in-feed exposures were categorized into four levels: no exposure, low exposure ($<25^{th}$ percentile), medium exposure (25^{th}-89^{th} percentile) and high exposure ($\geq 90^{th}$ percentile). To investigate the impact of temporality on the association between AMD exposures and AMR, we grouped AMD exposures based on the time of sample collection as either recent or non-recent, i.e., AMDs administered ≤6 d prior to sample collection and those administered ≥7 d prior to sample collection.

Two variables were included in all models as potential confounders: number of animals housed in the pen from which the composite faecal sample was obtained ("pen size") and DOF. Pen size was modeled as a 5-level ordinal variable due to non-linearity with the logit of the outcome (<101, 101-200, 201-300, 301-400, and >400 animals). The DOF variable exhibited a linear relationship with the logit of the outcome and was modeled as both a continuous and an ordinal variable (0-3, 4-70, 71-120, 121-180, and >180 DOF) for comparison purposes.

Population and study design factors created numerous issues related to data hierarchy, clustering and repeated measures. Resistance outcomes could be clustered within feedlot (n=4 feedlots), within pens (n=275 pens), and within samples, as multiple isolates were collected from each sample (n=564 samples). In addition, repeated measures were present at two levels: first, pens were sampled multiple times throughout the feeding period; and second, a subset of NTSEC isolates were tested by two different resistance testing methods.

Because there are a number of equally valid modeling approaches to analyze such data, we used and compared a variety of methods. In all model types, feedlot was included as a categorical fixed effect due to the small number of feedlots and the fact that none of the model predictors were considered to be feedlot-level effects. GEE with alternating logistic regression (ALR) was used to account for clustering of isolates within samples as well as repeat susceptibility testing on some isolates (132). For these models, sample identification number was specified as the repeated subject with an exchangeable correlation structure and susceptibility testing method was specified as the subcluster with a 1-nested log odds ratio structure. When data sparseness did not support GEE with ALR modeling (i.e., the correlation matrix was not positive definite or parameter estimates were unrealistically large), GEE without ALR was used (157), and only BM results were analyzed to avoid issues of non-independence.

Finally, generalized linear mixed modeling (GLMM) with random effects and Laplace estimation (158) was used to control for multiple levels of clustering. When model convergence was achieved, isolates, samples and pens were specified as random effects. When convergence could not be attained on all three levels of clustering, only susceptibility results from BM were used in the analysis and separate models specifying pens and samples as random effects were compared. To facilitate comparison of model results, subject-specific (SS) parameter estimates from GLMM models were converted to population-averaged (PA) parameter estimates using the equation (157):

$$\beta^{PA} \approx \frac{\beta^{SS}}{\sqrt{1 + 0.346\sigma_h^2}}$$

The modeling decisions made at the outset of this analysis, combined with the limitations imposed by model convergence and stability, yielded 10 permutations of modeling methods (models A-J) [Table 3.3].

These included various combinations of model type (GEE with ALR vs. GEE vs. GLMM), clustering specification, and methods for quantification of DOF and AMD exposures (continuous vs. categorical).

The same approach to model development was used for all 10 modeling methods (A-J). First, univariable screening models were used to analyze associations between each class of AMD exposure (n=8) and every resistance outcome (n=19 AMDs for models using both BM and DD, and n=15 AMDs for models using only BM results). The AMD exposures were split into recent and non-recent exposures as described above. Each screening model included fixed effect variables for feedlot, DOF, and pen size. The AMD exposure variables with a *P*-value ≤0.20 for

either recent or non-recent exposures were included in the initial candidate multivariable model. Backward elimination was used to refine multivariable models using a critical alpha for retention of 0.05 with recent and non-recent AMD exposures considered independently. Variables exhibiting confounding upon stepwise removal (defined as >20% change in any parameter estimate) were added back to the model. Once all variables met the critical alpha value, confounding was reassessed and variables were removed if confounding was no longer present.

Models for each resistance outcome were assessed independently using each of the modeling methods (A-J), resulting in development of 139 different multivariable models [Table 3.3 and Table 3.4].

. Model fit was evaluated using Akaike's Information Criterion (AIC) for GLMM models, and quasi-information criterion (QIC) for GEE models.

RESULTS

A total of 300 pens were sampled at least once. Twenty-five pens were excluded from analysis due to missing exposure or resistance information, resulting in 275 pens for use in multivariable analyses that ranged in size from 27 to 555 head (median =168). Of these 275 pens, 23% (64/275) were sampled once during the feeding period, 48% (132/275) twice, and 29% (79/275) three times, resulting in collection of 564 composite faecal samples, from which a total of 2,911 NTSEC isolates were cultured. Almost all isolates were tested using BM (2,903/2,911), while 41% (1,192/2,911) were tested by DD, for a total of 4,095 susceptibility test results for analysis. Of the 564 composite faecal samples, 40% (226/564) were collected between zero and three DOF, another 39% (214/564) between four and 120 DOF, and 22% (124/564) later in the feeding period (median = 152 DOF, range 121 – 244 DOF).

Prevalence of resistance to most AMDs was too low for valid estimation of least-square means for adjusted prevalence estimates [Table 3.4]. Only six of the 19 resistances tested attained crude prevalence >2%, namely ampicillin, chloramphenicol, streptomycin, sulfisoxazole, tetracycline and florfenciol [Figure 3.1]. DOF was significantly associated with resistance levels for all modeled AMDs except ampicillin [Figure 3.1].

Antimicrobial Drug Use

The most commonly used AMD was in-feed tetracycline, which was administered to all pens of cattle in this study population; however, because some of the pens were only sampled at arrival, and not again later in the feeding period, only 83% of pens were exposed prior to collection of the "last" sample [Table 3.2]. Pen-level prevalence of exposure to many parenterally administered AMD classes was below 50% [Table 3.2], and even among exposed pens, distribution of AMD exposures had a strong right skew because most pens contained only one or two exposed animals, while a handful of pens received pen-wide metaphylactic treatment. Over 96% of all AMD exposures occurred ≥7 d prior to sample collection and were classified as "non-recent". This was consistent across all AMD classes with the exception of in-feed macrolides, of which 31.3% of ADDs were classified as "recent", i.e., administered ≤6 d with respect to sample collection. This disparity stems from the AMD protocols utilized in the 4 study feedlots.

Results of Multivariable Modeling

For AMDs with resistance prevalence sufficient to support multivariable modeling [Table 3.5] we found several statistically significant associations between AMU and AMR. However, most of these associations were not consistent between different model specifications. Details of comparisons are given below.

Effect of Quantifying AMD Exposures as Continuous vs. Categorical Variables

The quantification of AMD exposures as continuous or categorical variables exerted the strongest influence over model results, best illustrated in the results for chloramphenicol resistance [Table 3.6]. When AMD exposures were quantified as continuous variables (models A, B and I), only non-recent parenteral macrolide exposures were significantly associated with an increase in the odds of isolating chloramphenicol-resistant NTSEC. However, this association was not seen when modeling the AMD exposures as categorical variables (models F and G), and instead there was a positive association with non-recent in-feed tetracycline and parenteral quinolone exposures, as well as a negative association with recent parenteral quinolone exposures. Quantification of AMD exposures produced a similar effect when modeling streptomycin resistance, as models in which exposures were treated continuously contained a different significant association than models in which exposures were treated categorically [Table 3.7, models B, D and I versus E, F and G, respectively].

Effects of Different Modeling Strategies

While GEE, GEE/ALR and GLMM are all valid methods for regression modeling of clustered data, our results show that choice of modeling strategy can result in substantial differences with respect to which subset of AMD exposures exhibit significant association with resistance outcomes, as seen in the case of ampicillin resistance [Table 3.8]. Models A and B indicate that recent parenteral exposures to macrolides, recent in-feed exposures to tetracyclines and recent in-feed exposures to macrolides were associated with decreased odds of recovering ampicillin-resistant NTSEC, while non-recent exposures to in-feed tetracycline were associated with increased odds. However, model I suggests that only the association between recent parenteral macrolide exposures and ampicillin resistance was statistically significant. The only

difference between these models was that models A and B used GEE for clustered data, while model I used GLMM.

Effects of clustering

In a few instances, the hierarchical level of non-independence accounted for in model specifications exerted a strong influence on model results, for example in the results of models F and G for sulfisoxazole resistance [Table 3.9]. These models were identical except that model F included a random effect at the pen level and model G at the sample level. This change resulted in vastly different final multivariable models, with model G showing only one significant association between AMD exposure and sulfisoxazole resistance, and model F showing four significant associations.

Effect of Modeling DOF as a Categorical vs. Continuous Variable

It is often considered best practice to model inherently continuous data as continuous. However, this may not be possible due to violation of the assumption of linearity, or categorization may be preferred in order to interpret model results in context of real-world practices. We modeled DOF as both a continuous and categorical variable and found that this difference, while less impactful than other modeling decisions, can substantially change model results [Table 3.10].

Models A and B for tetracycline resistance are specified identically except DOF is categorical in model A and continuous in model B. Model A identified only one statistically significant association between AMD exposures and resistance, while model B identified four [Table 3.10].

Model Comparisons

Despite large variability in models results, three AMU-AMR relationships were detected in all models. Recent exposures to parenteral tetracycline and sulfonamide were positively associated with odds of isolating NTSEC resistant to tetracycline and sulfisoxazole, respectively [Table 3.9 and 3.10], while recent parenteral macrolide exposures were associated with decreased odds of ampicillin-resistant NTSEC [Table 3.8].

The point estimates for ORs for tetracycline resistance with respect to the median increase of recent parenteral tetracycline exposures ranged from 1.12 to 1.19 for models in which exposures were modeled as continuous variables, and from to 3.12 to 3.23 for models in which exposures were categorized [Table 3.10]. Different model types (GEE/ALR, GEE, or GLMM) produced very similar odds ratios if the method for quantifying AMD exposures was the same (models A – D and J, respectively), despite the fact that only BM test results were used in GEE models and BM and DD susceptibility test results were used in GEE/ALR and GLMM models. Similar patterns emerged from model results for the association between recent parenteral sulfonamide exposure and resistance to sulfisoxazole [Table 3.9].

Results differ somewhat for the association between recent parenteral macrolide exposure and ampicillin resistance [Table 3.8]. Across all models, recent parenteral macrolide administration demonstrated a negative association with ampicillin resistance, with the magnitude of this effect strongest in GEE models in which macrolide exposures were quantified as a continuous variable (models A, B and I).

Model fit statistics were not useful in identifying an optimal modeling strategy across all resistance outcomes, as no single model type consistently exhibited better goodness-of-fit [Table 3.11]. Among GEE models, model E – in which AMD exposures were categorized – had the

lowest QIC for three of five resistances analyzed, suggesting that categorization improved model fit. However, among GLMM models, treatment of AMD exposures as a continuous variable (i.e., model I) resulted in the lowest AIC for three of five resistances analyzed. Interestingly, model G consistently exhibited lower AIC than model F, suggesting that all else being equal, sample-level clustering resulted in better model fit than pen-level clustering. Surprisingly, models H and J had a higher AIC than models F, G and I; the former models specified random effects at the pen, sample and isolate levels, while the latter models specified random effects at only one level. This finding could stem from decreased model parsimony due to inclusion of additional random effects, as marginal AIC statistics do not account for random effects and therefore their inclusion can decrease goodness-of-fit (159).

DISCUSSION

These results highlight the inherent complexity in modeling AMU-AMR associations from real-world data. Despite this complexity, we have uncovered several important relationships between AMU and AMR outcomes in faecal NTSEC. The direction and magnitude of these relationships remained consistent despite changes in modeling technique, suggesting that they are likely true drivers of NTSEC resistance in this study population. Specifically, use of parenteral tetracyclines and sulfonamides increased the odds of recovering NTSEC resistant to tetracyclines and sulfisoxazoles, respectively, while use of parenteral macrolides decreased the odds of recovering ampicillin-resistant NTSEC. Importantly, these three relationships apply only to AMD given within one week prior to sample collection, suggesting that shifts in the most predominant resistance phenotypes are short-lived (i.e., reverts back to a susceptible populations), or that other changes to the microbiota eventually "crowd out" these resistant populations. In addition, the shortest withdrawal times for injectable

tetracyclines, sulfonamides and macrolides are currently 18, 5 and 18 days, respectively. Given these withdrawal times and the short-lived relationship between exposures to these AMDs and AMR, it is questionable whether or not such use plays a significant role in dissemination of resistance through the food chain.

The three associations described above are consistent and easily interpretable; the rest of our results, however, highlight the crucial role that modeling decisions play in model results, the most obvious example being quantification of AMD exposures. In some instances, categorization of exposures produced models with wholly different subsets of significant predictors when compared to models in which exposures were not categorized. Given the highly skewed distribution of AMD exposures across pens, we believe that categorization is the more legitimate method for modeling AMU data. We attempted to evaluate linearity both by adding the quadratic term for AMD exposures and by modeling AMD exposures as quintiles and graphing resulting parameter estimates against the logit of the outcome. Unfortunately, these models either would not convergence or lacked of a positive definite Hessian matrix, and therefore we were unable to formally evaluate linearity. Ideally, goodness-of-fit statistics would provide generalizable guidelines for model selection. However, our results show that the "best fit" model depends greatly on the resistance outcome being modeled [**Table 3.**].

The issue of quantification applied to confounders as well as variables of primary interest. Our analysis accounted for DOF as a known confounder of the AMU-AMR relationship (21). The question is how to best account for DOF, i.e., as a categorical or continuous variable, and does this make a difference? Our results uncovered instances in which even this seemingly minor decision produced differential model results with respect to the set of significant predictors in the final model [Table 3.10, models A and B].

Clustering of subjects and samples occurs frequently in agriculture production settings (160) and modelers can choose from several techniques to account for this. Ideally, these techniques would result in similar model results; however, we have shown this is not always the case. GLMM may produce a final model with a wholly different subset of significant predictors than GEE, as seen in the final model results for tetracycline resistance in which AMDs were categorized [Table 3.10]. While this is not surprising, it is an important finding given that often times model selection is based on necessity rather than choice, for instance when data are sparse and mixed models do not converge. Furthermore, choice of modeling technique can be based on haphazard criteria such as researcher preference.

The role of timing of AMD exposures in driving or mitigating resistance is made clear in these results, and in some cases, there is a strong dichotomy in this regard. Recent parenteral quinolone exposures decrease the odds of recovering chloramphenicol-resistant NTSEC, while non-recent parenteral quinolone exposures strongly increase these odds [Table 3.6]. Multiple factors could be driving this finding, including co-selection of resistance genes on plasmids as well as microbial population dynamics in which the short-term, dramatic alteration of the microbiome caused by exposure to quinolone creates a long-term niche for chloramphenicol-resistant NTSEC bacterial subpopulations, perhaps due to the presence of unrelated traits. Other studies have found similar interactions between timing of antimicrobial exposure and development of resistance (78), and together these results highlight the importance of accounting for timing in models of AMU/AMR. We divided AMD exposures at 7 days relative to sampling; however, other schemes may be just as legitimate and should be based on treatment protocols employed in the study and/or target population.

The veterinary community has long recognized the difficulties of researching AMU/AMR in production settings. The analysis presented here is founded on a unique database in which many of these difficulties were overcome, including linkage of individual AMD exposures with susceptibility test results. The enrollment of 300 pens provided sufficient power to uncover associations that may have remained hidden in smaller studies and the longitudinal sampling design allowed exploration of resistance changes over time. Despite these advancements, the complex nature of AMU and AMR renders interpretation of results challenging.

The findings presented here add another layer of complexity by demonstrating that modeling decisions greatly impact model results, especially with respect to the set of significant predictors in the final model. Which results are we to believe? For example, some AMU-AMR associations were seen across all model types, while others were present in less than 25% of models assessed. Does this mean that the latter associations have less real-world applicability? Hill's criteria of causation state that consistency of results across different studies, times, circumstances and populations strengthens the evidence for a causal relationship (161), suggesting that those AMU-AMR associations identified in all models are likely causally linked. In addition, Hill's criterion of plausibility is readily apparent in the associations between sulfonamide exposures and sulfisoxazole resistance and tetracycline use and tetracycline resistance, lending more weight to the causal nature of these relationships. The negative association between macrolide use and ampicillin resistance is less directly understood, and further research is needed to understand microbiome-wide population genetic dynamics that may be influencing this relationship.

Importantly, however, most model results were not consistent, and the question then becomes how to determine which inconsistently identified AMU-AMR associations to consider valid. From a technical perspective, the most valid model is that which most appropriately handles the data structure and provides best data fit. However, there is often a gap between theory and application, and choice of modeling technique is not always dictated solely by mathematical appropriateness. Often, factors such as study design, the ability of the model to converge given sparse data, the need for a population-averaged estimate, and even researcher preference will weigh heavily in the decision (133). Therefore, while perhaps unsurprising, the finding that modeling technique can substantially change the set of significant predictors in the final model is disconcerting, particularly when the results have potential ramifications for public health and policy decisions, such as the case with AMU and AMR in livestock production. It is therefore our opinion that stakeholders in the AMU-AMR debate deserve to see results of all legitimate models, and that decisions about which model results to act on should be based on a careful assessment of the relative risk, the consistency and the plausibility of each AMU-AMR relationship being analyzed.

Table 3.1. Antimicrobial drugs used in this population.

AMD and Dosage	Primary Reason for Use	Class	ADD
Parenteral			
Ceftiofur sodium 1 mg/kg BW	BRD Treatment	Beta lactam	1
Ceftiofur crystalline free acid 6.6 mg/kg BW	BRD Treatment	Beta lactam	3
Ceftiofur hydrochloride 1.1 mg/kg BW	BRD Treatment	Beta lactam	1
Enrofloxacin 7.7 mg/kg BW	Relapse BRD Treatment	Quinolone	3
Florfenicol 40 mg/kg BW	BRD Treatment	Phenicol	3
Florfenicol 40 mg/kg BW & Flunixin meglumine 2.2 mg/kg BW	BRD Treatment	Phenicol	3
Oxytetracycline			
10 mg/kg BW	BRD Prevention/Treatment	Tetracycline	1
20 mg/kg BW	BRD Prevention/Treatment	Tetracycline	2
30 mg/kg BW	BRD Prevention/Treatment	Tetracycline	3
Tilmicosin 10 mg/kg BW	BRD Prevention/Treatment	Macrolide	3
Trimethoprim and sulfadoxine 16 mg/kg BW	BRD Treatment	Sulfonamide	1
Tulathromycin 2.5 mg/kg BW	BRD Prevention/Treatment	Macrolide	3
Tylosin tartrate 29 mg	Implant Site Abscess Prevention	Macrolide	1/275
In-Feed			
Chlortetracycline @			
35 mg/kg diet dry matter	Liver Abscess Prevention	Tetracycline	1/18[a]
1 g/head/day	Histophilosis Prevention/Treatment	Tetracycline	1/6
3 g/head/day	Histophilosis Prevention/Treatment	Tetracycline	1/2
6 g/head/day	Histophilosis Prevention/Treatment	Tetracycline	1
Tylosin phosphate @ 11 mg/kg diet dry matter	Liver Abscess Prevention	Macrolide	1/80[a]

ADD = Animal Defined Daily Dose; BW = body weight; BRD = Bovine Respiratory Disease
[a] Assuming 9 kg of dry matter intake per individual animal per day

Table 3.2. Pen-level exposure to AMDs, by class.

AMD Class	No. *(%)* of pens exposed prior to collection of last composite faecal sample	Median (interquartile range) ADD's per pen upon collection of last composite faecal sample[a]
Parenteral Betalactam	164 *(60%)*	9 (4-17)
Parenteral Quinolone	77 *(28%)*	6 (3-12)
Parenteral Phenicol	55 *(20%)*	3 (3-12)
Parenteral Macrolide	141 *(51%)*	21 (6-57)
Parenteral Sulfonamide	116 *(62%)*	4 (3-9)
Parenteral Tetracycline	153 *(56%)*	46 (8-418)
In-Feed Tetracycline	229 *(83%)*	1,002 (615-2,042)
In-Feed Macrolide	96 *(35%)*	17 (9-32)

[a]Among pens receiving at least 1 ADD of AMD prior to collection of last composite faecal sample

Table 3.3. Model specifications for all 10 models used to analyze associations between AMU and AMR in NTSEC isolates.

Model	Modeling Technique[a]	Test Results Included in Analysis	Clusters Accounted for in Analysis	Quantification of Days-on-Feed	Quantification of AMD Exposures
A	GEE	BM	Multiple isolates per sample	categorical	continuous
B	GEE	BM	Multiple isolates per sample	continuous	continuous
C	GEE/ALR	BM & DD	Multiple tests per isolate Multiple isolates per sample	categorical	continuous
D	GEE/ALR	BM & DD	Multiple tests per isolate Multiple isolates per sample	continuous	continuous
E	GEE	BM	Multiple isolates per sample	continuous	categorical
F	GLMM	BM	Multiple samples per pen	continuous	categorical
G	GLMM	BM	Multiple isolates per sample	continuous	categorical
H	GLMM	BM & DD	Multiple tests per isolate Multiple isolates per sample Multiple samples per pen	continuous	categorical
I	GLMM	BM	Multiple isolates per sample	continuous	continuous
J	GLMM	BM & DD	Multiple tests per isolate Multiple isolates per sample Multiple samples per pen	continuous	continuous

GEE = Generalized estimating equations
GEE/ALR = Generalized estimating equations with Alternating Logistic Regression
GLMM = Generalized linear mixed modeling
BM = broth microdilution
DD = disk diffusion

Table 3.4. Crude prevalence of antimicrobial resistance in NTSEC isolates (for antimicrobials with prevalence that would not support multivariable modeling of adjusted prevalence of estimates)[e].

Resistance Phenotype	No. of Resistant Isolates	% (95% CI)[d]
Amikacin[a]	0	0.0 (0.0 – 0.0)
Amoxicillin-clavulanate[b]	10	0.3 (0.1 – 0.5)
Cefoxitin[a]	4	0.2 (0.0 – 0.4)
Ceftazidime[c]	1	0.3 (0.0 – 0.5)
Ceftiofur[a]	9	0.3 (0.1 – 0.4)
Ceftriaxone[a]	3	0.1 (0.0 – 0.3)
Ciprofloxacin[a]	0	0.0 (0.0 – 0.0)
Enrofloxacin[c]	2	0.3 (0.0 – 0.7)
Gentamicin[b]	5	0.2 (0.1 – 0.4)
Kanamycin[a]	31	1.1 (0.8 – 1.5)
Nalidixic acid[a]	41	1.5 (1.0 – 1.9)
Neomycin[c]	7	0.8 (0.3 – 1.2)
Trimethoprim-sulfadioxine[b]	73	1.8 (1.4 – 2.2)

[a]Tested by broth microdilution only (n=2,903)
[b]Tested by broth microdilution and disk diffusion (n=1,192)
[c]Tested by disk diffusion only (N=4,095)
[d] Adjusted CI for binomial proportions (adding 2 successes and 2 failures) were estimated as previously described (131).
[e]Adjusted prevalence of resistance for other drugs can be found in Figure 3.1.

Table 3.5. Results of modeling process for all antimicrobial resistance outcomes.

Resistance Outcome	Model									
	A	B	C	D	E	F	G	H	I	J
Amikacin	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Ampicillin	✔	✔	--	--	✔	✔	✔	--	✔	--
Amoxicillin-Clavulanate	--	--	--	--	--	--	--	--	--	--
Cefoxitin	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Ceftazidine	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Ceftiofur	--	--	--	--	--	--	--	--	--	--
Ceftriaxone	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Chloramphenicol	✔	✔	n/n[b]	n/n[b]	✔	✔	✔	n/n[b]	✔	n/n[b]
Ciprofloxacin	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Enrofloxacin	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Florfenicol	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Gentamicin	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Kanamycin	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Nalidixic Acid	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Neomycin	--	--	n/n[b]	n/n[b]	--	--	--	n/n[b]	--	n/n[b]
Streptomycin	✔*	✔	✔*	✔	✔	✔	✔	--	✔	--
Sulfisoxazole	✔	✔	✔	✔	✔	✔	✔	--	✔	--
Tetracycline	✔	✔	✔	✔	✔	n/n[a]	n/n[a]	✔	n/n[a]	✔
Trimethoprim-Sulfamethoxazole	--	--	--	--	--	--	--	--	--	--

✔ model converged and results are presented

✔*model converged, but no results are presented because no AMD exposures were statistically significant in final model

-- model would not converge

n/n[a]: model not needed because models with random effects at all levels did converge

n/n[b]: model not needed because resistance was only tested with one susceptibility test (i.e., no repeated measures on isolates).

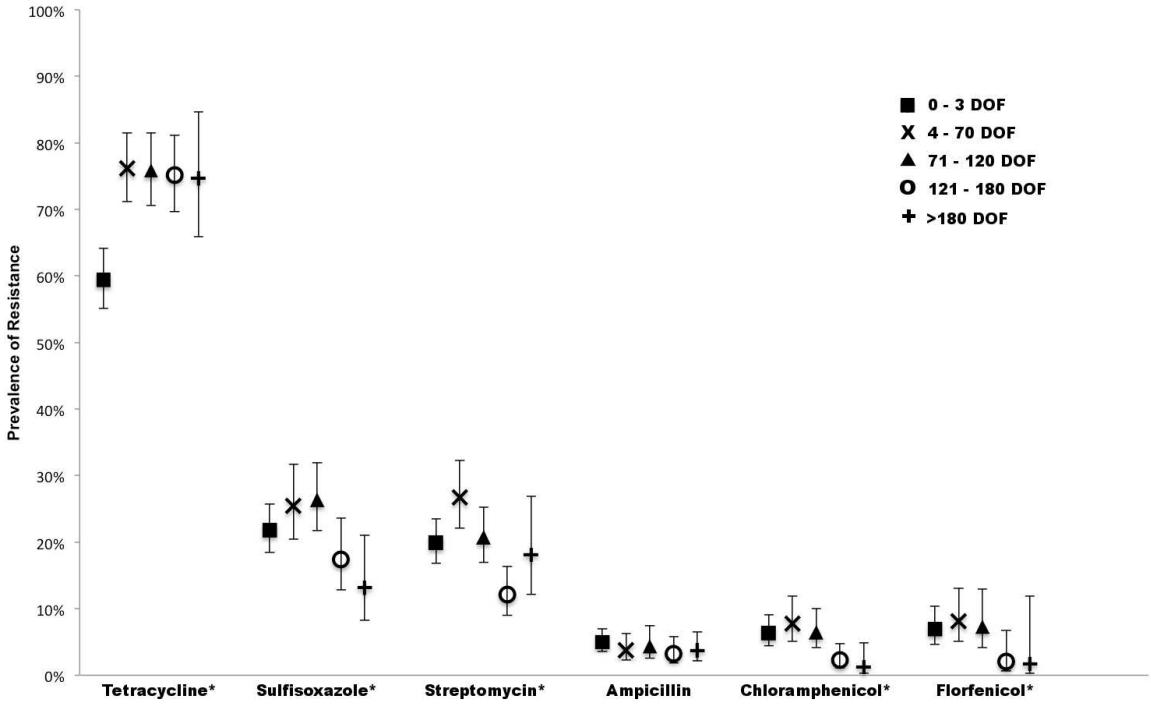

Figure 3.1. Adjusted[a] prevalence of resistance in NTSEC isolates for six AMDs.
[a]Adjusted for days-on-feed at sample collection, as well as pen size and feedlot. Clustering of isolates within samples was controlled for using multivariable GEE with a Poisson distribution.
*indicates AMDs for which there were statistically significant ($p<0.05$) differences in resistance prevalence by DOF.

Table 3.6. Comparison of multivariable model results (population-averaged odds ratio and 95% confidence interval) for the outcome of chloramphenicol resistance.

	Models in which AMD exposures were treated as continuous variables				Models in which AMD exposures were treated as categorical variables	
Variable	Model A[a]	Model B[a]	Model I	Exposure Category	Model F[b]	Model G[b]
Recent[c] parenteral quinolone exposure	[e]	[e]	[e]			
				Unexposed	REF	REF
				Exposed	0.16 *(0.02 – 0.52)*	0.22 *(0.00 – 0.79)*
Nonrecent[d] parenteral quinolone exposure	[e]	[e]	[e]			
				Unexposed	REF	REF
				Exposed	2.34 *(1.49 – 6.18)*	1.97 *(1.17 – 10.46)*
Nonrecent[d] parenteral macrolide exposure	1.04 *(1.02 – 1.06)*	1.03 *(1.01 – 1.06)*	1.07 *(1.04 – 1.09)*		[e]	[e]
Nonrecent[d] in-feed tetracycline exposure	[e]	[e]	[e]			
				Unexposed	REF	REF
				Low	1.18 *(0.69 – 2.67)*	1.29 *(0.53 – 4.87)*
				Medium	2.03 *(1.36 – 9.92)*	2.48 *(1.04 – 27.3)*
				High	3.80 *(2.71 – 50.51)*	6.66 *(2.95 – 366)*

[a]OR and 95% CI represent the change in odds of resistance for a median increase in ADD's for the pertinent exposure.
[b]OR and 95% CI have been converted from a subject-specific to a population-averaged estimate.
[c]Recent exposures are those that occurred ≤ 6 days prior to sample collection.
[d]Nonrecent exposures are those than occurred ≥7 days prior to sample collection.
[e]Variable was not significant in the final multivariable model, and therefore results are not shown.
REF = reference category

Table 3.7. Comparison of multivariable model results (population-averaged odds ratio and 95% confidence interval) for the outcome of streptomycin resistance.

	Models in which AMD exposures were treated as continuous variables				Models in which AMD exposures were treated as categorical variables		
Variable	Model B[a]	Model D[a]	Model I[a,b]	Exposure Category	Model E	Model F[b]	Model G[b]
Recent[c] in-feed tetracycline exposure	1.04 *(1.02 – 1.07)*	1.03 *(1.02 – 1.07)*	1.05 *(1.00 – 1.09)*		[e]	[e]	[e]
Nonrecent[d] in-feed tetracycline exposure	[e]	[e]	[e]		[e]		[e]
				Unexposed		REF	
				Low		1.48 *(1.09 – 2.14)*	
				Medium		1.85 *(1.20 – 3.12)*	
				High		2.46 *(1.26 – 5.45)*	
Nonrecent[d] in-feed macrolide exposure	[e]	[e]	[e]				
				Unexposed	REF	REF	REF
				Low	1.55 *(0.88 – 2.72)*	1.59 *(0.99 – 2.71)*	1.47 *(0.80 – 2.87)*
				Medium	0.66 *(0.43 – 1.02)*	0.70 *(0.43 – 1.07)*	0.57 *(0.32 – 0.93)*
				High	2.37 *(1.06 – 5.32)*	2.29 *(1.12 – 5.27)*	2.17 *(0.83 – 6.30)*

[a]OR and 95% CI represent the change in odds of resistance for a median increase in ADD's for the pertinent exposure.
[b]OR and 95% CI have been converted from a subject-specific to a population-averaged estimate.
[c]Recent exposures are those that occurred ≤ 6 days prior to sample collection.
[d]Nonrecent exposures are those than occurred ≥7 days prior to sample collection.
[e]Predictor was not significant in the final multivariable model, and therefore results are not shown.
REF = reference category

Table 3.8. Comparison of multivariable model results (population-averaged odds ratio and 95% confidence interval) for the outcome of ampicillin resistance.

	Models in which AMD exposures were treated as continuous variables			Models in which AMD exposures were treated as categorical variables			
Predictor	Model A[a]	Model B[a]	Model I[a,b]	Exposure Category	Model E	Model F[b]	Model G[b]
Recent[c] parenteral macrolide exposures	0.05 *(0.00 – 0.70)*	0.06 *(0.00 – 0.74)*	0.03 *(0.00 – 1.15)*				
				Unexposed	REF	REF	REF
				Exposed	0.12 *(0.03 – 0.48)*	0.18 *(0.05 – 0.65)*	0.11 *(0.02 – 0.72)*
Recent[c] in-feed tetracycline exposures	0.53 *(0.29 – 0.94)*	0.75 *(0.54 – 1.04)*	[e]		[e]	[e]	[e]
Non-recent[d] in-feed tetracycline exposures	1.30 *(1.09 – 1.55)*	1.30 *(1.09 – 1.42)*	[e]		[e]	[e]	[e]
Recent[c] in-feed macrolide exposures	0.40 *(0.21 – 0.75)*	0.59 *(0.36 – 0.97)*	[e]		[e]	[e]	[e]

[a]OR and 95% CI represent the change in odds of resistance for a median increase in ADD's for the pertinent exposure.
[b]OR and 95% CI have been converted from a subject-specific to a population-averaged estimate.
[c]Recent exposures are those that occurred ≤ 6 days prior to sample collection.
[d]Nonrecent exposures are those than occurred ≥7 days prior to sample collection.
[e]Predictor was not significant in the final multivariable model, and therefore results are not shown.
REF = reference category

Table 3.9. Comparison of multivariable model results (population-averaged odds ratio and 95% confidence interval) for the outcome of sulfisoxazole resistance.

	Models in which AMD exposures were treated as continuous variables						Models in which AMD exposures were treated as categorical variables		
Predictor	Model A[a]	Model B[a]	Model C[a]	Model D[a]	Model I[a,b]	Exposure Category	Model E[b]	Model F[b]	Model G[b]
Recent[c] parenteral sulfonamide exposures	1.45 *(1.19 – 1.76)*	1.50 *(1.21 – 1.85)*	1.38 *(0.59 – 0.88)*	1.44 *(1.20 – 1.73)*	1.52 *(1.03 – 2.26)*				
						Unexposed	REF	REF	REF
						Exposed	2.54 *(1.53 – 4.20)*	2.36 *(1.18 – 4.71)*	2.31 *(1.0 – 8.3)*
Recent[c] parenteral quinolone exposures	e	e	e	e	1.37 *(1.05 – 1.78)*		e	e	e
Non-recent[d] parenteral quinolone exposures	0.90 *(0.81 – 1.01)*	e	e	e	e		e	e	e
Recent[c] in-feed tetracycline exposures	e	e	e	e	e		e		e
						Unexposed	REF	REF	
						Low	0.61 *(0.41 – 0.90)*	0.54 *(0.38 – 0.77)*	
						Medium	0.59 *(0.37 – 0.94)*	0.56 *(0.36 – 0.86)*	
						High	0.75 *(0.37 – 1.51)*	0.78 *(0.40 – 1.51)*	
Recent[c] in-feed macrolide exposures	e	e	e	e	e		e		e
						Unexposed		REF	
						Low		1.14 *(0.61 – 2.13)*	
						Medium		0.49 *(0.26 – 0.90)*	
						High		0.26 *(0.07 – 0.91)*	
Non-recent[d] in-feed macrolide exposures	e	e	e	e	e				e
						Unexposed	REF	REF	
						Low	1.63 *(0.79 – 3.37)*	1.86 *(0.95 – 3.65)*	
						Medium	1.04 *(0.58 – 1.87)*	1.13 *(0.68 – 1.88)*	
						High	3.79 *(1.02 – 14.02)*	3.61 *(1.32 – 9.83)*	

[a]OR and 95% CI represent the change in odds of resistance for a median increase in ADD's for the pertinent exposure.
[b]OR and 95% CI have been converted from a subject-specific to a population-averaged estimate.
[c]Recent exposures are those that occurred ≤ 6 days prior to sample collection.
[d]Nonrecent exposures are those than occurred ≥7 days prior to sample collection.
[e]Predictor was not significant in the final multivariable model, and therefore results are not shown.

Table 3.10. Comparison of multivariable model results (population-averaged odds ratio and 95% confidence interval) for the outcome of tetracycline resistance.

Models in which AMD exposures were treated as continuous variables						Exposures Categories	Models in which AMD exposures were treated as categorical variables	
Predictor	Model A[a]	Model B[a]	Model C[a]	Model D[a]	Model J[a,b]		Model E[b]	Model H[b]
Recent[c] parenteral tetracycline exposures	1.14 *(1.01 – 1.29)*	1.19 *(1.06 – 1.35)*	1.12 *(1.02 – 1.24)*	1.16 *(1.05 – 1.29)*	1.14 *(1.02 – 1.27)*			
						Unexposed	REF	REF
						Exposed	3.12 *(1.94 – 5.02)*	3.23 *(1.99 – 5.25)*
Non-recent[d] parenteral tetracycline exposures	[e]	1.03 *(1.00 – 1.07)*	[e]	1.03 *(1.00 – 1.07)*	1.04 *(1.00 – 1.07)*		[e]	[e]
Non-recent[d] parenteral sulfonamide exposures	[e]	[e]	[e]	[e]	[e]		[e]	
						Unexposed		REF
						Exposed		1.53 *(1.12 – 2.12)*
Non-recent[d] parenteral quinolone exposures	[e]	0.86 *(0.75 – 0.99)*	0.87 *(0.76 – 1.00)*	0.85 *(0.74 – 0.98)*	0.83 *(0.71 – 0.97)*		[e]	[e]
Recent[c] parenteral phenicol exposures	[e]	[e]	2.99 *(1.34 – 6.65)*	3.55 *(1.50 – 8.38)*	[e]			[e]
						Unexposed	REF	
						Exposed	2.78 *(1.20 – 6.41)*	
Non-recent[d] parenteral phenicol exposures	[e]	1.13 *(1.00 – 1.28)*	[e]	[e]	1.13 *(1.00 – 1.27)*		[e]	[e]
Non-recent in-feed tetracycline exposures	[e]	[e]	[e]	[e]	[e]			[e]
						Unexposed	REF	
						Low	1.85 *(1.20 – 2.85)*	
						Medium	1.81 *(1.01 – 3.25)*	
						High	1.48 *(0.56 – 3.91)*	

[a]OR and 95% CI represent the change in odds of resistance for a median increase in ADD's for the pertinent exposure.
[b]OR and 95% CI have been converted from a subject-specific to a population-averaged estimate.
[c]Recent exposures are those that occurred ≤ 6 days prior to sample collection.
[d]Nonrecent exposures are those than occurred ≥7 days prior to sample collection.
[e]Predictor was not significant in the final multivariable model, and therefore results are not shown.
REF = reference level

Table 3.11. Comparison of final multivariable model goodness-of-fit statistics.

Model	Goodness-of-Fit Statistic[a]	Modeling Technique	Tetra-cycline	Strepto-mycin	Sulfisoxazole	Ampicillin	Chloram-phenicol
A	QIC	GEE	3323	[b]	2960	1318	1217
B	QIC	GEE	3334	2951	2975	1313	1221
C	QIC	GEE/ALR	4794	4261	4214	[b]	[b]
D	QIC	GEE/ALR	4781	[b]	4215	[b]	[b]
E	QIC	GEE	3298	2941	2970	1329	1203
F	AIC	GLMM	[b]	2889	2896	1277	1118
G	AIC	GLMM	[b]	2840	2801	1227	1055
H	AIC	GLMM	4145	[b]	[b]	[b]	[b]
I	AIC	GLMM	[b]	2846	2798	1222	1054
J	AIC	GLMM	4160	[b]	[b]	[b]	[b]

[a]QIC = Quasi-Akaike's Information Criterion; AIC = Akaike's Information Criterion.
[b]Final multivariable model results not available due to lack of model convergence.

CHAPTER 4: CHARACTERIZATION OF THE RESISTOME IN MANURE, SOIL AND WASTEWATER FROM DAIRY AND BEEF PRODUCTION SYSTEMS

INTRODUCTION

Livestock production effluent may be implicated in the transmission of antimicrobial resistant bacteria into aquatic, terrestrial and atmospheric ecosystems through uncontained wastewater runoff, lagoon seepage, land-applied manure, and airborne particulate matter (49,51–54,162). Current regulations regarding livestock manure and wastewater management practices were designed to achieve acceptable levels of organic nutrients and coliform bacteria within ground and surface waters and manured soils. Increasing evidence suggests that current management practices may not adequately consider the risk of transfer of both antimicrobial resistant bacteria and genetic determinants (ARDs) from livestock production systems to humans, particularly if there are antimicrobial drug (AMD) residues within these systems (95,96,98,99,163,164). However, AMD exposures may not be necessary for significant increases in ARD levels to be detected in soils after manure application (103,165), and some studies show that ARD increases in manure-amended soil only occur when application rates are high or manure is not properly stored (90). Furthermore, non-manured or "pristine" soil contains a diverse repertoire of ARDs, making it difficult to distinguish between "native" and anthropogenically impacted ARD content (166).

In North American cattle production systems, AMDs are used to treat, prevent and control disease, which also results in lower amount of food required per kilogram of weight gain. AMD use in extensive management settings (i.e., cow-calf production settings on pasture) is relatively low, with only 1.9% of U.S. and 2.7% of Canadian beef cows reportedly treated with AMDs (12,167). The primary reason for AMD use in beef cows is primarily to treat active

disease, such as foot infections or metritis (12,167). In contrast, >20% of cattle in U.S. feedlots receive AMDs for prevention of respiratory disease, >13% receive AMDs to treat active respiratory disease, and >70% are administered in-feed AMDs for purposes of liver abscess prevention and to optimize health and weight gain (11). Among AMDs classified as medically important (i.e., excluding ionophores) (9,168), macrolides are the most commonly administered in U.S. feedlots, both in-feed and injectable. In North American dairy production, >94% of operations administer AMDs to prevent development of mastitis during the dry period, with >97% of this use attributed to betalactam AMDs, including first- and third-generation cephalosporins (169). Pre-weaned dairy calves are most commonly treated for respiratory disease and diarrhea, with phenicols, cephalosporins, macrolides, sulfonamides, tetracyclines and aminoglycosides all being used in approximately the same proportions (169).

Given these AMD use practices and evidence that AMD residues in livestock effluent can select for elevated AMR levels, it is important to improve our understanding of how current management systems for cattle and their environment may impact AMR transmission to the public. Culture-independent methods provide an ideal method for developing this understanding, as the genetic processes that give rise to and maintain AMR in a bacterial population function within a complex microbial community and often affect multiple resistance classes in a networked fashion (170–172). Antimicrobial resistance determinants (ARDs) can be exchanged between non-pathogenic and pathogenic and distantly-related bacteria (104,173–175), and bacteria increase rates of horizontal gene transfer (HGT) of ARDs under conditions that induce the stress response, including antibiotic exposure (115,116). Therefore, studies that investigate 1 or 2 culturable organisms, taxa or resistance mechanisms may produce a myopic view of the AMR ecology within livestock waste management systems. In order to more fully understand

dynamics of AMR in this complex setting, a pan-microbial approach is needed to interrogate the entire microbial population (the microbiome) in order to characterize all aspects of antimicrobial resistance (the resistome) in livestock production waste management systems. Such an analysis would enable quantification of the risk of AMR transmittance posed by different livestock practices, and could provide a foundation for development of practical mitigation strategies for minimizing risk of ARD transfer from livestock production to aquatic, terrestrial and atmospheric ecosystems.

Despite the unique insight that can be gained from such a culture-independent approach, very little is known about the livestock production resistome, especially for dairy and beef production. The few studies that have been published are largely descriptive in nature and are restricted to rectal grab fecal samples that have been obtained from <5 individual research cattle (119,120). One reason for the dearth of larger, inferential studies is that basic information is not available to guide complex investigative efforts. For instance, estimating necessary sample size to detect differences between operations that utilize disparate AMD strategies is impossible without knowledge of normal variability of the resistome among different cattle, or in cattle raised in different settings. In addition, many factors may confound the relationship between AMD use and differences in the resistome, but to-date these potential factors have not been identified for cattle production. This makes it difficult to design well-controlled field studies to investigate strategies for modulating the livestock production resistome in desirable and public health-protecting ways.

Therefore, the goal of this study was to begin to fill this knowledge gap by providing a description of the dairy and beef production resistome in raw feces, soil and wastewater, as well as to identify factors that may commonly influence this resistome. Dairy and beef systems were

chosen in order to understand potential resistome differences between livestock production systems while precluding confounding by commodity host species (e.g., poultry and swine). In addition, a conventional and an organic dairy were chosen to investigate the potential impact of AMD use practices on the resistome. A U.S. and a Canadian feedlot were chosen to explore whether differences in diet impact the resistome of cattle production, as U.S. feedlots typically feed corn-based diets, while Canadian feedlots commonly feed barley-based diets. And finally, a cow-calf ranch was selected in order to compare resistome differences between an intensive (i.e., feedlot) and extensive (i.e., pastured) cattle production system.

MATERIALS AND METHODS

Study Overview

Fecal, soil and water samples were collected from each of the operations described above. Total DNA was extracted, sequenced and aligned to a custom database of ARD sequences. Alignments within each sample were summed by resistance gene, mechanism and class. Resistome composition, diversity, richness and abundance were compared between matrix type (feces, soil or water), production system (beef vs. dairy), cattle age (preweaned vs. adult), AMD use practices (organic vs. conventional), diet type (barley-based vs. corn-based) and management system (extensive vs. intensive).

Study population and sampling sites

The conventional and organic dairies as well as the US feedlot were located in northeastern Colorado, while the Canadian feedlot and ranch were located in southern Alberta. The conventional dairy milked 990 cows on one location at the time of sampling. Cows and calves were treated with a variety of AMDs for clinical illness and dry-off. The organic dairy milked ~16,500 mature cows daily and managed ~23,000 animals across five locations at the

time of sampling. No AMDs were used on the organic dairy. The US feedlot had a one-time holding capacity of ~90,000 head and fed cattle a steam-flaked corn-based diet. The Canadian feedlot, located in Alberta, had a one-time holding capacity of 17,000 cattle and fed a barley-based diet. Both the U.S. and Canadian feedlots administered in-feed macrolides and ionophores to all cattle. The cow-calf ranch was located at an Agriculture Canada research station in Onefour, Alberta and grazed approximately 600 cattle across several grazing locations. Pastured cattle only received AMDs for treatment of clinical illness, which was a relatively rare event.

Sample collection and processing

Feces

At each of the 5 operations described above, 2 composite fecal samples were collected from adult cattle, for a total of 10 adult fecal samples. Additionally, 2 composite fecal samples were collected at the 2 dairies (conventional and organic) from preweaned calves, resulting in 4 calf fecal samples and 14 total fecal samples for the entire study. At the cow-calf ranch, the 2 composite fecal samples were collected from adult cattle in an area where animals congregated at the time of sampling. Composite fecal samples were collected at the feedlots and dairies from 2 purposively selected pens. Feedlot pens were chosen based on slaughter schedule such that samples represented cattle that were nearly ready for slaughter (cattle had been in the feedlot for >165 days). At dairies, cattle were grouped by level of production, and the pens with the highest production were chosen for sampling. To collect composite samples, personnel walked through the selected pens or pasture area in a prescribed pattern, collecting approximately 1-2 g of feces from 20 locations. These 20 individual samples were placed in a sterile bag and thoroughly mixed by hand for 1 min, after which enough fecal material was removed to fill a sterile 50mL Falcon tube. Dairy calves were housed in individual hutches, and therefore 20 tablespoon-sized

fecal samples were collected from the ground of 20 randomly selected individual hutches and mixed together as described above.

Soil

The same feedlot and dairy pens that were sampled for feces were also sampled for soil, using the same methods as described for compositing of feces. Composite soil samples were collected at the cow-calf ranch from the same area in which fecal samples were collected, using the same methods. Soil was not collected from dairy calf hutches, and therefore a total of 10 composite soil samples were collected (2 at each operation).

Wastewater

At each operation, 2 wastewater samples were collected, for a total of 10 wastewater samples. In the feedlots and dairies, wastewater samples were collected from the holding lagoon that was closest to the pens that were sampled during fecal collection. If lagoons were separated into "high-solids" and "low-solids" holding systems, then a single sample was collected from each. At each lagoon, samples were collected near the bank from opposite sides. However, winter weather conditions at the US feedlot prevented access to the entire lagoon, and therefore both samples were collected from the same side. At the cow-calf ranch, samples were taken from opposite sides of a dugout that was used to collect free-flowing water in order to maintain a constant supply of water for cattle.

Sample processing and sequencing

Samples were placed on ice for transport from the field back to the laboratory. Fecal and soil samples were stored at -80°C until processed. Water samples were stored at 4°C for a maximum of 24 h, and then centrifuged at 15,000 x g for 20 min. The resulting supernatant was decanted and each pellet was stored in a 15mL Falcon tube at -80C° until processed.

After thawing, DNA extraction from the fecal (10g) and soil samples (10g) was performed using the Mo Bio PowerMax Soil DNA Isolation Kit, while DNA extraction from water samples (250mg) was performed using the Mo Bio PowerFecal DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to manufacturer's instructions. Extracted and purified DNA for fecal and soil samples was eluted in 400µl and 200µl of the kit elution buffer, respectively, while wastewater samples were eluted in 50µl of the kit elution buffer. After extraction, DNA concentration was measured at 260 nm using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific). Samples that were not sufficiently concentrated (defined as <20 ng/µl) were subjected to ethanol precipitation. To the final DNA sample, 1/10 volume of 3M sodium acetate, pH 5.2, was added. Two volumes of cold 100% molecular grade ethanol was added and the sample was mixed several times by inversion before incubating at -20°C for 1 hour. Samples were centrifuged at 11,000 x g for 20 minutes at 4°C. Supernatants were carefully discarded and 150 µL 70% cold ethanol was added and mixed by inversion. Samples were centrifuged a final time at 11,000 x g for 10 minutes at 4°C. Supernatants were again discarded and the DNA pellets allowed to air dry before resuspending in ¼ the original DNA volume with Solution C6 included in the Mo Bio DNA extraction kits.

After DNA extraction, 100 µl DNA of each fecal and soil sample and 30 µl DNA of each wastewater sample was delivered for single-end sequencing on the Ion Proton™ platform using the P1 chip (Thermo Fisher Scientific). Sequencing libraries were prepared according to the manufacturer's protocol.

Bioinformatics

Reads were trimmed and filtered for quality using Trimmomatic (176) in the following manner: first, the leading 3 and trailing 3 nucleotides were removed from each read, then a

sliding window of 4 nucleotides was used to remove nucleotides from the 3' end until the average Phred score across the window was at least 15. Trimmomatic's "ILLUMINACLIP" command was used to remove adapters supplied in the TruSeq3 adapter sequence file. A maximum of 2 mismatches were allowed in the initial seed, and adapter clipping occurred if a match score of 30 was reached. In addition, we specified that both reads be retained upon clipping, despite probable complete sequence redundancy, to supply more reads for downstream applications. Reads classified as host (i.e., bovine) were removed from further analysis by alignment to the UMD 3.1 *Bos taurus* genome with BWA (177). Non-host reads were then aligned to a custom non-redundant database of 3,111 unique ARD sequences compiled using CD-HIT (178) from ARG-ANNOT, Resfinder and CARD, all of which are actively-curated, AMR-dedicated databases (179–181). ARDs with >80% gene fraction (i.e., >80% of the nucleotides in the ARD sequence were covered by at least one read) were considered to be positively identified in a sample. Alignments to each positively identified ARD were then summed within each sample, and ARDs were further classified into a mechanism and class of resistance.

Data Analysis

Samples without any identified ARDs (n=4) were removed from further analysis due to an inability to normalize and ordinate such samples. For all remaining samples, the number of alignments to each positively identified ARD within each sample were summed and normalized to the total number of aligned reads within each sample using a data-driven approach based on shifts in count distributions (182,183). Because conventions for identifying unique ARDs vary between resistance classes, normalized counts were aggregated to the mechanism and class level to allow for less biased comparisons among samples.

Primary comparisons of interest included resistome differences between beef and dairy systems; conventional and organic dairy samples; calves and adult cattle; pasture and intensively-management operations; and between the Canadian (barley-based diet) and U.S. (corn-based diet) feedlots. In addition, we investigated differences in sample matrix (e.g., feces vs. soil vs. wastewater) as potential confounders of these primary comparisons.

To assess differences in ARD abundance at the mechanism and class levels while taking into account the potential for shallow sequencing depth, zero-inflated Gaussian mixture models were used to account for distinct processes for zero-count data (i.e., true absence vs. incomplete sequencing depth) (182). When possible, sample matrix was added to the model as a potential confounder. Pairwise comparisons of log-fold change in abundance between groups were conducted using limma, while adjusting for multiple comparisons using the Benjamini-Hochberg procedure and using a critical α of 0.05 (184).

Non-metric multidimensional scaling (NMDS) using Euclidean distances between Hellinger-transformation normalized counts of resistance genes, mechanisms and classes was used to ordinate samples based on resistome composition (185). Significant dissimilarity of ordination between groups was assessed using the Analysis of Similarities statistic (186).

To compare Shannon's diversity and richness across groups, Kruskal-Wallis test statistic with Nemenyi post-hoc comparisons adjusted for rank ties was used. Comparisons were made at the gene, mechanism and class levels, and differences between system (beef vs. dairy), sample matrix (feces vs. soil vs. water), dairy production type (organic vs. conventional) and feedlot diet type (corn vs. barley) were assessed separately.

RESULTS

Overall resistome composition is dominated by tetracycline resistance ARDs

Sequencing produced over 1.05 billion sequencing reads across all samples, at an average of 30.8 million reads per sample (range 14.9 – 48.9M). Trimming low-quality sequences resulted in removal of 20.9% of all reads (range 15% - 29%) and filtering of *Bos taurus* removed a further 0.41% (range 0.007% - 5.4%).

Across all samples, we identified 214,196 reads that aligned to 248 ARDs in the master non-redundant database, comprising 35 resistance mechanisms within 16 classes of resistance. The majority of these reads aligned to ARDs that confer resistance to tetracyclines (69.3%), and within this class, 98% of reads aligned to ribosomal protection proteins with the largest representation for TetQ and TetW (45.6% and 23.2% of all reads aligning to tetracycline ribosomal protection proteins, respectively). TetQ and TetW were also the most widely distributed ARD groups across all samples. Of the 30 samples containing ARDs, 27 contained TetQ and 22 contained TetW. Interestingly, these ARD groups were also the most prevalent in fecal samples collected from over 1,000 humans as part of two large metagenomic studies (Forslund *et al.*, 2013; Hu *et al*., 2013), suggesting that they may be common in both agricultural and human populations. Studies across diverse agricultural ecosystems also document the ubiquity of tetracycline resistance genes (187,188).

Reads aligning to the macrolide-lincosamide-streptogramin (MLS) class of antimicrobials comprised 11.1% of all ARD-aligned reads. Among MLS-aligned reads, 60.0% aligned to macrolide efflux pumps, 21.7% to Erm 23s rRNA methyltransferases, and 16.4% aligned to lincosamide nucleotidyltransferases. The mefA efflux pump was the most frequently identified

mechanism within the macrolide class, with 96.8% of all reads aligning to macrolide efflux pumps.

Few ARDs were identified in samples collected from the cow-calf ranch

No ARDs were identified in the soil and wastewater samples collected from the cow-calf ranch (n=4). This finding is unexpected given previous reports of a diverse and abundant resistome in soils and water not impacted by human activity (61,189). However, these studies utilized functional metagenomics and/or PCR to detect ARDs. Such assays may be more sensitive than shotgun metagenomics, and indeed it is likely that deeper sequencing would have revealed the presence of ARDs in the pasture soil and water samples. However, sequencing depth was relatively even across samples, and therefore the difference in ARD identification between the pasture and feedlot/dairy water and soil samples suggests that extensive rearing methods (including less confined rearing environments, lower stocking density, and much lower AMD use) may result in lower ARD levels compared to more intensive rearing methods. Alternatively, soil and wastewater ARDs on ranches may not be as geographically concentrated and therefore more samples may be needed to gain a representative portrait of the ranch environmental resistome. However, soil samples were collected from areas where cattle commonly congregated, and water was collected from a dugout, and therefore these samples should represent the resistome of locations where ARDs were likely to be most concentrated. In addition, the two fecal samples collected from the pasture contained 16 ARDs total, compared to 24, 35, 34 and 90 in the conventional dairy, organic dairy, US feedlot and Canadian feedlot, respectively, again suggesting that extensive rearing promotes presence of fewer ARDs overall. Previous studies have documented a significantly lower level of integrons (which commonly carry ARDs) in grass-fed compared to lot-fed cattle, although the content of the integrons did not

differ substantially between the two groups (190). Culture-based studies also document lower levels of resistance in generic *Escherichia coli* isolated from beef cows on pasture compared to feedlot cattle (17–19,28). However, there are many other differences between these populations, including genetics, sex and age of the cattle, and therefore more controlled studies are needed to determine whether differences in both phenotypic and genotypic resistance levels are due to AMD use practices or other factors.

Resistome composition and diversity differ between calves and adult cattle

The resistome of fecal samples collected from preweaned dairy calves was different from the fecal resistome of mature cattle of all types (Stress < 0.10 and ANOSIM $P < 0.05$, Figure 4.1A). Shannon's diversity and richness at the gene, mechanism and class levels were all lower in adult cattle feces compared to calf feces (Kruskal-Wallis $P = 0.02$, 0.02 and 0.04 for richness and $P = 0.02$, 0.09 and 0.07 for Shannon's diversity, respectively). While low sample numbers precluded making formal statistical comparisons of the resistome characteristics of feces collected from dairy calves and adult dairy cows, descriptive analysis suggests that this overall pattern of resistome diversity was not impacted by the potentially confounding effects of production system (i.e., beef versus dairy), as the calf feces, both organic and conventional, contained many more resistance mechanisms than feces from adult dairy cattle (Figure 4.2). Previous studies in calves have documented significant changes in fecal microbial diversity and composition during the pre-weaning period of calf development (191,192), and these changes could be driving resistome composition. However, a comparison of the microbiome of mature versus pre-weaned cattle has yet to be conducted, and furthermore it is unknown whether patterns of change in resistome diversity parallel those of the microbiome. While some studies have documented tight correlation between resistome and microbiome composition, others have

reported a decoupling of the two (104,105). Interestingly, the dairy calves that were reared according to certified organic practices (including absence of antimicrobial treatments) exhibited an abundant and diverse resistome, suggesting that factors other than AMD use are at play. Similar findings have been reported in 6-month old infants that were unexposed to AMDs (193).

Trimethoprim ARDs and aminoglycoside acetyltransferase ARDs were identified in calf feces, but not in mature cattle feces. This finding could stem from use practices on the conventional dairy in this study, which administered trimethoprim-sulfadiazine and sulfamethazine to calves for treatment of scours, but did not administer any AMDs of these classes to adult dairy cattle. In addition, aminoglycoside adenyltransferases and phosphotransferases were found in higher abundance in calf than adult feces (Figure 4.1B). These findings concur with culture-based results in generic *E. coli*, which found higher prevalence of resistance to trimethoprim-sulfamethoxazole and the aminoglycosides streptomycin, kanamycin and gentamycin in beef calves compared to their corresponding dam (18). However, aminoglycosides were not used in either dairy operation in this study, and therefore AMD use practices do not directly explain these findings. We did not identify any tetracycline major facilitator superfamily (MFS) ARDs in calf feces, but we did in adult cattle feces, and macrolide efflux pumps and lincosamide nucleotidyltransferases were significantly more abundant in adult compared to calf feces. Interestingly, lincosamides were utilized to treat mastitis on the conventional dairy farm in this study, which could account for the increased lincosamide resistance in relation to the preweaned calves.

Comparison of the resistomes of conventionally- and organically-reared calves may shed light on the role of AMD use practices in driving resistome differences. In this study population, organically raised calves did not receive any AMDs, while the conventional dairy operation

utilized betalactams (including cephalosporins), florfenicols, tetracyclines, macrolides and quinolones for the treatment of calf respiratory disease, trimethoprim-sulfadiazine and sulfamethazine for the treatment of scours, and macrolides for prophylaxis against respiratory disease. Low sample number precluded a formal comparison of the resistomes of organic and conventional calf fecal samples, but descriptive results show that the two fecal samples collected from conventionally raised dairy calves contained slightly more unique ARD mechanisms (29/30) than those from organically raised dairy calves (25/30, Figure 4.2). Among the 29 ARD mechanisms identified in feces from conventionally raised calves, 20 were identified in both samples, while 7 of the 25 identified in feces from organically raised calves were identified in both samples. These findings suggest that the resistome of organically raised calves may contain fewer unique ARDs and at lower frequency than the resistome of conventionally raised calves; however, it is important to note that the resistome of organically raised calves contained a diverse set of ARDs, despite the naiveté of these calves to AMDs. Culture-based studies of generic *E. coli* isolated from preweaned calf feces support the difference in AMR in conventional and calf feces, but larger sample numbers are needed to confirm this finding across the entire microbiome (194–197). Due to the stark difference in calf feces versus mature cattle feces, including presence of many unique resistance mechanisms and classes, calf samples were removed from further analyses to enable a direct comparison of adult cattle feces.

The beef feedlot resistome differs from the dairy resistome

The resistome compositions from samples collected in beef vs. dairy management settings were significantly different when compared across all sample matrices (ANOSIM $P < 0.05$, Figure 4.5A). However, Shannon's diversity and richness indices were not significantly different between beef and dairy. In order to understand system differences in isolation from

potential effects of sample matrix, we attempted to ordinate samples separately for feces, soil and wastewater. Within fecal samples, ordination showed clear separation of beef (feedlot and pasture) and dairy resistomes (Figure 4.5B); analysis of soil and wastewater resistomes could not be compared between systems due to data sparseness.

Of the 10 classes of ARDs identified among soil, wastewater and fecal samples collected from adult cattle, 8 differed in abundance when compared between dairy and beef samples. Of these, 6 were more abundant in beef samples versus dairy, while 2 were more abundant in dairy samples. Due to low overall abundance of some of these classes, however, estimates of differences in abundance may not be reliable and therefore we will restrict this discussion to ARD classes present in at least 10 of the 30 soil, wastewater and adult fecal samples; this list comprised resistance to AMD classes tetracyclines, MLS, aminoglycosides, betalactams, and general-purpose mechanisms. Tetracycline ARDs (the most abundant class overall) were more abundant in feedlots than in dairies. Aminoglycoside acetyltransferases and phosphotransferases, which were also relatively abundant, were significantly more abundant in dairy samples. These differences could reflect differential AMD use practices in feedlots and dairies. Although the American Association of Bovine Practitioners strongly discourages dairy and beef veterinarians from administering aminoglycosides to cattle, nationwide surveys suggest that they are still being used with some frequency in dairies (169), but not beef feedlots (11). In addition, tetracyclines are reportedly used more frequently by U.S. feedlots than U.S. dairies, both in-feed and parenterally (11,169). However, there are many other factors that could account for these differences, including frequency of pen cleaning (which could influence levels of mixing between soil and feces), lagoon construction and management (which could impact the lagoon resistome), and feed composition (which could affect the fecal resistome). In addition,

betalactam ARDs were more abundant in feedlot samples, even though these AMDs are reported to be much more widely used in dairy production (11,198). These findings highlight the complexity of dynamics between AMD use and AMR, and suggest that AMD use practices do not solely or directly influence AMR patterns in livestock production.

Overall, MLS ARDs were not differentially abundant between beef and dairy saples, although lincosamide nucleotidyltransferases were significantly more abundant in beef samples while Erm 23S rRNA methyltransferases were significantly more abundant in dairy samples. These contrasting differences may therefore have offset each other, reducing the ability to detect an overall difference at the class level.

The resistomes of feces, soil and wastewater are distinct

The resistome composition of feces, soil and wastewater were significantly different (NMDS ordination stress < 0.10, ANOSIM $P < 0.05$). Shannon's diversity and richness were significantly higher in soil versus wastewater and feces at the gene level, and in soil versus wastewater at the mechanism level (Figure 4.3). The differentiation of soil from feces is especially interesting given that pen floors in dairies and feedlots are often a mixture of dried, compacted feces and underlying soil, rather than undisturbed soil matrix. In this context, the differentiation of "soil" (i.e., compacted feces) from fresh feces could stem from simple mixing of the two components, or from changes that occur within feces over time and under varying environmental exposures. Alexander *et al.* reported significant changes in the levels of ampicillin and tetracycline resistant generic *E. coli* isolated from feces subjected to ambient field conditions for 175 days, and this finding could be representative of the entire microbiome and resistome (73).

Ordination biplot results indicated that aminoglycoside, spectinomycin, phenicol and tetracycline resistance ARDs strongly influenced separation of soil samples from wastewater and feces (Figure 4.4). Phenicol and spectinomycin ARDs were not identified in any adult cattle fecal samples, and aminoglycoside ARDs of all types were less abundant in adult cattle fecal samples compared to soil samples (log-fold change = -4.6, adjusted $P < 0.0001$). Tetracycline ARDs, which were highly abundant in all samples, were more abundant in adult feces than in soil (log-fold change = 1.50, adjusted $P = 0.006$), and this change was driven overwhelmingly by the abundance of ribosomal protection proteins, as major facilitator superfamily (MFS) efflux pumps were less abundant in feces compared to soil (log-fold change = -2.1, adjusted $P = 0.02$). Interestingly, the discrimination of feces from soil samples based on the relative abundance of tetracycline ribosomal protection proteins versus tetracycline MFS efflux pumps has been previously described for human gut samples and agricultural soils (Gibson *et al*., 2015). Betalactam ARDs were also significantly more abundant in fecal samples than in soil, which in turn contained significantly more than wastewater samples. Differences in betalactam ARD abundance were driven primarily by class C betalactamases. However, betalactam AMDs have been shown to degrade relatively quickly in ambient conditions, and this could account for significantly lower levels in soil and wastewater if AMD residues are responsible for a significant portion of ARDs found in these environments (199).

Unfortunately, the confounding effect of sample type (i.e., feces vs. soil vs. wastewater) may have obscured associations between resistome composition and other external factors such as antimicrobial use practices and diet formulations, as sample numbers did not permit formal analysis of these associations within sample type. However, this finding can guide future sampling efforts, and indeed may provide a logical rubric on which to base future study designs.

Fortuitously, wastewater, manure and soils are often managed separately in large livestock production systems, and therefore research efforts can focus within one matrix without forfeiting real-world applicability.

Antimicrobial exposures on dairies did not significantly impact resistome composition

The resistomes of conventional and organic dairy samples (excluding calf fecal samples) were not significantly different upon ordination, and there were no significant differences in Shannon's diversity or richness. Abundance of ARD classes and mechanisms did not differ significantly between organic and conventional dairy samples, with the exception of macrolide resistance efflux pumps, which were more abundant in the samples collected from the conventional dairy (log-fold change = 9.4, adjusted $P < 0.0001$). The lack of major differences between these samples may, however, be due to insufficient study power to detect true differences, as descriptive analysis suggests major structural differences. One stark descriptive finding was the near absence of any identified ARDs in the lagoon samples collected from the organic dairy. Indeed, across both lagoon samples collected at this location, only 3 unique ARDs were identified: a lincosamide resistance gene (a LnuC with 18 reads aligning) and two tetracycline resistance genes (a TetQ and a TetW with 133 and 52 reads aligning, respectively). These samples clearly differed from the wastewater samples collected from the conventional dairy, which contained 32 unique ARDs across 12 mechanisms of resistance. Previous studies have described significantly higher concentrations of TetO, TetW and Sul1 in lagoons at conventional dairies compared to organic dairies (91), as well as higher absolute and relative levels of TetO, TetQ, TetW and TetM in feedlot lagoons that drained pens that were exposed to moderate and high levels of antimicrobials compared to lagoons that drained pens in which antimicrobials were not used (200). Taken together, these findings suggest that AMD use

practices may substantially impact the resistome of stored wastewater, and therefore regulations regarding lagoon construction and management as well as wastewater use/disposal should be tailored to specific livestock operations (201).

Of the 24 mechanisms identified within non-calf dairy samples, 14 were identified in samples taken from the conventional operation, while 19 were identified in samples taken from the organic operation. ARDs aligning to chloramphenicol active extrusion genes, passive efflux proteins, and efflux pumps, as well as class D betalactamases (bla-OXA), the TetX inactivation enzyme and glycopeptide resistance Van operons were all identified in conventional but not organic samples. Conversely, organic samples contained several general-purpose resistance mechanisms not found in conventional samples (e.g., MATE, MFS efflux pumps and RND efflux pumps, porin modification genes, and regulators of resistance mechanisms). The presence of resistance mechanisms with broad substrate specificity has been reported on organically-grown vegetables, and our own research suggests that these types of "general purpose" resistance mechanisms may be more abundant in cattle populations when AMD use pressures are absent (202). Under this theory, general purpose resistance mechanisms are favored in the absence of AMD selective pressure as a means for bacteria to defend against numerous and varied detrimental compounds, while resistance mechanisms with a more targeted purpose are favored in the presence of AMD. Further studies with larger sample sizes are needed to test this hypothesis.

Resistome composition did not differ between U.S. and Canadian feedlot samples

There were no significant differences identified when comparing the resistomes of samples collected at the U.S. and Canadian feedlots, which may indicate that diet type (i.e., primary carbohydrate source being corn or barley) did not have a major impact (ANOSIM P =

0.13, 0.32, 0.24 for NMDS ordination at the gene, mechanism, and class levels, respectively). Diversity and richness were also not significantly different between samples collected at the two feedlots. However, descriptive results seem to indicate that perhaps this lack of statistical difference stems from low study power, as the soil and fecal samples collected at the Canadian feedlot appeared to be much different than those collected at the US feedlot (Figure 4.6). Samples collected at the Canadian feedlot (which feed a barley-based diet) contained ARDs that confer resistance to 8 different classes of antimicrobials via 32 mechanisms. In comparison, ARDs aligning to 5 classes and 10 mechanisms of resistance were identified in samples collected at the U.S. feedlot. In general, this analysis provides strong support that larger sample sizes are needed to differentiate resistomes based on factors that may impact resistance in more subtle ways. Previous studies suggest that diet significantly impacts the cattle fecal microbiome (203,204). Alternatively, AMD exposures in these cattle could effectively decouple the microbiome and resistome, rendering the resistome impervious to dietary differences, while maintaining microbiome sensitivity.

Among mechanisms of resistance that were present in samples from both feedlots, only the MLS mechanisms differed significantly in abundance. Specifically, reads aligning to lincosamide nucleotidyltransferases were more abundant in Canadian samples, while reads aligning to Erm 23s rRNA methyltransferases and macrolide resistance efflux pumps were more abundant in US samples. Overall, reads aligning to MLS resistance-associated ARDs were less abundant in samples collected from the Canadian feedlot (log-fold change = -1.01, adjusted P = 0.04). This difference could reflect differential use practices regarding in-feed antimicrobials, as U.S. feedlots tend to use in-feed macrolides more frequently than Canadian feedlots (Calvin Booker, Feedlot Health Management Services, Okotoks, Alberta, personal communication).

Utility of resistome analysis and implications for future studies

This study is the first published comparison of the fecal, soil and wastewater resistomes of beef and dairy production systems. Using a shotgun metagenomics approach, we reveal the diversity of the beef and dairy resistome through identification of 248 unique ARDs within 16 classes of resistance. Results indicate that resistome composition is likely influenced by sample matrix, stage of animal development (i.e., age), and production system. These findings suggest that future research, regulations and decisions regarding livestock waste management systems should be tailored to each effluent as well as to different production systems. Furthermore, antimicrobial use practices on dairies and diet formulation in feedlots were not identified as statistically significant drivers of resistome composition. However, this finding must be interpreted in the context of relatively low sample number (only 2 biological replicates per sample matrix per production system) and perhaps insufficient power to uncover true differences. Importantly, the findings in this study highlight the utility of resistome analysis in producing actionable guidance on complex AMR issues, but also reveal the nascent state of resistome research in agriculture and the need for larger, hypothesis-driven studies.

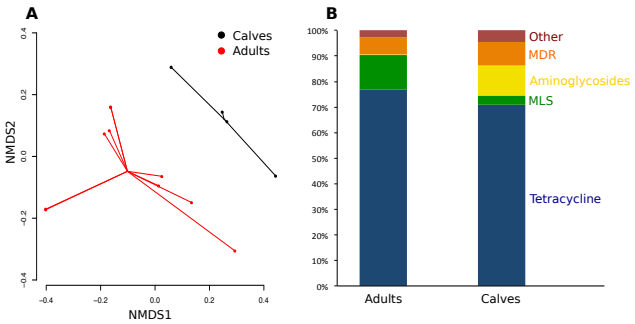

Figure 4.1. A comparison of fecal samples collected from calves and adult cattle. **A)** NMDS ordination of Euclidean distances between Hellinger-transformed normalized counts of ARDs in fecal samples collected from calves vs. adults. **B)** Proportion of all aligned reads that aligned to ARDs within different classes of resistance, in adult cattle feces versus calf feces.

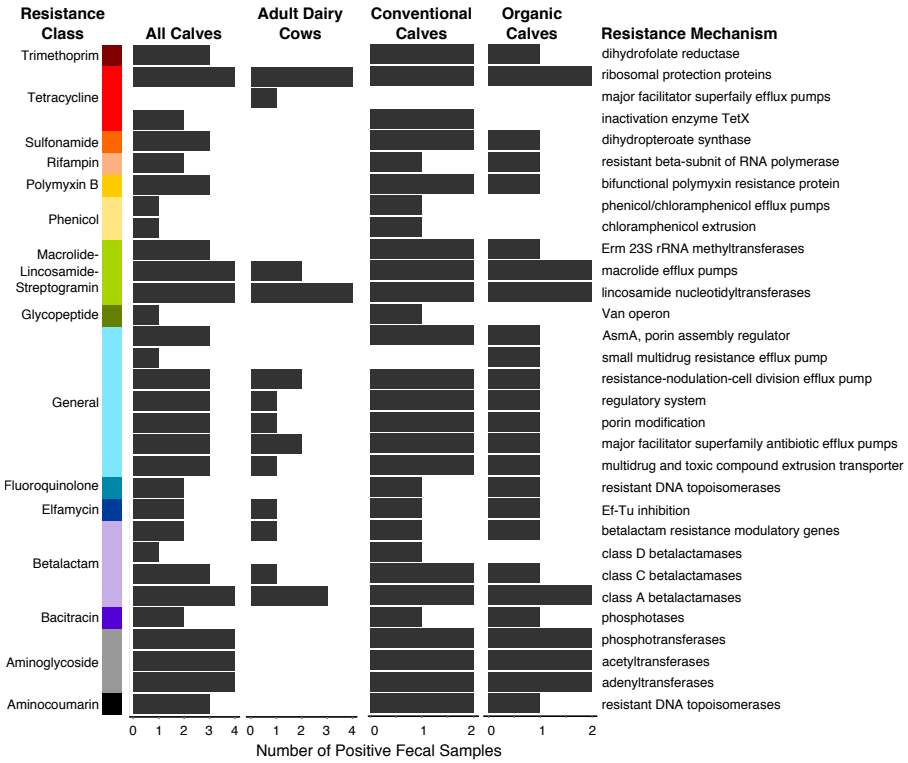

Figure 4.2. The calf fecal resistome is significantly more diverse and rich than the adult fecal resistome. Number of fecal samples containing ARDs within each mechanism and class of resistance, separated by calves vs. adults, and conventionally raised vs. organically raised calves.

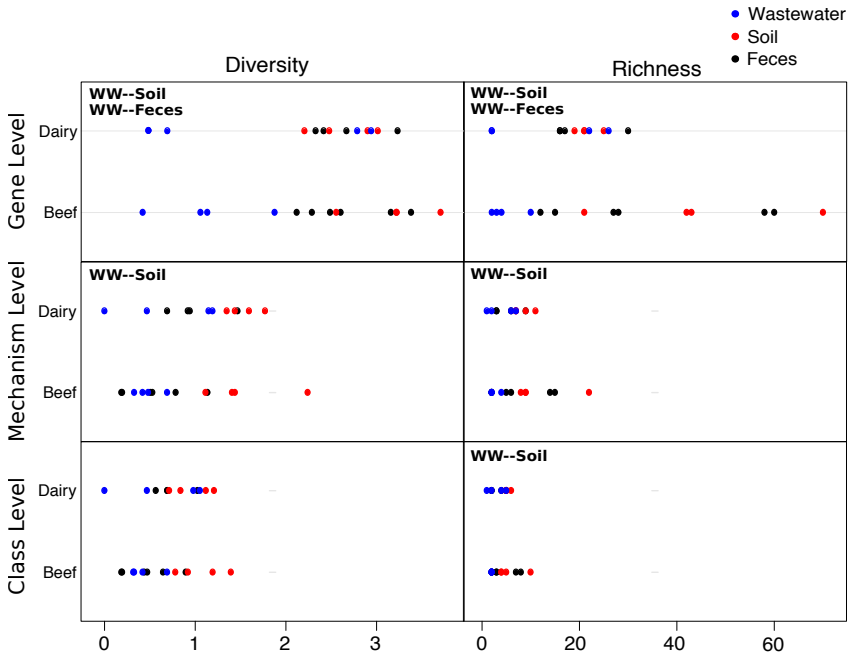

Figure 4.3. Soil samples are significantly more diverse and rich than wastewater. Dotplots showing Shannon's diversity and richness at the gene, mechanism and class levels, separated by system (beef vs. dairy) and colored by sample matrix, i.e., feces (black), soil (red) and wastewater (blue). Bolded text within each panel indicates which matrices different based on Nemenyi post-hoc pairwise comparisons (WW = wastewater). Diversity and richness were not significant different between beef and dairy at any level.

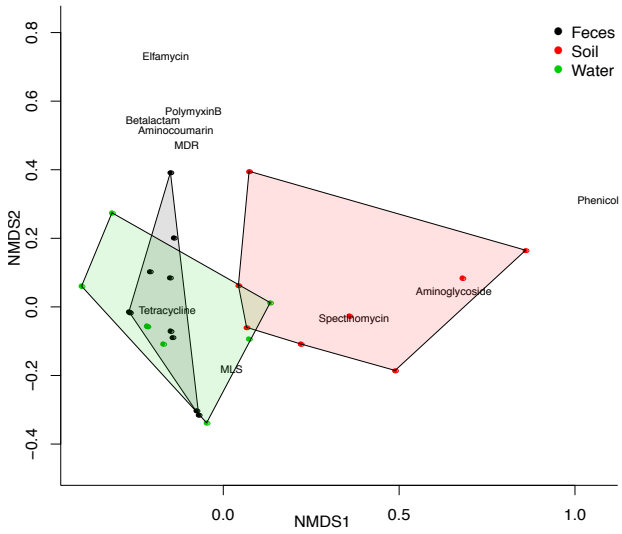

Figure 4.4. Specific resistance classes drive separation of soil from fecal and lagoon resistomes. NMDS ordination depicting Hellinger-transformed Euclidean distances between samples from feces (black), soil (red) and lagoon water (green) based on normalized counts of ARDs aggregated at the resistance class level. Biplot coordinates of resistance classes are labeled with the class name, and show that aminoglycoside, phenicol and spectinomycin resistances differentiate the soil from the fecal and lagoon resistomes.

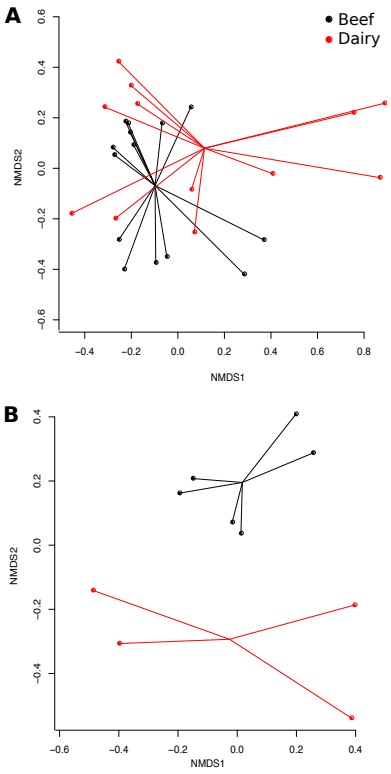

Figure 4.5. Beef and dairy systems have different resistomes. NMDS ordination at the ARD level of **A)** (adult) fecal, soil and lagoon samples and **B)** only adult fecal samples were both significantly different based on system, e.g., beef vs. dairy (ANOSIM $P < 0.05$).

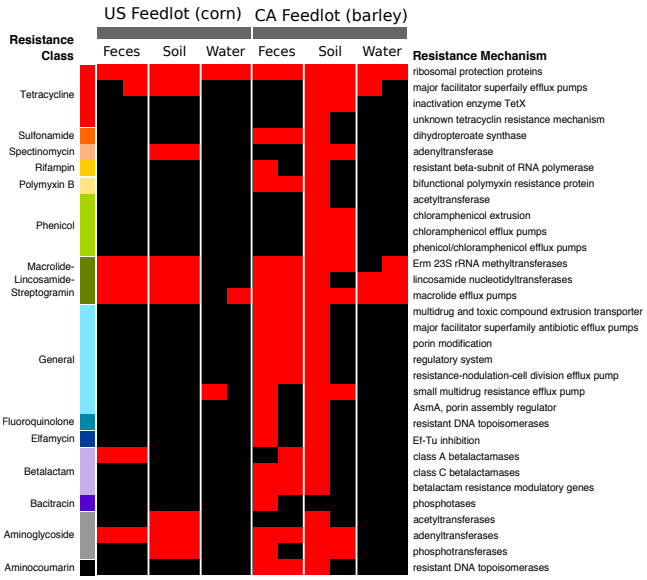

Figure 4.6. Binary heatmap of resistance mechanisms and classes identified in fecal, soil and lagoon wastewater samples collected from a U.S. and a Canadian feedlot. Black = absent, red = present.

CHAPTER 5: RESISTOME ABUNDANCE AND DIVERSITY DECREASE DURING BEEF PRODUCTION IN CATTLE, THE ENVIRONMENT AND BEEF PRODUCTS

SUMMARY

Antimicrobial-resistant bacteria can be transmitted to humans through the food chain via beef and other meat products. However, no study to date has traced antimicrobial resistance genes (collectively known as the resistome) through the beef production process. We followed 1,741 commercially reared beef cattle from the time they entered 4 feedlots to the point at which the resulting beef products were market-ready. We collected antimicrobial drug exposures and utilized shotgun metagenomics to interrogate the resistome at critical points in the beef supply chain. We identified more than 300 unique antimicrobial-resistance genes, 74% of which coded for resistance to tetracyclines. We also found that resistome diversity, but not microbiome diversity, decreased while cattle were in the feedlot, indicating selective pressure from antimicrobial drugs, which in beef production are used primarily in feedlots. We did not identify resistance genes in market-ready beef products, and also observed a significant reduction in the microbial population during slaughter and processing, suggesting that interventions currently utilized for pathogen reduction can also reduce the risk of resistance genes being transmitted to beef consumers via market-ready beef. Finally, we identified genes coding for resistance to critically important antimicrobials, including carbapenems, which are not used in beef production and therefore likely arose from co-selection or environmental introduction from external sources. Our findings highlight the complexity of microbial ecology, including antimicrobial resistance, in beef production and demonstrate that current practices appear to minimize microbial abundance, which essentially eliminates the resistome.

MAIN TEXT

The production of food products, including beef, is an important potential source of antimicrobial resistant (AMR) infections in humans. Beef is a widely consumed protein commodity, and production and consumption of beef is expected to increase (205). Several critically important antimicrobial drugs (AMDs) such as third-generation cephalosporins are used in beef production, while others are not, e.g., carbapenems (168). Use of these AMDs is thought to increase the risk of AMR being transmitted to humans both through environmental exposures (i.e., air, water and soil), as well as through consumption of beef products (206). While surveillance for foodborne AMR pathogens has been part of food safety systems for decades (207), we have yet to fully understand and quantify the public health risk posed by transmission of non-pathogenic bacteria that carry antimicrobial resistance determinants (ARDs). These ARDs, while not present in disease-causing agents, could pose a risk to human health if the bacteria carrying them become established within the microbiome of the human host, subsequently enabling horizontal gene transfer of these ARDs to pathogens (104,208). Establishment within an individual's microbiome could occur either through ingestion of contaminated beef products or through environmental exposures disseminated from beef production settings such as feedlots (206). The rate at which this occurs is unknown, largely due to an historical reliance on culture and isolation of pathogens and an inability to access the microbial community and its complete repertoire of ARDs (i.e., the resistome).

Several steps in the beef production system could play crucial roles in the transmission of antimicrobial resistance (AMR) from beef production to humans via environmental interfaces (e.g., air, soil and water) and beef products. Feedlots utilize AMDs more frequently than any other phase of beef production (19,20), a fact that has raised concern that these operations may

represent the main step in beef production at which AMR is acquired or maintained. Furthermore, feedlots are intricately linked to environmental exposure pathways such as air, manure, soil and water, enabling indirect human exposure to feedlot effluents (162). Slaughterhouses are a potential control point in AMR transmission, as they employ multiple sequential antimicrobial interventions to reduce pathogen contamination in beef products; routine verification and validation testing of these interventions has demonstrated that they are effective in reducing not only pathogen, but also total bacterial contamination (41). We hypothesized that the antimicrobial measures used in feedlots and slaughterhouses would exert a measurable effect on the presence, abundance and composition of ARDs in the bacterial populations of cattle, the feedlot environment and market-ready beef products. Furthermore, we hypothesized that use of a metagenomics approach would enable us to quantify these changes at an ecological level and therefore better understand the risk to public health, compared to use of a culture-based approach.

In order to understand how feedlots and slaughterhouses affect ARD transmission, it is imperative to track cattle through the beef production system, documenting AMD use and antimicrobial interventions and describing resistome changes over time. However, research in this area has been constrained by the challenges of tracking beef products and environmental effluents from individual or pens of cattle and collecting detailed records of AMD exposure for the cattle being studied. Specific challenges include lack of unique animal identification, use of non-computerized or otherwise hard-to-access AMD treatment records, uncontrolled effluents that are difficult to follow (e.g., air and runoff water), disassembly of the slaughtered carcass into hundreds of non-linked parts, and the sheer difficulty of handling and sampling feedlot steers, which weigh over 1,000 pounds. Because of these complexities, to our knowledge no studies

have specifically tracked antimicrobial use in livestock with antimicrobial resistance in retail products or consumers. Studies in this area are either descriptive (209) or ecological in scale (84). This dearth of evidence greatly complicates efforts to develop effective policies related to antimicrobial use in livestock with the goal of protecting public health (210), both through the food chain and through indirect environmental exposures such as dust, run-off and manure. The objective of this study was to perform a prospective longitudinal analysis of antimicrobial use and resistance in beef production and to exploit shotgun metagenomics to characterize resistome dynamics in the environment and the products of cohort cattle from feedlot through to the finished product.

In North America, cattle raised for beef production are typically born on variably sized ranches and are sold and shipped to feedlot operators and aggregated in the feedlot into pens. In this study, the size of pens ranged from 150 to 281 cattle, while the total feedlot capacity was ~100,00 cattle at each feedlot. In the feedlot, cattle are fed a high-energy diet until they reach a weight of ~1200 pounds, typically after 3-6 months depending on the weight and age at which they enter the feedlot. Cattle in this study were in the feedlot between 93 and 185 days. At this point, they are shipped to an abattoir and held for <12 hours in a pen with access to water before going through the slaughter process, which involves euthanasia and then disassembly of the carcass into beef products that are then packaged and marketed for human consumption.

A convenience sample of beef feedlots in Texas (n=2) and Colorado (n=2) was selected for study participation, and 2 pens of cattle were randomly enrolled from each feedlot, with a total of 1,741 cattle from 8 pens. Antimicrobial exposures in these pens were recorded throughout the feeding period, i.e., the total time that animals are housed in the feedlot (Table S5.1). In order to assess the resistome throughout the feedlot and slaughter processes, we

collected pooled, ecological-level samples from pens of cattle and their environment as they moved through the beef production system (Figure 5.1). Pooled fecal, soil and drinking water samples from each pen were collected soon after arrival of the cattle at the feedlots ("arrival samples", n=24, one per pen per sample type), as well as just before the same pens of cattle were shipped for slaughter ("exit samples", n=24, one per pen per sample type). Cattle were then transported (<8 h transport time) by truck to 2 slaughter plants and the walls, floors and ceilings of the trucks were swabbed ("truck samples", n=8, one per pen) immediately after the cattle were unloaded at the slaughterhouses. Cattle were placed in holding pens outside the slaughterhouses, where pooled fecal and drinking water samples were collected after cattle had been moved into the slaughterhouse ("holding pen samples", n=16, one per pen per sample type). Cattle were then euthanized and the carasses were disassembled into beef products (Figure 5.1). At the end of this process, pooled swab samples were taken from the conveyor used to transport and disassemble carcass parts (n=8, one per pen). In addition, beef trimmings were collected and rinsed to obtain a pooled sample of the highest-risk part of the carcass (n=8, one per pen). The table and trimming samples represent the microbiome and resistome after all antimicrobial interventions have been applied to the carcass, and just before the beef products are packaged for retail distribution ("market-ready samples") (211).

Total DNA was extracted from 88 samples and sequenced on an Illumina HiSeq 2000, resulting in 407.7 Gb of sequence data (average 46.3 M reads per sample, range 12.0 M – 93.4 M). One sample (a drinking water sample) did not contain enough DNA (i.e., <1ng) to be sufficiently sequenced. Reads were trimmed and filtered for quality, and reads classified as host genome (*Bos taurus*) were removed from further analysis (Table S5.2) (211). Non-host reads were then aligned to a custom non-redundant database of ARD sequences compiled from

publicly available sources (211). ARDs with a gene fraction of >80% across all alignments were considered to be positively identified in a sample (211). We identified 319 unique ARDs across all 87 samples (Table S5.3). The majority of ARDs were present in low numbers (Figure S1), and the median number of unique ARDs identified per sample was 33 (range: 0 to 136; Figure S5.2). The 319 ARDs identified represent 42 antimicrobial resistance mechanisms within 17 classes of resistance (Table S5.4). Reads aligning to genes that encode resistance to tetrayclines and the macrolide-lincosamide-streptogramin class of antimicrobials were most abundant.

To assess systematic changes in resistome composition during the feeding period (i.e., from arrival to exit, truck and holding samples), non-metric multidimensional scaling (NMDS) ordination using Hellinger transformation and Euclidean distances was performed at the ARD level (212). Samples with only one ARD (n = 2) were removed for clustering purposes. The pre-slaughter samples clustered by sample matrix (i.e., feces, soil, water and sponges, the latter of which comprised all truck samples) based on ARD composition (Adonis $P = 0.001$, Figure 5.2A). Therefore, to avoid confounding, we performed resistome ordination separately on fecal, soil and water samples (the truck resistome could not be compared owing to complete confounding between sampling location and matrix type), all of which exhibited a significant shift from arrival to exit or holding (Figure 5.2B-D). While this shift could result from AMD exposures, culture-based studies of phenotypic resistance have reported mixed results when looking for associations between feedlot AMD use and AMR (20,21). In addition to AMD exposures, cattle undergo numerous changes during the feeding period, including maturation and a gradual shift from forage-based to high-energy rations, all of which have been shown to affect the fecal microbiome in swine, although little is known about these factors in beef cattle (213–

215). Therefore, changes in the resistome could also be driven by changes in bacterial community composition, a phenomenon recently reported for a set of functionally confirmed metagenomic soil samples (105). The procrustes analysis of NMDS ordination results confirmed the correlation between the resistome (ARD level) and the microbiome (species level) in arrival and exit samples (Figure 5.4B-C). However, there was tighter correlation on arrival than on exit, hinting that additional factors such as AMD exposures may have influenced resistome changes independently of the microbiome. In addition, Shannon's diversity at the ARD level decreased while cattle were in the feedlot, while diversity of microbial species did not, suggesting a decoupling of the resistome from the microbiome.

Interestingly, the decrease in ARD diversity was driven primarily by a reduction in ARD richness (Figure 5.3). Richness, in turn, occurred primarily through the loss of general-purpose (i.e., multiclass) resistance mechanisms, as well as loss of resistance to classes of drugs that were not used on cattle in this study population or in the feeding systems in which they were raised (e.g., phenicols, aminocoumarins, elfamycins, rifampin, bacitracin and polymyxin B). In contrast, resistance to macrolides and tetracyclines remained prevalent in exit and holding pen samples, and these were the two most commonly used classes of antimicrobials (Table S5.1). Indeed, all cattle received in-feed tylosin (a macrolide) throughout the entire feeding period to prevent liver abscesses. Use of other antimicrobial agents was sparse on the individual animal level, but pen-level exposure was higher and all 8 pens contained at least one animal that was treated with injectable macrolides and tetracyclines (Table S5.1). Five of the 8 pens contained cattle that received fluoroquinolones, and 3 of the 8 contained cattle that received β-lactam AMDs. One hypothesis for these findings is that antimicrobial exposure during the feeding period created pressure on the microbial population such that the fitness costs of maintaining a

diverse resistome (i.e., the resistome detected in arrival samples) were outweighed by the need to respond to specific stimuli; i.e., pen-level exposure to macrolides and tetracyclines. A result of this shift is decreased ARD richness due to the loss of general-purpose and unnecessary resistance mechanisms.

Interestingly, the aminoglycoside class was an exception to this pattern, as aminoglycoside resistance remained prevalent throughout the feeding period despite absence of these drugs in this study population and in beef production in general; spectinomycin and sulfonamides, which were also identified post-arrival, can be used in beef cattle, but were not used in this study population. In addition, we identified several ARDs that have been associated with phenotypic resistance to critically important antimicrobial drugs in humans when expressed in disease-causing agents (Table S5.5); however, these AMDs were not used in this study population and most are not labeled for use in cattle at all. A soil sample collected at feedlot arrival contained the carbapenemase (bla)OXA-235 (216)as well as vgaD and vatG ARDs, which together confer resistance to quinupristin-dalfopristin (217). Another soil sample collected at arrival and a water sample from a holding pen contained the carbapenemase ARD (bla)OXA-347, which has only been shown to confer resistance to ampicillin but is classified as a carbapenemase based on 53% amino acid identity (218). Of the 7 analyzed water samples collected from the holding pens, 3 contained reads aligning to the strict carbapenemase class bla(cphA). Additionally, the 4 truck samples collected in Texas all contained a CfrA 23S rRNA methyltransferase, which confers resistance to phenicols, lincosamides, oxazolidinones (linezolid), pleuromutilins, and streptogramin A ($PhLOPS_A$). Despite the presence of these ARDs, study cattle were not exposed to aminoglycosides, carbapenems, streprogramins, phenicols, lincosamides, linezolid, or pleuromutilins while in the feedlot (Table S5.1). In

addition, carbapenems, streptogramins, pleuromutilins and linezolid are not approved for use in cattle production. Therefore, antimicrobial use practices in these study cattle or in other cattle populations cannot directly explain the presence of these important ARDs. It is important to note that these ARDs were present in extremely low relative abundance; fewer than a dozen of the 1.2M ARD-assigned reads aligned to each of these critically important ARDs. While high sequence homology and the resistance-conferring functional residues were intact (Table S5.6), we cannot infer phenotypic expression from these data. Furthermore, while this is the first published report of these ARDs in the feedlot setting, it is also the first study to utilize a shotgun metagenomics approach on feedlot samples; therefore, we cannot contextualize these findings with respect to previous research and we cannot determine whether presence of these ARDs in feedlot samples is a novel or long-standing phenomenon. Identifying these ARDs in metagenomic data may provide important insight above and beyond a culture- or PCR-based approach, but additional work is needed to understand the biological, ecological and public health consequences of these findings. For instance, the presence of these ARDs in the feedlot setting, even in the absence of corresponding AMD exposures, could be explained by transfer into the feedlot environment through either cattle or fomites (e.g., feedlot workers, feedlot working dogs and horses, or environmental exposures such as air or water). Alternatively, or in combination, use of other AMD classes could co-select for these ARDs within the cattle population. In either case, these findings highlight the complexity of the AMU-AMR relationship, as well as the fact that food production in general (and beef production specifically) is intrinsically linked to other ecosystems via diffuse environmental contacts. Given these complexities, we believe an ecological and metagenomic approach is necessary to aggressively research this important public and human health issue.

We also identified bla(TEM)-116, an extended-spectrum β-lactamase (ESBL), in 1 water holding pen sample. Third-generation cephalosporins (a class of β-lactams) are used in cattle production, although use is limited, as reflected in the use data collected in this study (Table S5.1). We did not identify ARDs from the bla(NDM), bla(SHV) or bla(CTX-M) or bla(CMY) classes of ESBLs, or the carbapenemases bla(SME), bla(IMI), bla(NMC), bla(GES) or bla(KPC) (Table S5.5).

Interestingly, ARD composition did not differ significantly between pens of cattle (n=8) or feedlots (n=4) when ordinated using NMDS. This was somewhat surprising given the geographic separation of the 4 feedlots and the fact that pens of cattle within feedlots do not have contact with one another. However, common management and AMD use practices between all 4 feedlots could explain this lack of difference. In addition, water runoff, windborne dust and fomites within feedlots could contribute to a mixing of pen resistomes, despite differential AMD exposures and cattle populations between pens. Furthermore, resistome composition at the ARD level, but not the mechanism and class levels, did differ significantly by geography (e.g., Colorado versus Texas feedlot, truck and holding pen samples, ANOSIM $P < 0.05$). These findings support the idea of environmental connectivity within feedlots and even within regions, but suggests that the "connected environment" is limited at larger geographic spans. The fact that the resistome differed by state at the ARD level, but not the mechanism and class level, suggests that while individual gene content differs between distant feedlots, the overall resistance potential (and by extension functionality) of the microbial population is similar; this, in turn, may be reflective of management and AMD use practices that are common among most North American feedlots. In effect, the bacterial populations in the Texas and Colorado feedlots may have taken a different evolutionary path to respond to these common threats, but the general

mechanisms by which they respond, as well as the classes of AMDs to which they respond, are consistent.

When examining the 16 post-slaughter samples obtained from the belts, tables and meat trimmings, no ARDs were identified in any of these market-ready samples (n = 8 pooled belt/table samples and n=8 trimming rinses). These samples yielded large amounts of DNA, but >99% of the reads aligned to the bovine genome (Table S5.2); therefore, the lack of detection of ARDs could be attributable to low sequencing coverage of bacterial DNA. However, there are also plausible biological explanations for the lack of bacterial DNA (and thus ARDs) in these samples. The bacterial contamination of beef during slaughter occurs primarily during removal of the hide and gastrointestinal tract (GIT), at which point the surfaces of carcasses can routinely be contaminated with aerobic bacterial counts of 6.1 – 9.1 log CFU/100 cm^2 (41). To decrease this contamination, it is standard in North America for carcasses to undergo several highly effective antibacterial interventions after hide and GIT removal, including steam vacuuming, carcass washing, the application of organic acid rinses and thermal pasteurization (41). All carcasses in this study underwent each of these interventions sequentially, a process that has been shown to reduce bacterial loads by >5 log CFU/100 cm^2 total plate count (219).

To differentiate between incomplete sequencing and true reduction of the microbial population, we conducted a microbiome analysis of samples pre- and post-slaughter, the former comprising all arrival, exit, truck and holding pen samples. Reads that did not align to the bovine genome were classified into a phylogenetic clade using exact-match *k*-mer alignment to the NCBI RefSeq database of complete microbial genomes (211,220). Exact matches were summed at the species and genus levels within each sample, and totals were normalized to the number of mapped reads within each sample using a data-driven approach based on shifts in

count distributions (221). We found that microbiome (i.e., total microbial community) diversity was significantly reduced in the post-slaughter samples compared to the pre-slaughter samples, suggesting a dramatic alteration in the composition of the microbial community, which could reflect the impact of antibacterial interventions during slaughter (Figure 5.1A). However, low diversity could also be the result of low-coverage sequencing of microbial DNA in the post-slaughter samples (222). Therefore, we conducted a closer analysis of differential microbial abundance between pre- and post-slaughter samples using zero-inflated Gaussian mixture models to account for distinct processes for zero-count data (i.e., true absence vs. incomplete sequencing depth) (182). Pairwise comparisons between pre- and post-slaughter samples were performed using limma's makeContrasts function (223), and pen ID was added to all models as a covariable to account for repeated measurements. We identified 416 (of 763) differentially abundant genera and 840 (of 1,821) differentially abundant species, the vast majority of which were more abundant in the pre-slaughter samples (Figure 5.1B). Of the 19 genera and 68 species that were more abundant in post-slaughter samples, many are known to be heat-tolerant and/or environmentally hardy; e.g., *Staphylothermus*, *Pyrococcus*, *Pseudomonas* and *Pleurocapsa*, suggesting that they were able to withstand the high heat and other harsh conditions utilized as part of the multiple antimicrobial interventions that occur during slaughter and processing of beef carcasses. This is the first report of survival of these bacteria during slaughter, as many are difficult to culture. Furthermore, the lack of ARDs in the post-slaughter samples suggests that there is no (or very low) resistance in these bacteria, but the incomplete sequencing cannot be fully discounted. Interestingly, culture-based resistance surveillance in beef trimmings (207) reported >30% prevalence of resistance amongst *Salmonella* isolates (207). However, *Salmonella* was recovered from <1% of these trimming samples (22 of 1,791 samples collected

in 2011), suggesting that recovery of resistant *Salmonella* is a very rare event; in this respect, the results of these culture-based efforts concur with our findings. Furthermore, samples taken as part of this surveillance program were enriched prior to isolation, likely resulting in increased sensitivity compared to the shotgun metagenomics approach.

To our knowledge, this is the first metagenomic investigation of AMR that followed specific pens of cattle from feedlot entry through slaughter to market-ready product in a longitudinal fashion. The use of shotgun metagenomics provided a novel view of resistance dynamics within the beef production system. These results are largely generalizable to North American beef production, particularly within geographic regions, as resistome composition did not vary between feedlots or pens. While our results suggest that slaughter-based intervention systems minimize the likelihood of intact ARDs being passed through the food chain, our results highlight the potential risk posed by indirect environmental exposures to the pre-slaughter resistome. This concern is especially salient given evidence in this study that ARDs may be "shared" between pens of cattle and between feedlots within a geographic region, indicating environmental connectivity that could also extend to human habitats through wastewater run-off, manure application on cropland, and windborne particulate matter. The pattern of resistome change during the feeding period suggests that AMD use practices may be the driving force behind the feedlot resistome, but more research is warranted. Furthermore, the scientific community urgently needs to develop a better understanding of the risk of different resistomes (224), which would inform an assessment of the risk of the "exit" versus the "arrival" resistome. Finally, this study highlights the utility of an ecological, metagenomics and systems approach to investigating AMR in food production, and provides a glimpse of the unique insights that can be gained in order to better inform public health-related policy

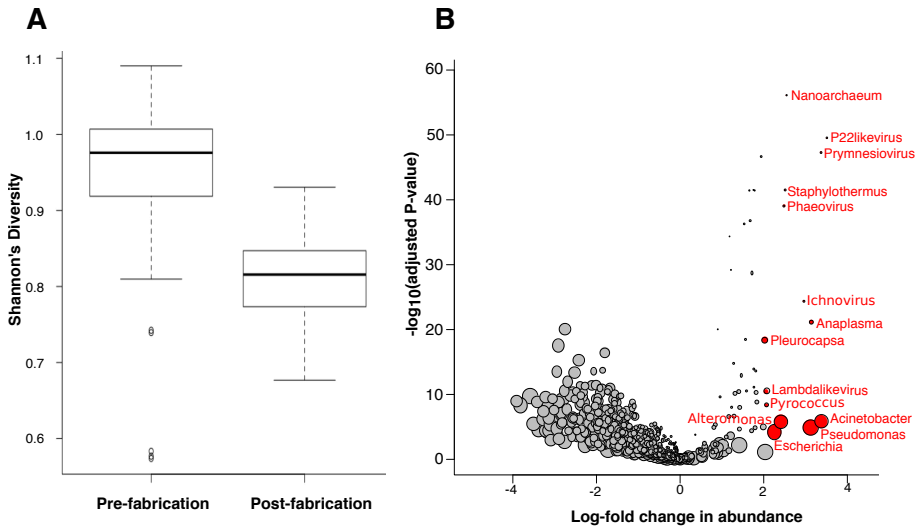

Figure 5.1. (A) Boxplot of Shannon's diversity at the species level, pre- vs. post-slaughter samples. Shannon's diversity was significantly lower in post-slaughter samples when tested using generalized linear modeling (P<0.0001). (B) Volcano plot of log-fold change in abundance of genus from pre- to post-slaughter. Red dots with red labels have a log-fold change >2 and an adjusted P-value <0.05, whereas gray dots do not. Dot size is proportional to the average abundance of the genus across all samples.

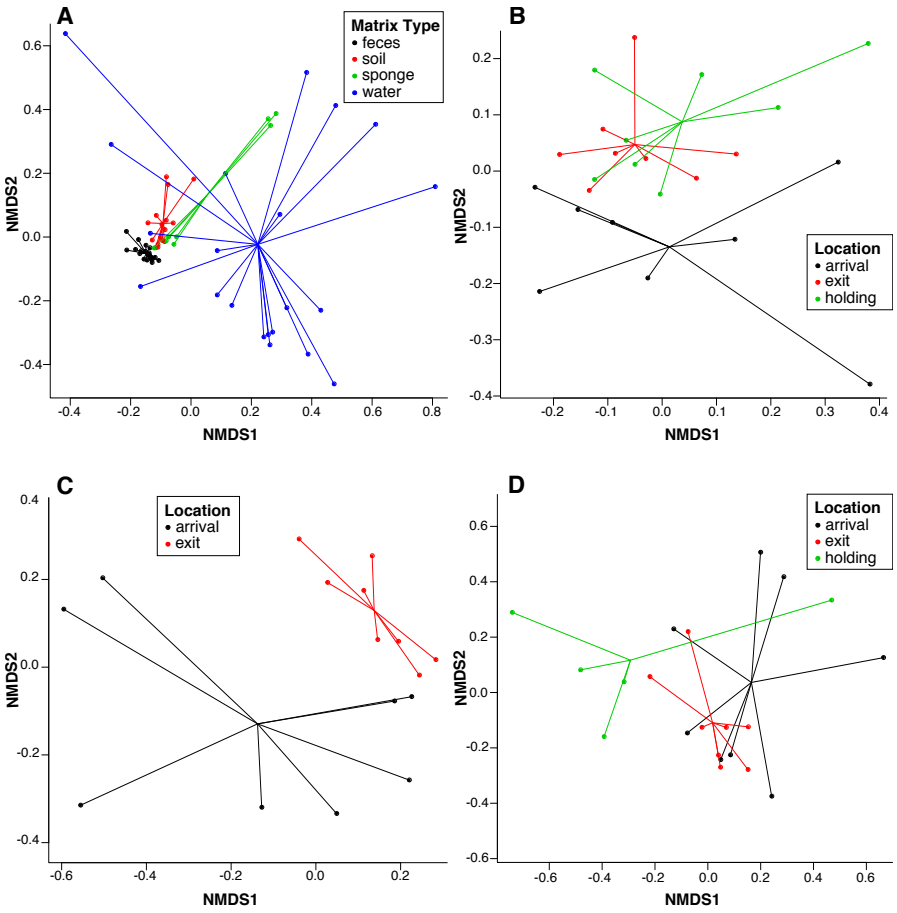

Figure 5.2. Non-metric multidimensional scaling (NMDS) ordination plots of pre-slaughter sample ARD composition, depicting significant sample separation by (A) matrix ($P = 0.001$), and location within (B) feces ($P = 0.03$), (C) soil ($P = 0.004$) and (D) water ($P = 0.002$).

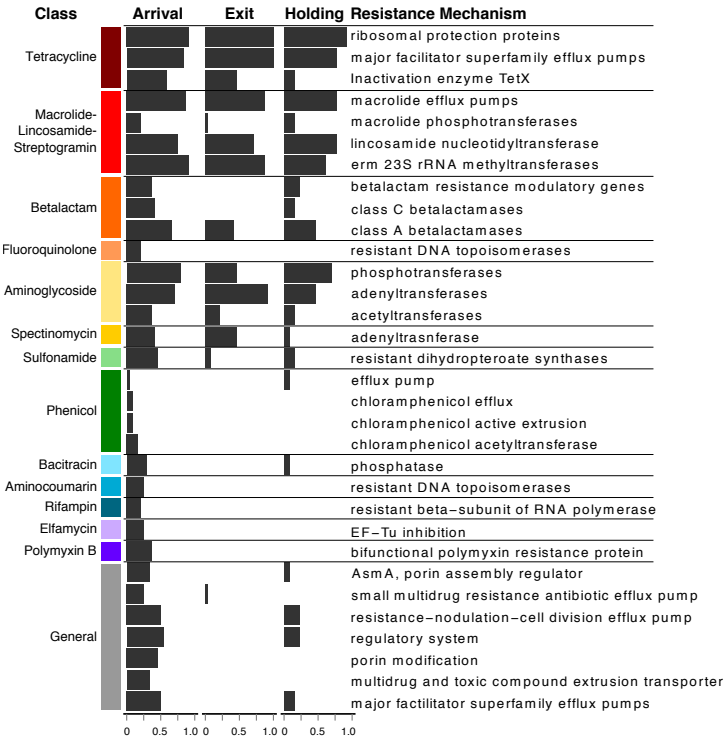

Figure 5.3. Proportion of arrival (n=24), exit (n=24) and holding (n=15) samples that contained at least one ARD in each resistance mechanism (n=33), grouped by resistance class.

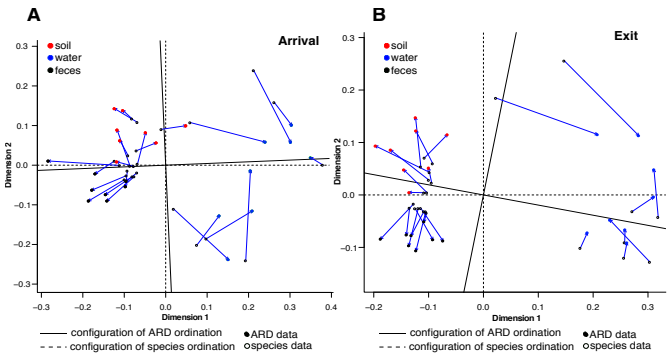

Figure 5.4. Procrustes analysis of ARD content (filled circles) and species composition (open circles) at arrival (A) and exit (B) using Hellinger transformation and NMDS ordination (212). Soil (red), water (blue) and fecal (black) samples clustered significantly in the microbiome and resistome data. Procrustes configurations were significantly correlated in the arrival and exit samples, but less so in the exit samples ($M^2 = 0.29$ and 0.18, respectively, $P<0.001$).

MATERIALS AND METHODS

Antimicrobial use data

AMU data were aggregated and analyzed at the pen level because samples were collected as composites and an ecological perspective on the resistome was desired. In order to standardize drug exposures across cattle and across differing dosages, AMD treatments were reported in units of "Animal Defined Dose" (ADD), or the number of days that a given AMD is expected to persist at therapeutic concentrations in the target tissue (156).

Sample collection and processing

All fecal, soil, sponge and trimming samples were collected using sterile gloves sprayed with alcohol and placed into sterilized Whirl-Pak bags (Nasco). Water samples were collected into bottles and centrifuge tubes that had been submerged in bleach for 5 min, rinsed with sterile water and then autoclaved. Pooled fecal samples representing all the cattle in a single pen were collected from feedlot pen and holding pen floors; pooled soil samples representing all the cattle in a single pen were also collected from feedlot pen floors, but not holding pen floors, which were concrete. Investigators walked through pens on diagonal lines, collecting ~30 g of feces or soil from 12 approximately equally spaced locations. The 12 soil and 12 fecal samples from each pen were then placed in one Whirl-Pak bag each (Nasco) and mixed thoroughly to combine. The contents of the water trough in each pen were thoroughly mixed, and drinking water samples (1 L each) were collected and placed into sterile containers. Truck samples were collected using an EZ Reach™ polyurethane sponge pre-hydrated with 10 mL Dey/Engle neutralizing broth (World Bioproducts LLC). Sponges were used to swab the internal walls of each truck (sides, door and floor); each surface was swabbed 20 times on the front and back of the sponge. For each pen of cattle, 3 of 5 trucks were randomly selected for sampling, and 1 sponge was used for

each truck. After slaughter, carcasses in each pen were grouped and processed by USDA quality grade. Post-slaughter samples were obtained when the USDA grading group with the greatest number of carcasses was being disassembled and processed. In the slaughter room, EZ Reach™ polyurethane sponges pre-hydrated with 10 mL Dey/Engle neutralizing broth were used to collect swab samples at the end of the conveyor belts used to process the chuck and round primal cuts, as well as the trimmings. The end of these belts represents the last stage in the slaughter and disassembly process, immediately prior to beef being packaged for retail distribution. To obtain belt samples, sponges were held on each running belt for one minute per side. Beef trimming samples were collected from the trim conveyor belt immediately prior to the spraying of the last antimicrobial solution in the slaughter process.

All samples were transferred on ice to the Center for Meat Safety & Quality at Colorado State University. Samples collected in Colorado arrived within one hour of collection, and samples collected in Texas arrived within 48 hours. Upon arrival, fecal, soil, sponge and trimming samples were immediately frozen at -80°C. Water samples were centrifuged at 15,000x*g* for 20 min at 4°C, and 5 mL of the pellet was collected for DNA extraction.

Sample Processing

All samples were thawed prior to DNA extraction. Four hundred grams of thawed meat from each trimming sample were rinsed in 90 mL of buffered peptone water (BPW) and then placed at 4°C to separate and harden the lipid content. After hardening, the liquid portion of the sample was removed and centrifuged at 4280x*g* at 4°C for 20 minutes, after which the supernatant was discarded and the pellet re-suspended in 5 mL of cold, sterile saline. The cold saline wash was repeated, and after the final centrifugation, 250 mg of the resulting pellet was utilized for extraction. Sponge samples were squeezed with a Brayer squeegee to remove the

broth liquid. The sponges were then rinsed in 10 mL of BPW and squeegeed again. The rinsate from both rounds of squeezing were then centrifuged at 4300x*g* at 4°C for 20 minutes, the supernatant was removed, and the pellets were re-suspended in phosphate buffered saline (PBS), at which point the pellets from samples collected from the same pen were combined, resulting in 1 pooled truck sample and 1 pooled belt sample per pen. The combined samples were then centrifuged again at 4280x*g* at 4C for 20 minutes, and 250 mg of the resulting pellet was weighed and set aside for DNA extraction.

The Mo Bio PowerSoil DNA Isolation Kit was used to extract DNA from 250 mg/sample of water, sponge and trimming pellets, whereas the Mo Bio PowerMax Soil DNA Isolation Kit was used to extract DNA from 10 g/sample of pooled feces and soil (Mo Bio Laboratories, Inc.). Different kits were used to accommodate different sample volumes (as recommended by the manufacturer); however, they utilize identical reagents and chemistries. A sedimentation step was used to process the feces and soil prior to DNA extraction, allowing for the simultaneous sedimentation of heavier soil/fecal debris and the release of bacterial cells into the upper supernatant. This step made it possible to process a greater volume of sample matrix (up to 10 g) while removing additional PCR inhibitors known to be present in soil and feces (225), resulting in a more complete representation of bacterial DNA presence. Briefly, 30 mL of BPW was added to 10 g soil or feces in a 50 mL conical tube, and the samples were shaken vigorously to mix well before being allowed to sediment on the bench for 10 min. Supernatants, including limited soil/fecal debris, were transferred to a new 50 mL conical tube and centrifuged for 10 min at 4,300x*g*. The BPW was removed, and the resulting sample pellet was rinsed with 5 mL of molecular grade sterile PBS and centrifuged again at 4,300x*g* for 10 min. The supernatant was removed, and the resulting pellet was re-suspended in 15 mL of PowerBead solution before

being transferred to the PowerMax Bead Solution Tube provided in the kit and proceeding with the DNA extraction protocol.

DNA extraction was performed using the MoBio PowerMax Soil DNA Isolation Kit or Mo Bio PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc.) according to the manufacturer's protocol. DNA for fecal and soil samples was eluted in 5 mL of the kit elution buffer, and water, sponge and trimming rinsate samples were eluted in 50 µl of the kit elution buffer to maximize DNA concentration. After extraction, DNA concentration was measured at 260 nm using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Inc.). Samples that did not have a concentration of at least 20 ng/µl (1 µg total in 50 µl) were precipitated using a traditional ethanol precipitation procedure. To the final DNA sample, 1/10 volume of 3M sodium acetate, pH 5.2, was added. Two volumes of cold 100% molecular grade ethanol was added and the sample was mixed several times by inversion before incubating at -20°C for 1 hour. Samples were centrifuged at 11,000 x g for 20 minutes at 4°C. Supernatants were carefully discarded and 150 µL 70% cold ethanol was added and mixed by inversion. Samples were centrifuged a final time at 11,000 x g for 10 minutes at 4°C. Supernatants were again discarded and the DNA pellets allowed to air dry before resuspending in ¼ the original DNA volume with Solution C6 included in the Mo Bio DNA extraction kits.

After DNA extraction or concentration, 100 µl of each fecal and soil DNA and 30 µl of each water, sponge and trimming rinsate DNA were delivered on ice to the Genomics and Microarray Core at the University of Colorado Denver. Libraries were constructed using the Illumina TruSeq® DNA Library Kit (Illumina, Inc.) for samples that contained at least 1 µg of DNA and using the NuGEN Ultra Low DNA Library Preparation (NuGEN Technologies Inc.) for samples that contained less than 1 µg of DNA, following the manufacturer's protocols.

Paired-end sequencing was performed on the Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA).

Creation of master, non-redundant ARD database

Resfinder (180), ARG-ANNOT (179) and CARD (181) databases were chosen for the foundation of the master database because they are specific to antimicrobial resistance genes, and the databases are actively curated and frequently updated. Redundant sequences between ARG-ANNOT and Resfinder were identified using CD-HIT-EST-2D (178) with local alignment (-G 0) and the following parameters: -c 1.0 -AS 0 -AL 0 -aL 1.0 -aS 1.0. A single representative sequence was selected from each resulting doubleton cluster (n=1,427), and these sequences were appended to the list of unique gene sequences in ARG-ANNOT (n=261) and Resfinder (n=715). This process was then repeated for the CARD database using the combined ARG-ANNOT/Resfinder non-redundant database. Seven hundred and eight sequences were unique to CARD, resulting in a final non-redundant database containing 3,111 unique ARD sequences. Each ARD sequence identified in the metagenomic sample data was assigned to a class and mechanism of resistance (Table S5.4).

Bioinformatics pipeline used to identify ARDs

Raw sequence data were obtained from the Genomics and Microarray Core at the University of Colorado Denver. Reads were filtered for quality using Trimmomatic (176) in the following manner: first, the leading 3 and trailing 3 nucleotides were removed from each read, then a sliding window of 4 nucleotides was used to remove nucleotides from the 3' end until the average Phred score across the window was at least 15. Trimmomatic's "ILLUMINACLIP" command was used to remove adapters supplied in the TruSeq3 adapter sequence file. A maximum of 2 mismatches were allowed in the initial seed, and adapter clipping occurred if a

match score of 30 was reached. In addition, we specified that both reads be retained upon clipping, despite probable complete sequence redundancy, to supply more reads for downstream applications.

After clipping and trimming, reads were matched to the *Bos taurus* reference genome (UMD_3.1) using Kraken (220) in "quick operation" mode; reads with <5 13-mers matching to the *Bos taurus* genome ("non-host" reads) were extracted for further analysis. Non-host reads were then aligned to the master, non-redundant ARD database using BWA with default settings (177). A custom-developed Java-based script was used to parse the resulting SAM file such that the gene fraction was calculated for each ARD identified in each sample. Gene fraction is defined as the proportion of nucleotides in the ARD that aligned with at least one read. ARDs with gene fraction of >80% were defined as present in the sample and were included in further analyses. For each such gene in each sample, the total number of aligned reads was summed to create a count matrix with samples in columns and genes in rows. This count matrix was used to analyze changes in the log-fold abundance of resistance mechanisms and classes as well as for ordination and heatmap generation.

Analysis of log-fold change in abundance

Raw count data at the gene level were normalized using cumulative-sum scaling, a method that has been shown to introduce less bias than total-sum scaling or rarefying to the smallest library size (221). Due to the sparseness of count data, a default percentile of 0.5 was chosen for normalization, based on published recommendations (182). Normalized counts were then aggregated to the mechanism and class level using the "aggTax" function in metagenomeSeq. Multivariate, zero-inflated Gaussian mixture models were fit to mechanism- and class-level normalized counts using metagenomeSeq's "fitZig" function, with

"useCSSoffset" set to "FALSE" as aggregation was performed with normalized counts. All models included a pen identification number as a covariable to account for the potential clustering of response variable within pens. Models that compared arrival and exit samples also included matrix type (soil, feces and water) as a covariable to account for potential confounding. The output of fitZig was then transferred into limma's "makeContrasts" and "eBayes" functions to conduct pairwise comparisons of log-fold change in abundance between sample groups (223), adjusting for multiple comparisons using the Benjamini-Hochberg procedure and using a critical α of 0.05.

Ordination

All ordinations were conducted with "vegan" (226), using the Hellinger transformation of normalized counts of aligned reads per gene within each sample (212). Euclidean distances were calculated, and vegan's "metaMDS" function was used to perform non-metric dimensional scaling, enabling the discovery of a stable ordination solution using many random starts. Ordination results were tested for statistical significance using the analysis of similarities ("anosim") as implemented in vegan.

Richness and diversity comparisons

Richness was defined for all analyses as the number of unique features (genes, mechanisms, classes, species or genuses) in a sample. Diversity was calculated using Shannon's diversity index, and therefore incorporated a relative evenness of features within samples. The comparisons of richness and diversity between sample groups were conducted using paired Wilcoxon signed rank test due to the non-parametric nature of the data and the presence of repeated measures when comparing different sampling locations (e.g., arrival vs. exit).

Microbiome classification

Kraken was used to classify reads phylogenetically, and output was converted into MPA format for further analysis using Kraken's "Kraken-mpa-report" program (220). The number of matches to each taxon was normalized within samples, and normalized counts were aggregated to the species and genus levels using the "aggTax" function in the R package "metagenomeSeq" (182). The analysis of changes in abundance was conducted as described in the "Analysis of log-fold change in abundance" section above.

Table S5.1. Antimicrobial drug usage in the study population.

Drug (dosage)	Drug Class	Primary Reason for Use	Number of Animals treated *(%)*	ADD[a]	Total ADDs	ADD/ Animal[b]
Tylosin phosphate (11 mg/kg diet dry matter)[c]	Macrolide	Liver abscess prevention	1,741 *(100%)*	1/80	2,780	1.62
Tulathromycin (2.5 mg/kg BW[d])	Macrolide	BRD[e] Treatment	81 *(4.7%)*	3	243	0.14
Oxytetracycline (9 mg/lb BW)	Tetracycline	BRD Treatment	85 *(4.8%)*	1	85	0.06
Oxytetracycline and Flunixin meglumine (30 mg/kg BW and 2 mg/kg BW)	Tetracycline	BRD Treatment	1 *(0.06%)*	5	5	0.02
Danofloxacin mesylate (8 mg/kg BW)	Fluoroquinolone	BRD Treatment	18 *(1.03%)*	3	36	0.03
Enrofloxacin (7.7 mg/kg BW)	Fluoroquinolone	BRD Treatment	2 *(0.11%)*	3	6	0.02
Ceftiofur sodium (1 mg/kg BW)	β-lactam	BRD Treatment	2 *(0.11%)*	1	2	0.01
Ceftiofur crystalline free acid (6.6 mg/kg BW)	β-lactam	BRD Treatment	5 *(0.29%)*	3	15	0.03

[a]ADD = Animal defined daily dose, or the number of days that a single treatment of drug remains at therapeutic concentrations in the target tissue (156)
[b]This is the ADD per animal calculated as the total ADDs administered to animals within the pen divided by the total number of animals in the pen
[c]Assuming average daily intake of 9 kg of dry matter per day
[d]BW = body weight
[e]BRD = bovine respiratory disease

Table S5.2. Percentage of reads classified as *Bos taurus*, by sample type

Sample Type	**Average (min – max)** ***(%)***
Feces	1.54 (0.32 – 4.10)
Soil	2.54 (0.10 – 15.5)
Water	9.72 (0.04 – 57.9)
Truck sponges	1.34 (0.16 – 6.90)
Belt/table sponges	99.3 (97.7 – 99.8)
Carcass trimmings	99.6 (99.5 – 99.7)

Table S5.3: List of 319 ARDs identified across all 87 samples.

ARD ID	Resistance Class	Resistance Mechanism	Originating Database
(AGly)Aac)-IVa:X01385:244-1029:786	Aminoglycoside	Aminoglycoside acetyltransferases	ARG-ANNOT
(AGly)AadA1-pm:JQ690540:7968-8798:831	Aminoglycoside	Aminoglycoside nucleotidyltransferases	ARG-ANNOT
(AGly)AadA1:M95287:3320-4111:792	Aminoglycoside	Aminoglycoside nucleotidyltransferases	ARG-ANNOT
(AGly)AadA11:AJ567827:1-792:792	Aminoglycoside	Aminoglycoside nucleotidyltransferases	ARG-ANNOT
(AGly)AadA24:HQ123586:88-768:781	Aminoglycoside	Aminoglycoside nucleotidyltransferases	ARG-ANNOT
(AGly)AadA5:AF137361:64-852:789	Aminoglycoside	Aminoglycoside nucleotidyltransferases	ARG-ANNOT
(AGly)AadA7:AF224733:32-829:798	Aminoglycoside	Aminoglycoside adenyltransferases	ARG-ANNOT
(AGly)AadA9:AJ420072:26773-27609:837	Aminoglycoside	Aminoglycoside nucleotidyltransferases	ARG-ANNOT
(AGly)AadD:AF181950:3176-3946:771	Aminoglycoside	Aminoglycoside nucleotidyltransferases	ARG-ANNOT
(AGly)Ant6-Ia:AF330699:22-930:909	Aminoglycoside	Aminoglycoside nucleotidyltransferases	ARG-ANNOT
(AGly)Ant6-Ib:FN594949:27482-28339:858	Aminoglycoside	Aminoglycoside nucleotidyltransferases	ARG-ANNOT
(AGly)Aph3-Ia:HQ840942:23569-24384:816	Aminoglycoside	Aminoglycoside phosphotransferases	ARG-ANNOT
(AGly)Aph3-III:V01547:535-1329:795	Aminoglycoside	Aminoglycoside phosphotransferases	ARG-ANNOT
(AGly)Aph3Ia:FJ172370:38668-39483:816	Aminoglycoside	Aminoglycoside phosphotransferases	ARG-ANNOT
(AGly)Aph4-Ia:V01499:231-1256:1026	Aminoglycoside	Aminoglycoside phosphotransferases	ARG-ANNOT
(AGly)Sat-2A:X51546:518-1042:525	Aminoglycoside	Aminoglycoside acetyltransferases	ARG-ANNOT
(AGly)Sat4A:X92945:38870-39412:543	Aminoglycoside	Aminoglycoside acetyltransferases	ARG-ANNOT
(AGly)Spc:X02588:331-1113:783	Spectinomycin	Spectinomycin adenyltrasnferase	ARG-ANNOT
(AGly)Str:X92946:18908-18060:849	Aminoglycoside	Aminoglycoside adenyltransferases	ARG-ANNOT
(AGly)StrA:AB366441:22458-23261:804	Aminoglycoside	Aminoglycoside phosphotransferases	ARG-ANNOT
(AGly)StrB:FJ474091:264-1100:837	Aminoglycoside	Aminoglycoside phosphotransferases	ARG-ANNOT
(Bla)ACI-1:AJ007350:240-1094:855	β-lactam	Class A β-lactamases	ARG-ANNOT
(Bla)AmpC1_Ecoli:FN649414:2765051-2766355:1302	β-lactam	Class C β-lactamases	ARG-ANNOT
(Bla)AmpC2_Ecoli:CP002970:332756-333889:1134	β-lactam	Class C β-lactamases	ARG-ANNOT
(Bla)AMPH_Ecoli:AP012030:395554-396711:1158	β-lactam	Class C β-lactamases	ARG-ANNOT
(Bla)CARB-10:EU850412:2270-3166:897	β-lactam	Class A β-lactamases	ARG-ANNOT
(Bla)CARB-4:AY913772:1600-2466:867	β-lactam	Class A β-lactamases	ARG-ANNOT
(Bla)CARB-5:AF135373:12-908:897	β-lactam	Class A β-lactamases	ARG-ANNOT
(Bla)cfxA:U38243:150-1115:966	β-lactam	Class A β-lactamases	ARG-ANNOT
(Bla)cfxA4:AY769933:1-966:966	β-lactam	Class A β-lactamases	ARG-ANNOT
(Bla)cfxA5:AY769934:28-993:966	β-lactam	Class A β-lactamases	ARG-ANNOT
(Bla)cfxA6:GQ342996:798-1793:966	β-lactam	Class A β-lactamases	ARG-ANNOT
(Bla)cphA5:AY227051:1-765:765	β-lactam	Class B (metallo-)β-lactamases	ARG-ANNOT
(Bla)OXA-235:JQ820240:591-1421:831	β-lactam	Class D β-lactamases	ARG-ANNOT
(Bla)OXA-347:JN086160:1583-2407:825	β-lactam	Class D β-lactamases	ARG-ANNOT

(Bla)Penicillin_Binding_Protein_Ecoli:CP002291:664439-666340:1902	β-lactam	Genes modulating β-lactam resistance	ARG-ANNOT
(Bla)ROB-1:AF022114:303-1220:918	β-lactam	Class A β-lactamases	ARG-ANNOT
(Bla)TEM-116:AY425988:6-866:861	β-lactam	Class A β-lactamases	ARG-ANNOT
(Gly)VanA-G:AY271782:157-606:450	Glycopeptide	Van	ARG-ANNOT
(MLS)CfrA:AM408573:10028-11077:1050	MLS	Cfr 23S rRNA methyltransferase	ARG-ANNOT
(MLS)Erm33:AJ313523:163-894:732	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)Erm35:AF319779:33-833:801	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)Erm42:FR734406:1-906:906	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)ErmA:X03216:4551-5282:732	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)ErmB:M11180:714-1451:738	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)ErmC:M19652:988-1722:735	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)ErmF:M14730:241-1041:801	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)ErmG:M15332:672-1406:735	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)ErmQ:L22689:262-1035:774	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)ErmT:M64090:168-902:735	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)ErmX:M36726:296-1150:855	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)ErmY:AB014481:556-1290:735	MLS	Erm 23S rRNA methyltransferases	ARG-ANNOT
(MLS)LnuB:AJ238249:127-930:804	MLS	Lincosamide nucleotidyltransferase (Lin)	ARG-ANNOT
(MLS)LnuC:AY928180:1150-1644:495	MLS	Lincosamide nucleotidyltransferase (Lin)	ARG-ANNOT
(MLS)LsaB:AJ579365:4150-5628:1479	MLS	Streptogramin resistance ATP-binding cassette (ABC) efflux pumps	ARG-ANNOT
(MLS)MefA:U70055:314-1531:1218	MLS	Macrolide resistance efflux pumps	ARG-ANNOT
(MLS)MefB:FJ196385:11084-12313:1230	MLS	Macrolide resistance efflux pumps	ARG-ANNOT
(MLS)MphB:D85892:1159-2067:909	MLS	Macrolide phosphotransferases (MPH)	ARG-ANNOT
(MLS)MphE:JF769133:8777-9661:885	MLS	Macrolide phosphotransferases (MPH)	ARG-ANNOT
(MLS)MsrD:AF274302:2462-3925:1464	MLS	Macrolide resistance efflux pumps	ARG-ANNOT
(MLS)MsrE:JF769133:7246-8721:1476	MLS	Macrolide resistance efflux pumps	ARG-ANNOT
(MLS)VgaD:GQ205627:1394-2971:1578	Efflux pumps conferring antibiotic resistance	ABC antibiotic efflux pump	ARG-ANNOT
(Phe)CatB10:AJ878850:1197-1829:633	Phenicol	Chloramphenicol acetyltransferase (CAT)	ARG-ANNOT
(Phe)CatQ:M55620459-1118:660	Phenicol	Chloramphenicol acetyltransferase (CAT)	ARG-ANNOT
(Phe)Cmr:U85507:3518-4693:1172	Phenicol	Chloramphenicol active extrusion	ARG-ANNOT
(Phe)FexA:JQ041372:14024-15451:1428	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	ARG-ANNOT
(Phe)FloR:AKLJ01000508:383-1597:1215	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	ARG-ANNOT
(Sul)SulII:EU360945:1617-2432:816	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	ARG-ANNOT
(Tet)Tet-31:AJ250203:1651-2883:1233	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	ARG-ANNOT
(Tet)Tet-32:DQ647324:181-2100:1920	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)Tet-33:AJ420072:22940-24163:1224	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	ARG-ANNOT

(Tet)Tet-36:AJ514254:2534-4456:1923	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)Tet-39:AY743590:749-1936:1188	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	ARG-ANNOT
(Tet)Tet-40:AM419751:14211-15431:1221	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)Tet-44:FN594949:25245-27167:1923	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)TetA-P:L20800:	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	ARG-ANNOT
(Tet)TetA:JX424423:94438-95712:1275	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	ARG-ANNOT
(Tet)TetB-P:L20800:2309-4267:1959	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)TetC:EU751612:1-1191:1191	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	ARG-ANNOT
(Tet)TetE:DQ366299:36-1253:1218	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	ARG-ANNOT
(Tet)TetL:FN435329:1-1377:1377	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	ARG-ANNOT
(Tet)TetM:U08812:1981-3900:1920	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)TetO:M18896:207-2126:1920	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)TetQ:Z21523:362-2287:1926	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)TetR:HF545434:53576-54226:651	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)TetS:L09756:447-2372:1926	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)TetT:L42544:478-2433:1956	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)TetW:AJ222769:3687-5606:1920	Tetracycline	Tetracycline resistance ribosomal protection proteins	ARG-ANNOT
(Tet)TetX:M37699:586-1752:1167	Tetracycline	Tetracycline inactivation enzyme TetX	ARG-ANNOT
(Tet)TetY:AF070999:1680-2855:1176	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	ARG-ANNOT
(Tet)TetZ:AF121000:11880-13034:1155	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	ARG-ANNOT
(Tmt)DfrA3:J03306:103-591:489	Trimethoprim	dihydrofolate reductase	ARG-ANNOT
aadA1_1_X02340	Aminoglycoside	Aminoglycoside adenyltransferases	Resfinder
aadA1_2_JN815078	Aminoglycoside	Aminoglycoside adenyltransferases	Resfinder
aadA1_3_JQ414041	Aminoglycoside	Aminoglycoside adenyltransferases	Resfinder
aadA1_5_JQ480156	Aminoglycoside	Aminoglycoside adenyltransferases	Resfinder
aadA1_5_JX185132	Aminoglycoside	Aminoglycoside adenyltransferases	Resfinder
aadA11_1_AY144590	Aminoglycoside	Aminoglycoside adenyltransferases	Resfinder
AB104852.1.gene4	Aminoglycoside	Aminoglycoside adenyltransferases	CARD
AF024666.2.gene33	Phenicol	Chloramphenicol efflux	CARD
AF047479.2.gene12	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	CARD
AF118107.1.gene1	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	CARD

AF118110.1.gene1	β-lactam	Class A β-lactamases	CARD
AF205943.1.gene15	Aminoglycoside	Aminoglycoside adenyltransferases	CARD
AF231986.2.gene2	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	CARD
AF252855.1.gene1	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	CARD
AF313472.2.gene15	Aminoglycoside	Aminoglycoside phosphotransferases	CARD
AF313472.2.gene16	Aminoglycoside	Aminoglycoside phosphotransferases	CARD
AF332662.1.gene1	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	CARD
AJ223604.1.gene9	Efflux pumps conferring antibiotic resistance	Small multidrug resistance (SMR) antibiotic efflux pump	CARD
AJ295238.gene	Tetracycline	Tetracycline resistance ribosomal protection proteins	CARD
AJ518835.1.gene6	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	CARD
AJ549214.1.gene2	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	CARD
AM180355.1.gene636	Tetracycline	Tetracycline resistance ribosomal protection proteins	CARD
AM296480.1.gene1	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	CARD
AM399080.1.gene2	MLS	Lincosamide nucleotidyltransferase (Lin)	CARD
AP009048.1.gene1101	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD
AP009048.1.gene1113	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD
AP009048.1.gene1178	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene1179	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene1295	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene1587	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene1721	Efflux pumps conferring antibiotic resistance	Multidrug and toxic compound extrusion (MATE) transporter	CARD
AP009048.1.gene2132	Efflux pumps conferring antibiotic resistance	Resistance-nodulation-cell division (RND) efflux pump	CARD
AP009048.1.gene2133	Efflux pumps conferring antibiotic resistance	Resistance-nodulation-cell division (RND) efflux pump	CARD
AP009048.1.gene2134	Efflux pumps conferring antibiotic resistance	Resistance-nodulation-cell division (RND) efflux pump	CARD
AP009048.1.gene2135	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD
AP009048.1.gene2136	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene2137	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene2287	Fluoroquinolone	Fluoroquinolone-resistant DNA topoisomerases	CARD
AP009048.1.gene2312	Polymyxin B	bifunctional polymyxin resistance protein	CARD
AP009048.1.gene2427	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD

AP009048.1.gene2430	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene2520	Efflux pumps conferring antibiotic resistance	Resistance-nodulation-cell division (RND) efflux pump	CARD
AP009048.1.gene2734	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD
AP009048.1.gene2735	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD
AP009048.1.gene3066	Fluoroquinolone	Fluoroquinolone-resistant DNA topoisomerases	CARD
AP009048.1.gene3077	Aminocoumarins	Aminocoumarin-resistant DNA topoisomerases	CARD
AP009048.1.gene3103	Bacitracin	phosphatase	CARD
AP009048.1.gene3222	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	CARD
AP009048.1.gene3307	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene3308	Efflux pumps conferring antibiotic resistance	Resistance-nodulation-cell division (RND) efflux pump	CARD
AP009048.1.gene3309	Efflux pumps conferring antibiotic resistance	Resistance-nodulation-cell division (RND) efflux pump	CARD
AP009048.1.gene3341	Rifampin	Rifampin-resistant beta-subunit of RNA polymerase (RpoB)	CARD
AP009048.1.gene3414	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene3415	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene3616	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD
AP009048.1.gene3819	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene3822	Efflux pumps conferring antibiotic resistance	Resistance-nodulation-cell division (RND) efflux pump	CARD
AP009048.1.gene3823	Efflux pumps conferring antibiotic resistance	Resistance-nodulation-cell division (RND) efflux pump	CARD
AP009048.1.gene3929	Genes reducing permeability to antibiotics	Porin modification	CARD
AP009048.1.gene3975	Genes modulating antibiotic efflux	Regulatory system	CARD
AP009048.1.gene596	Efflux pumps conferring antibiotic resistance	Small multidrug resistance (SMR) antibiotic efflux pump	CARD
aph(3)-Ia_1_V00359	Aminoglycoside	Aminoglycoside phosphotransferases	Resfinder
AY034138.1.gene10	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	CARD
AY115475.1.gene3	Aminoglycoside	Aminoglycoside adenyltransferases	CARD
AY171578.gene	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	CARD
AY216678.1.gene1	Aminoglycoside	Aminoglycoside acetyltransferases	CARD
AY339625.2.gene5	Aminoglycoside	Aminoglycoside phosphotransferases	CARD
AY712687.1.gene1	Aminoglycoside	Aminoglycoside adenyltransferases	CARD
AY758206.1.gene7	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	CARD

blaCARB-8_1_AY178993	β-lactam	Class A β-lactamases	Resfinder
blaROB-1_1_DQ840517	β-lactam	Class A β-lactamases	Resfinder
blaTEM-1A_4_HM749966	β-lactam	Class A β-lactamases	Resfinder
cat_2_M35190	Phenicol	Chloramphenicol acetyltransferase (CAT)	Resfinder
cfxA3_1_AF472622	β-lactam	Class A β-lactamases	Resfinder
CP000034.1.gene2186	Genes modulating antibiotic efflux	Regulatory system	CARD
CP000034.1.gene2198	Genes reducing permeability to antibiotics	Unknown	CARD
CP000034.1.gene2328	β-lactam	Mutant porin proteins conferring antibiotic resistance	CARD
CP000034.1.gene2879	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD
CP000034.1.gene3210	Aminocoumarins	Aminocoumarin-resistant DNA topoisomerases	CARD
CP000034.1.gene3741	Rifampin	Rifampin-resistant beta-subunit of RNA polymerase (RpoB)	CARD
CP000034.1.gene3834	Genes modulating antibiotic efflux	Regulatory system	CARD
CP000647.1.gene3761	Elfamycins	EF-Tu inhibition	CARD
CP000647.1.gene4394	Elfamycins	EF-Tu inhibition	CARD
cphA1_4_AY261376	β-lactam	Class B (metallo-)β-lactamases	Resfinder
D78168.1.gene1	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD
D78168.1.gene2	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD
DQ464881.1.gene4	Aminoglycoside	Aminoglycoside phosphotransferases	CARD
DQ516954.1.gene1	Efflux pumps conferring antibiotic resistance	ABC antibiotic efflux pump	CARD
DQ516970.1.gene1	Efflux pumps conferring antibiotic resistance	ABC antibiotic efflux pump	CARD
DQ677333.1.gene1	Aminoglycoside	Aminoglycoside adenyltransferases	CARD
EF118171.1.gene7	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	CARD
erm(42)_2_AB601890	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(A)_3_EU348758	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(B)_1_JN899585	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(B)_12_U18931	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(B)_16_X82819	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(B)_17_X64695	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(B)_18_X66468	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(B)_7_AF368302	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(B)_9_AF299292	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(C)_1_V01278	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(C)_12_Y09003	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(C)_13_M13761	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(C)_15_U82607	MLS	Erm 23S rRNA methyltransferases	Resfinder

erm(F)_3_M17808	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(F)_4_M62487	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(G)_2_L42817	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(T)_2_AY894138	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(T)_3_AF310974	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(X)_2_X51472	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(X)_3_U21300	MLS	Erm 23S rRNA methyltransferases	Resfinder
erm(X)_4_NC_005206	MLS	Erm 23S rRNA methyltransferases	Resfinder
EU434751.1.gene2	Tetracycline	Tetracycline resistance ribosomal protection proteins	CARD
fexA_1_AJ549214	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	Resfinder
FJ768952.1.gene1	Efflux pumps conferring antibiotic resistance	Resistance-nodulation-cell division (RND) efflux pump	CARD
floR_1_AF071555	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	Resfinder
floR_2_AF118107	Phenicol	Florfenicol and chloramphenicol resistance efflux pump	Resfinder
FQ312006.1.gene706	Aminoglycoside	Aminoglycoside phosphotransferases	CARD
GQ149347.1.gene3	Glycopeptide	BRP	CARD
GQ205627.2.gene3	MLS	Streptogramin Vat acetyltransferase	CARD
GQ465831.1.gene2	Genes reducing permeability to antibiotics	Porin modification	CARD
GU060319.1.gene3	Aminoglycoside	Aminoglycoside adenyltransferases	CARD
GU371926.1.gene94	β-lactam	Class A β-lactamases	CARD
JQ364968.1.gene6	β-lactam	Class A β-lactamases	CARD
JQ394987.1.gene1	Efflux pumps conferring antibiotic resistance	MFS antibiotic efflux pump	CARD
JQ740052.1.gene2	Tetracycline	Tetracycline resistance ribosomal protection proteins	CARD
JQ861959.1.gene11	MLS	Lincosamide nucleotidyltransferase (Lin)	CARD
JX560992.1.gene10	Spectinomycin	Spectinomycin adenyltrasnferase	CARD
JX560992.1.gene8	Aminoglycoside	Aminoglycoside adenyltransferases	CARD
L22689.1.gene1	MLS	Erm 23S rRNA methyltransferases	CARD
M12730.1.gene3	MLS	Erm 23S rRNA methyltransferases	CARD
M58408.gene	Fluoroquinolone	Fluoroquinolone-resistant DNA topoisomerases	CARD
M62487.1.gene1	MLS	Erm 23S rRNA methyltransferases	CARD
M86701.1.gene1	Aminoglycoside	Aminoglycoside phosphotransferases	CARD
M86913.1.gene3	Aminoglycoside	Aminoglycoside adenyltransferases	CARD
mph(E)_3_EU294228	MLS	Macrolide phosphotransferases (MPH)	Resfinder
msr(D)_3_AF227520	MLS	Macrolide resistance efflux pumps	Resfinder
NC_002695.1.914983	Genes modulating antibiotic efflux	Regulatory system	CARD
NC_002695.1.915041	Genes modulating antibiotic efflux	Regulatory system	CARD
NC_002695.1.915651	Genes modulating antibiotic efflux	Regulatory system	CARD

NC_002695.1.916016	Genes modulating antibiotic efflux	Regulatory system	CARD
NC_002695.1.916103	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	CARD
NC_002695.1.916234	Bacitracin	phosphatase	CARD
NC_002695.1.916274	Fluoroquinolone	Fluoroquinolone-resistant DNA topoisomerases	CARD
NC_002695.1.917339	Genes modulating antibiotic efflux	Regulatory system	CARD
NC_004973.1.1449625	Aminoglycoside	Aminoglycoside phosphotransferases	CARD
NC_004973.1.1449626	Aminoglycoside	Aminoglycoside phosphotransferases	CARD
NC_011595.7072242	Elfamycins	EF-Tu inhibition	CARD
NC_023287.1.18156494	MLS	Macrolide resistance efflux pumps	CARD
str_2_FN435330	Aminoglycoside	Aminoglycoside adenyltransferases	Resfinder
strA_2_M28829	Aminoglycoside	Aminoglycoside phosphotransferases	Resfinder
strA_3_AF024602	Aminoglycoside	Aminoglycoside phosphotransferases	Resfinder
strA_4_NC_003384	Aminoglycoside	Aminoglycoside phosphotransferases	Resfinder
strA_5_AF321550	Aminoglycoside	Aminoglycoside phosphotransferases	Resfinder
strB_3_AF024602	Aminoglycoside	Aminoglycoside phosphotransferases	Resfinder
sul1_2_CP002151	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul1_9_AY963803	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_1_AF542061	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_10_AM183225	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_11_AY232670	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_12_AF497970	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_13_AJ289135	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_14_AJ514834	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_15_FJ968160	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_17_U57647	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_18_AJ830714	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_19_AJ319822	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_20_AJ830710	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_3_HQ840942	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_6_FN995456	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_7_HM486907	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_8_AJ877041	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
sul2_9_FJ197818	Sulfonamide	Sulfonamide-resistant dihydropteroate synthases	Resfinder
tet(31)_1_GQ283908	Tetracycline	Unknown	Resfinder
tet(32)_1_EU722333	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(33)_1_DQ077487	Tetracycline	Tetracycline resistance major	Resfinder

		facilitator superfamily (MFS) efflux pumps	
tet(33)_3_DQ390458	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(39)_2_EU495991	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(40)_1_FJ158002	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(40)_2_FJ158002	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(44)_1_NZ_ABDU01000081	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(A)_2_X00006	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(A)_3_AY196695	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(A)_4_AJ517790	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(A)_5_AJ419171	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(A)_6_AJ313332	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(B)_3_AP000342	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(C)_1_NC_002109	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(C)_2_NC_003123	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(C)_6_Y19114	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(C)_9_AY046276	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(G)_2_AF133139	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(H)_1_Y16103	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(H)_2_AJ245947	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(H)_3_Y15510	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(H)_4_U00792	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(L)_1_HM235948	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(L)_4_M11036	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(L)_5_D00006	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tet(M)_1_X92947	Tetracycline	Tetracycline resistance ribosomal	Resfinder

		protection proteins	
tet(M)_11_JN846696	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(M)_12_FR671418	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(M)_13_AM990992	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(M)_2_X90939	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(M)_4_X75073	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(M)_5_U58985	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(M)_6_M21136	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(M)_7_FN433596	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(M)_8_X04388	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(M)_9_X56353	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(O)_2_M20925	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(O)_3_Y07780	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(Q)_1_L33696	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(Q)_2_X58717	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(Q)_3_U73497	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(S)_1_DQ377340	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(S)_3_X92946	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(T)_2_AY660530	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(W)_1_DQ060146	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(W)_2_AY049983	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(W)_5_AJ427421	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(W)_6_FN396364	Tetracycline	Tetracycline resistance ribosomal protection proteins	Resfinder
tet(X)_1_GU014535	Tetracycline	Tetracycline inactivation enzyme TetX	Resfinder
tet(X)_3_AB097942	Tetracycline	Tetracycline inactivation enzyme TetX	Resfinder
tetA(P)_1_AB054980	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
tetA(P)_4_AB001076	Tetracycline	Tetracycline resistance major facilitator superfamily (MFS) efflux pumps	Resfinder
U01945.1.gene1	Aminoglycoside	Aminoglycoside acetyltransferases	CARD

Table S5.4: Resistance classification by class and mechanism.

Classes of Resistance -- Specific	**Mechanisms of Resistance**
Aminocoumarins	Aminocoumarin-resistant DNA topoisomerases
Aminoglycosides	Aminoglycoside acetyltransferases Aminoglycoside adenyltransferases Aminoglycoside phosphotransferases
Bacitracin	Bacitracin phosphatase
Elfamycins	EF-Tu inhibition
Fluoroquinolones	Fluoroquinolone-resistant DNA topoisomerases
Glycopeptides	Bleomycin resistance protein Vancomycin resistance proteins
Macrolide-Lincosamide-Streptogramin	Macrolide phosphotransferases Macrolide resistance efflux pumps Erm 23S rRNA methyltransferases Cfr 23S rRNA methyltransferase Lincosamide nucleotidyltransferase Streptogramin resistance ATP-binding cassette efflux pump Streptogramin acetyltransferase
Phenicol	Florfenicol and chloramphenicol resistance efflux pump Chloramphenicol acetyltransferase Chloramphenicol active extrusion Chloramphenicol efflux
Polymyxin B	Bifunctional polymyxin resistance protein
Rifampin	Rifampin-resistant beta-subunit of RNA polymerase
Spectinomycin	Spectinomycin adenyltrasnferase
Sulfonamide	Sulfonamide-resistant dihydropteroate synthases
Tetracycline	Tetracycline inactivation enzyme (TetX) Tetracycline major facilitator superfamily efflux pumps Tetracycline ribosomal protection proteins Unknown (Tet31)
Trimethoprim	Trimethoprim dihydrofolate reductase
β-Lactam	Class A β-lactamases Class B β-lactamases Class C β-lactamases Class D β-lactamases Genes modulating β-lactam resistance Mutant porin proteins conferring antibiotic resistance
Classes of Resistance -- General	**Mechanism**
Genes modulating antibiotic efflux	Regulatory system
Genes reducing permeability to antibiotics	Porin modification Unknown
Efflux pumps conferring antibiotic resistance	Major facilitator superfamily antibiotic efflux pump Multidrug and toxic compound extrusion transporter Resistance-nodulation-cell division efflux pump Small multidrug resistance antibiotic efflux pump ATP-binding cassette antibiotic efflux pump

Table S5.5: List of classes of resistance determinants to critically important antimicrobials searched for across all 87 samples.

Classification	Group	Result
Carbapenemases	bla(OXA)	identified
	bla(SME)	not identified
	bla(IMI)	not identified
	bla(NMD)	not identified
	bla(GES)	not identified
	bla(KPC)	not identified
	bla(cphA)[a]	identified
Extended-spectrum β-lactamases	bla(TEM)	identified
	bla(SHV)	not identified
	bla(CTX-M)	not identified
	bla(CMY)	not identified
	bla(NDM)	not identified
Quinupristin-dalfopristin resistance	vga/vat	identified
Linezolid resistance	cfr	identified

[a]bla(cphA) is a strict carbapenemase

Table S5.6: Critically important ARDs identified in the metagenomic data.

Gene ID (database)	Resistance Type	Sample Type, Location	Characteristics
(Bla)TEM-116:AY425988:6-866:861 (ARG-ANNOT)	ESBL (TEM)	1 x water, holding	100% nucleotide homology with reference across at least 80% gene fraction (minimum 1x coverage)
(Bla)OXA-347:JN086160:1583-2407:825 (ARG-ANNOT)	Carbapenemase (OXA)[a]	1 x water, holding 1 x soil, arrival	100% nucleotide homology with reference across at least 80% gene fraction (minimum 1x coverage)
(Bla)OXA-235:JQ820240:591-1421:831 (ARG-ANNOT)	Carbapenemase (OXA)	1 x soil, arrival	100% nucleotide homology to KSG, FGN and STFK motifs
cphA1_2_AYAY261377 (Resfinder)	Strict carbapenemase (cphA)	3 x water, holding	100% amino acid homology to reference (4 silent substitutions)
GQ205627.2.gene3 (CARD) AND (MLS)VgaD:GQ205627:1394-2971:1578	Quinuspristin-dalfopristin resistance (vatG and VgaD)	1 x soil, arrival	100% nucleotide homology to Walker A and B motifs; silent substitution in RSGG motif 100% nucleotide homology to LβH hexapeptide repeat domain
(MLS)CfrA:AM408573:10028-11077:1050 (ARG-ANNOT)	Multi-drug resistance to $PhLOPS_A$ (phenicol, lincosamide, oxazolidinones (linezolid), pleuromutilins, and streptogramin A)	4 x sponge, truck (all in Texas)	100% nucleotide homology with reference across at least 95% gene fraction (minimum 1x coverage)

[a]Note that phenotypic resistance to carbapenems has not been confirmed for this ARD

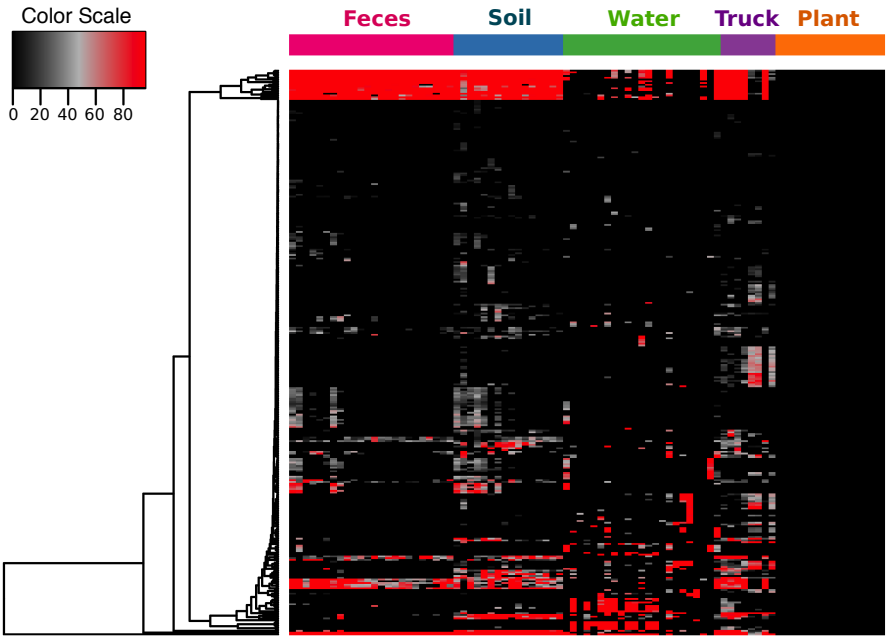

Figure S5.1. Heatmap of the 319 ARDs (rows) identified in 87 samples (columns) collected from the beef production system. Samples are grouped by sampling location. Normalized ARD counts are clustered using Euclidean distances with complete linkage.

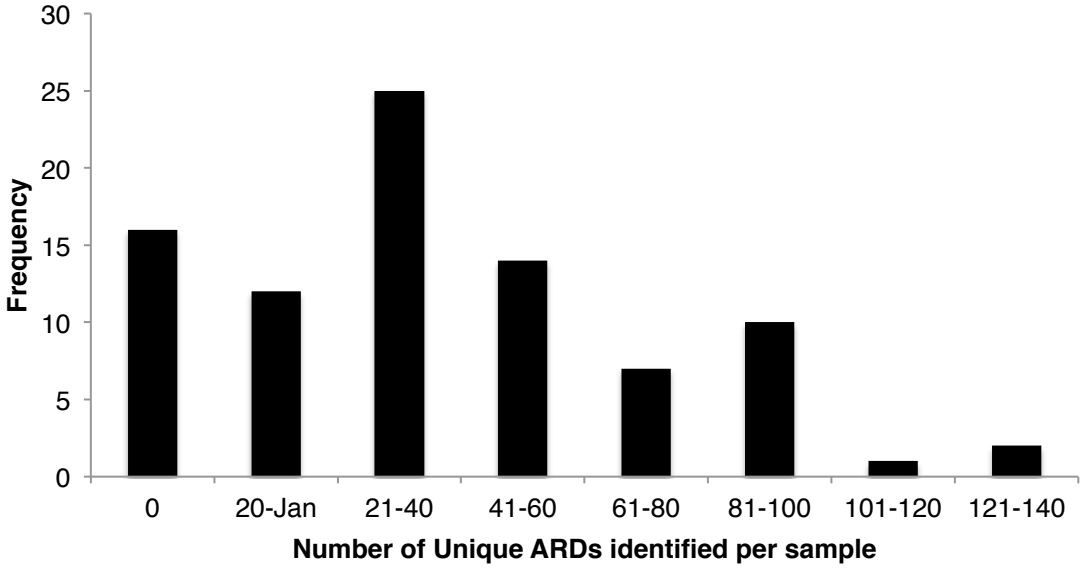

Figure S5.2. Histogram of unique ARDs identified per sample (N=87).

CONCLUSIONS

The overall goal of this dissertation was to advance our collective understanding of the epidemiology and ecology of AMR within beef production, and in particular to better understand how AMU practices influence AMR dynamics in the context of beef production as an integrated, multi-level system. Unfortunately, it is this crucial phrase – "as an integrated, multi-level system" – that makes this area of research so challenging. Understanding, reproducing and even predicting AMU-AMR associations *in vitro* or in tightly controlled populations is a tractable endeavor. The insights gained from such research, however, rarely extend to commercial or community settings. As a result, observational studies and field trials are necessary to generate externally valid findings with respect to AMU and AMR. The 4 projects that comprise this dissertation were conducted in large commercial beef production operations. The findings are representative of AMU-AMR dynamics under standard management conditions within the operations in the studies, which in general are representative of conditions in many large North American feedlots and fabrication plants.

The primary goal of chapter 1 was to understand how AMU and AMR interact within a pathogen of major economic and animal health impact, *Mannheimia haemolytica*. We found relatively low prevalence of AMR *M. haemolytica* overall, and no associations between AMU and AMR to any single drug. However, parenteral AMU greatly increased the odds of isolating MDR *M. haemolytica*, which comprised over half of all resistant isolates. Interestingly, parenteral AMU in both the sampled cattle themselves and in their pen mates increased these odds, suggesting that the shared pen environment and/or animal-to-animal contact play a major role in the epidemiology of AMR *M. haemolytica* within feedlots. This points to the inherently

multi-level nature of AMR, which necessitates a research strategy that can assess interaction between hosts and their environment, as well as between hosts within a shared environment.

In chapter 2, our goal was to characterize associations between AMU and AMR in a widely used "indicator" organism, non-type specific *Escherichia coli*. We found relatively high prevalence of resistance, particularly to tetracycline, across all sampling time points including arrival in the feedlot. In the short term, use of parenteral tetracycline and sulfonamides increased the odds of recovering tetracycline and sulfisoxazole resistant NTSEC, respectively, while use of parenteral macrolides decreased the odds of recovering ampicillin resistant NTSEC. These results demonstrate the non-intuitive nature of AMU and AMR, and indicate the need for a better understanding of how different AMD selection pressures modify resistance profiles under real-world feedlot conditions. This, in turn, necessitates a deeper understanding of the microbial population genetics of AMR in the context of real-world AMU and other management practices. In addition, the 3 AMU-AMR associations identified in chapter 2 only applied to AMDs administered within 7 days prior to sampling, highlighting the temporal nature of AMU and AMR. In order to understand the course of AMR during beef production, we need to investigate AMR in the context of production as a longitudinally integrated system that employs multiple, successional interventions that can substantially alter the microbial community and AMR through intensive antimicrobial pressures on the microbial population and its collective genetics.

When interpreted as a whole, the results of chapters 1 and 2 also demonstrate the potential danger of extrapolating AMR-related findings from one bacterial species or group to the entire system. Importantly, the same cattle and pens were sampled for chapters 1 and 2, and they were sampled for *M. haemolytica* and NTSEC at the same time. In this context, it becomes readily apparent that different organisms exhibit different AMR patterns, even within the same

host and under the same external conditions. For instance, 88% of *M. haemolytica* isolates were pan-susceptible, compared to <19% of NTSEC isolates. This casts serious doubt on the representativeness of any single bacterial species or group as an "indicator" for AMR dynamics within the larger microbial community, or for AMR within pathogens or other organisms of interest. Culture-based methods are appropriate for the purposes of quantifying, monitoring or tracking AMR within specific pathogens in beef production; however, they are inadequate if we need to understand the total risk posed by different beef production management practices on the transmittance of AMR bacteria and genetic determinants to humans.

Chapter 4 was designed to describe the genetic AMR potential (the "resistome") of beef production waste and its variability across management systems and effluents. We found that the resistome of feedlot wastes differed significantly from that of dairy and cow-calf wastes, and that the AMR profile of fecal and water wastes were significantly different from one another. AMU practices on dairies and ration composition in feedlots did not significantly impact resistome composition, although samples sizes were likely too small to provide sufficient power. This suggests that production system and microbial environment may exert a larger influence over AMR dynamics than any single management practice, and highlights the necessity of investigating AMU-AMR associations in commercial production units operating under standard management protocols.

The goal of chapter 5 was to provide a description of the resistome of pens of cattle and their environment as the cattle moved through the beef production process. We found that the resistome is fundamentally altered during the feeding period, during transport from the feedlot to slaughter, and during the slaughter and fabrication process. The diversity of the resistome, but not the microbiome, decreased significantly during the feeding period, indicating selective

pressure specifically on the genetic AMR potential of the microbial population. Resistome diversity then increased in the transport truck and holding pens prior to slaughter, likely reflecting the mixing of cattle from diverse sources in these confined areas. And finally, we demonstrated that the multi-hurdle interventions utilized in the slaughter and fabrication process drastically reduced the number of unique microbial species and AMR genetic determinants within beef products destined for retail. Overall, this project highlighted the utility and promise of investigating AMR in the context of entire microbial communities, and also emphasized the importance of framing AMR dynamics within the longitudinal and systems-based nature of beef production.

Together, chapters 4 and 5 also provide a general comparison of the risk posed by the two AMR transmission routes historically considered of primary importance, i.e., the food supply and environmental contamination with wastes. Presently, a much larger proportion of governmental regulation and surveillance is dedicated to the food supply as compared to environmental dissemination. This makes sense given foodborne pathogen concerns, the structure of our food industry and the widespread distribution of many food items. However, the results of chapters 4 and 5 suggest that wastewater or agricultural soil contamination with livestock production wastes may also represent sources of risk of AMR transmission. This interpretation also makes sense from the perspective of beef production as an integrated system with intensive microbial interventions applied during slaughter and fabrication, but comparatively few interventions applied to wastewater and manure.

Chapters 4 and 5 also highlight the infancy of the metagenomics approach to investigating AMR within complex, multi-level systems. One major knowledge gap pertains to evaluating the resistome in relation to its overall risk to human health. What constitutes a

"risky" resistome versus a "non-risky" one (224)? A resistome with more resistance genes is probably riskier than one with fewer, but what about the composition of the AMR profile? Certainly some AMR genes are more of a human health concern than others, but how do you assign a number to this? Furthermore, how can we differentiate AMR genes that are part of the "baseline" resistome, and those that arise and persist due to anthropogenic activities (227)? Finally, and most importantly, how do we measure the success or failure of a management intervention in terms of its impact on the AMR composition in a given environment, host or sample? The microbiome field is also grappling with such questions, and there is a growing consensus that there is no single "healthy" microbiome, but rather multiple "community types" that can be associated with different life histories (228,229). This can make it difficult to discern the microbiome's role in disease processes in individuals. Resistome researchers in the agricultural field will have an even more difficult time trying to define the "unhealthy" resistome because of all of the traditional epidemiological challenges of linking agricultural production practices with disease in humans. And this leads to an important, closing remark: the ability to access the resistome does not obviate the need to conduct epidemiologically sound studies; rather, it adds a crucial and long-missing piece to the overall puzzle of AMU-AMR associations in livestock production. The challenge now is to integrate this newfound knowledge with what we already know, and to design scientifically sound studies that incorporate this novel approach.

REFERENCES

1. Smith R, Coast J. The true cost of antimicrobial resistance. BMJ. 2013;346:f1493.

2. Tadesse DA, Zhao S, Tong E, Ayers S, Singh A, Bartholomew MJ, et al. Antimicrobial Drug Resistance in Escherichia coli from Humans and Food Animals, United States, 1950-2002. Emerg Infect Dis. 2012 May;18(5):741–9.

3. Depoorter P, Persoons D, Uyttendaele M, Butaye P, De Zutter L, Dierick K, et al. Assessment of human exposure to 3rd generation cephalosporin resistant E. coli (CREC) through consumption of broiler meat in Belgium. Int J Food Microbiol. 2012 Sep 17;159(1):30–8.

4. Mayrhofer S, Paulsen P, Smulders FJM, Hilbert F. Antimicrobial resistance in commensal Escherichia coli isolated from muscle foods as related to the veterinary use of antimicrobial agents in food-producing animals in Austria. Microb Drug Resist Larchmt N. 2006;12(4):278–83.

5. Heuer H, Kopmann C, Binh CTT, Top EM, Smalla K. Spreading antibiotic resistance through spread manure: characteristics of a novel plasmid type with low %G+C content. Environ Microbiol. 2009 Apr;11(4):937–49.

6. Li J, Wang T, Shao B, Shen J, Wang S, Wu Y. Plasmid-mediated quinolone resistance genes and antibiotic residues in wastewater and soil adjacent to swine feedlots: potential transfer to agricultural lands. Environ Health Perspect. 2012 Aug;120(8):1144–9.

7. Roberts RR, Hota B, Ahmad I, Scott RD, Foster SD, Abbasi F, et al. Hospital and Societal Costs of Antimicrobial-Resistant Infections in a Chicago Teaching Hospital: Implications for Antibiotic Stewardship. Clin Infect Dis. 2009 Oct 15;49(8):1175–84.

8. Dantes R, Mu Y, Belflower R, Aragon D, Dumyati G, Harrison LH, et al. National burden of invasive methicillin-resistant Staphylococcus aureus infections, United States, 2011. JAMA Intern Med. 2013 Nov 25;173(21):1970–8.

9. FDA. Guidance for Industry #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 [Internet]. FDA Center for Veterinary Medicine; 2013 [cited 2015 Jun 24]. Available from: http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM299624.pdf

10. Feedlot 2011 Part I: Management Practices on U.S. Feedlots with a Capacity of 1,000 or More Head. USDA Animal and Plant Health Inspection Service; 2013.

11. USDA. Feedlot 2011. Part IV: Health and Health Management on U.S. Feedlots with a Capacity of 1,000 or More Head [Internet]. USDA Animal and Plant Health Inspection Service; 2013 [cited 2015 Jun 20]. Available from: http://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot2011/Feed11_dr_PartIV.pdf

12. USDA. Beef 2007–08. Antimicrobial Drug Use and Antimicrobial Resistance on U.S. Cow-calf Operations, 2007–08 [Internet]. USDA Animal and Plant Health Inspection Service; 2012 [cited 2015 Jun 20]. Available from: http://www.aphis.usda.gov/animal_health/nahms/beefcowcalf/downloads/beef0708/Beef0708_ir_Antimicrobial.pdf

13. Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) - 2013 Annual Report [Internet]. Government of Canada, Public Health Agency of Canada; [cited 2015 Jun 18]. Available from: http://www.phac-aspc.gc.ca/cipars-picra/2013/annu-report-rapport-eng.php

14. DANMAP 2013 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark [Internet]. The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme; [cited 2015 Jun 18]. Available from: http://www.danmap.org/~/media/Projekt%20sites/Danmap/DANMAP%20reports/DANMAP%202013/DANMAP%202013.ashx

15. SVARM 2014 - Consumption of antibiotics and occurrence of antibiotic resistance in Sweden. [Internet]. Public Health Agency of Sweden and National Veterinary Institute; [cited 2015 Jun 18]. Available from: http://www.sva.se/globalassets/redesign2011/pdf/om_sva/publikationer/swedres_svarm2014.pdf

16. Salmonella in U.S. Cattle Feedlots [Internet]. USDA Animal and Plant Health Inspection Service, Center for Epidemiology and Animal Health, Veterinary Services; 2014 [cited 2015 Jun 18]. Available from: http://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot2011/Feed11_is_Salm.pdf

17. Carson CA, Reid-Smith R, Irwin RJ, Martin WS, McEwen SA. Antimicrobial resistance in generic fecal Escherichia coli from 29 beef farms in Ontario. Can J Vet Res. 2008 Mar;72(2):119–28.

18. Gow SP, Waldner CL, Rajić A, McFall ME, Reid-Smith R. Prevalence of antimicrobial resistance in fecal generic Escherichia coil isolated in western Canadian beef herds. Part II--cows and cow-calf pairs. Can J Vet Res Rev Can Rech Vét. 2008;72(2):91–100.

19. Gow SP, Waldner CL, Rajić A, McFall ME, Reid-Smith R. Prevalence of antimicrobial resistance in fecal generic Escherichia coil isolated in western Canadian cow-calf herds. Part I--beef calves. Can J Vet Res Rev Can Rech Vét. 2008;72(2):82–90.

20. Rao S, Van Donkersgoed J, Bohaychuk V, Besser T, Song X-M, Wagner B, et al. Antimicrobial drug use and antimicrobial resistance in enteric bacteria among cattle from Alberta feedlots. Foodborne Pathog Dis. 2010 Apr;7(4):449–57.

21. Morley PS, Dargatz DA, Hyatt DR, Dewell GA, Patterson JG, Burgess BA, et al. Effects of restricted antimicrobial exposure on antimicrobial resistance in fecal Escherichia coli from feedlot cattle. Foodborne Pathog Dis. 2011 Jan;8(1):87–98.

22. Checkley SL, Campbell JR, Chirino-Trejo M, Janzen ED, Waldner CL. Associations between antimicrobial use and the prevalence of antimicrobial resistance in fecal Escherichia coli from feedlot cattle in western Canada. Can Vet J Rev Vét Can. 2010 Aug;51(8):853–61.

23. Checkley SL, Campbell JR, Chirino-Trejo M, Janzen ED, McKinnon JJ. Antimicrobial resistance in generic fecal Escherichia coli obtained from beef cattle on arrival at the feedlot and prior to slaughter, and associations with volume of total individual cattle antimicrobial treatments in one western Canadian feedlot. Can J Vet Res. 2008 Mar;72(2):101–8.

24. Dargatz DA, Fedorka-Cray PJ, Ladely SR, Kopral CA, Ferris KE, Headrick ML. Prevalence and antimicrobial susceptibility of Salmonella spp. isolates from US cattle in feedlots in 1999 and 2000. J Appl Microbiol. 2003;95(4):753–61.

25. Khaitsa ML, Kegode RB, Bauer ML, Gibbs PS, Lardy GP, Doetkott DK. A longitudinal study of Salmonella shedding and antimicrobial resistance patterns in North Dakota feedlot cattle. J Food Prot. 2007 Feb;70(2):476–81.

26. Inglis GD, Morck DW, McAllister TA, Entz T, Olson ME, Yanke LJ, et al. Temporal prevalence of antimicrobial resistance in Campylobacter spp. from beef cattle in Alberta feedlots. Appl Environ Microbiol. 2006 Jun;72(6):4088–95.

27. Riley DG, Loneragan GH, Phillips WA, Gray JT, Fedorka-Cray PJ. Fecal shedding of foodborne pathogens by Florida-born heifers and steers in U.S. beef production segments. J Food Prot. 2008 Apr;71(4):807–10.

28. Berge AC, Hancock DD, Sischo WM, Besser TE. Geographic, farm, and animal factors associated with multiple antimicrobial resistance in fecal Escherichia coli isolates from cattle in the western United States. J Am Vet Med Assoc. 2010 Jun 15;236(12):1338–44.

29. Sharma R, Munns K, Alexander T, Entz T, Mirzaagha P, Yanke LJ, et al. Diversity and Distribution of Commensal Fecal Escherichia coli Bacteria in Beef Cattle Administered Selected Subtherapeutic Antimicrobials in a Feedlot Setting. Appl Environ Microbiol. 2008 Oct 15;74(20):6178–86.

30. Hurd HS, Doores S, Hayes D, Mathew A, Maurer J, Silley P, et al. Public health consequences of macrolide use in food animals: a deterministic risk assessment. J Food Prot. 2004 May;67(5):980–92.

31. Hurd HS, Malladi S. A stochastic assessment of the public health risks of the use of macrolide antibiotics in food animals. Risk Anal Off Publ Soc Risk Anal. 2008 Jun;28(3):695–710.

32. Hurd HS, Vaughn MB, Holtkamp D, Dickson J, Warnick L. Quantitative risk from fluoroquinolone-resistant Salmonella and Campylobacter due to treatment of dairy heifers with enrofloxacin for bovine respiratory disease. Foodborne Pathog Dis. 2010 Nov;7(11):1305–22.

33. Zhao S, Blickenstaff K, Bodeis-Jones S, Gaines SA, Tong E, McDermott PF. Comparison of the prevalences and antimicrobial resistances of Escherichia coli isolates from different retail meats in the United States, 2002 to 2008. Appl Environ Microbiol. 2012 Mar;78(6):1701–7.

34. Aslam M, Checkley S, Avery B, Chalmers G, Bohaychuk V, Gensler G, et al. Phenotypic and genetic characterization of antimicrobial resistance in Salmonella serovars isolated from retail meats in Alberta, Canada. Food Microbiol. 2012 Oct;32(1):110–7.

35. Bosilevac JM, Guerini MN, Kalchayanand N, Koohmaraie M. Prevalence and Characterization of Salmonellae in Commercial Ground Beef in the United States. Appl Environ Microbiol. 2009 Apr 1;75(7):1892–900.

36. Zhao S, Young SR, Tong E, Abbott JW, Womack N, Friedman SL, et al. Antimicrobial resistance of Campylobacter isolates from retail meat in the United States between 2002 and 2007. Appl Environ Microbiol. 2010 Dec;76(24):7949–56.

37. Kelman A, Soong Y-A, Dupuy N, Shafer D, Richbourg W, Johnson K, et al. Antimicrobial susceptibility of Staphylococcus aureus from retail ground meats. J Food Prot. 2011 Oct;74(10):1625–9.

38. Xia X, Meng J, Zhao S, Bodeis-Jones S, Gaines SA, Ayers SL, et al. Identification and antimicrobial resistance of extraintestinal pathogenic Escherichia coli from retail meats. J Food Prot. 2011 Jan;74(1):38–44.

39. Kilonzo-Nthenge A, Rotich E, Nahashon SN. Evaluation of drug-resistant Enterobacteriaceae in retail poultry and beef. Poult Sci. 2013 Apr;92(4):1098–107.

40. Greig JD, Waddell L, Wilhelm B, Wilkins W, Bucher O, Parker S, et al. The efficacy of interventions applied during primary processing on contamination of beef carcasses with Escherichia coli: A systematic review-meta-analysis of the published research. Food Control. 2012 Oct;27(2):385–97.

41. Bacon RT, Belk KE, Sofos JN, Clayton RP, Reagan JO, Smith GC. Microbial populations on animal hides and beef carcasses at different stages of slaughter in plants employing multiple-sequential interventions for decontamination. J Food Prot. 2000 Aug;63(8):1080–6.

42. Arslan S, Eyi A. Occurrence and antimicrobial resistance profiles of Salmonella species in retail meat products. J Food Prot. 2010 Sep;73(9):1613–7.

43. Miranda JM, Mondragón A, Vázquez BI, Fente CA, Cepeda A, Franco CM. Influence of farming methods on microbiological contamination and prevalence of resistance to antimicrobial drugs in isolates from beef. Meat Sci. 2009 Jun;82(2):284–8.

44. Zhao S, White DG, Friedman SL, Glenn A, Blickenstaff K, Ayers SL, et al. Antimicrobial Resistance in Salmonella enterica Serovar Heidelberg Isolates from Retail Meats, Including Poultry, from 2002 to 2006. Appl Environ Microbiol. 2008 Nov 1;74(21):6656–62.

45. Mayrhofer S, Paulsen P, Smulders FJM, Hilbert F. Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. Int J Food Microbiol. 2004 Dec 1;97(1):23–9.

46. USDA, FSIS. National Prevalence Estimate of Pathogens in Domestic Beef Manufacturing Trimmings (Trim). 2011.

47. Aslam M, Service C. Antimicrobial resistance and genetic profiling of Escherichia coli from a commercial beef packing plant. J Food Prot. 2006 Jul;69(7):1508–13.

48. Beier RC, Poole TL, Brichta-Harhay DM, Anderson RC, Bischoff KM, Hernandez CA, et al. Disinfectant and antibiotic susceptibility profiles of Escherichia coli O157:H7 strains from cattle carcasses, feces, and hides and ground beef from the United States. J Food Prot. 2013 Jan;76(1):6–17.

49. Sura S, Degenhardt D, Cessna AJ, Larney FJ, Olson AF, McAllister TA. Transport of three veterinary antimicrobials from feedlot pens via simulated rainfall runoff. Sci Total Environ. 2015 Jul 15;521:191–9.

50. McEachran AD, Blackwell BR, Hanson JD, Wooten KJ, Mayer GD, Cox SB, et al. Research. Antibiotics, Bacteria, and Antibiotic Resistance Genes: Aerial Transport from Cattle Feed Yards via Particulate Matter. Environ Health Perspect. 2015 Apr;123(4):337–43.

51. McNab Walt W., Singleton MJ, Moran JE, Esser BK. Assessing the Impact of Animal Waste Lagoon Seepage on the Geochemistry of an Underlying Shallow Aquifer. Environ Sci Technol. 2007 Feb 1;41(3):753–8.

52. Korom SF, Jeppson RW. Nitrate Contamination from Dairy Lagoons Constructed in Coarse Alluvial Deposits. J Environ Qual. 1994;23(5):973.

53. Van der Schans ML, Harter T, Leijnse A, Mathews MC, Meyer RD. Characterizing sources of nitrate leaching from an irrigated dairy farm in Merced County, California. J Contam Hydrol. 2009 Nov 3;110(1-2):9–21.

54. Mackie RI, Koike S, Krapac I, Chee-Sanford J, Maxwell S, Aminov RI. Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. Anim Biotechnol. 2006;17(2):157–76.

55. Literature Review of Contaminants in Livestock and Poultry Manure and Implications for Water Quality [Internet]. EPA Office of Water; 2013 [cited 2015 Jun 18]. Available from: http://water.epa.gov/scitech/cec/upload/Literature-Review-of-Contaminants-in-Livestock-and-Poultry-Manure-and-Implications-for-Water-Quality.pdf

56. Jaros P, Cookson AL, Campbell DM, Besser TE, Shringi S, Mackereth GF, et al. A prospective case–control and molecular epidemiological study of human cases of Shiga toxin-producing Escherichia coli in New Zealand. BMC Infect Dis. 2013 Sep 30;13(1):450.

57. Ravva SV, Sarreal CZ, Mandrell RE. Altered protozoan and bacterial communities and survival of Escherichia coli O157:H7 in monensin-treated wastewater from a dairy lagoon. PloS One. 2013;8(1):e54782.

58. Oliveira M, Viñas I, Usall J, Anguera M, Abadias M. Presence and survival of Escherichia coli O157:H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water. Int J Food Microbiol. 2012 May 15;156(2):133–40.

59. Holvoet K, Sampers I, Callens B, Dewulf J, Uyttendaele M. Moderate Prevalence of Antimicrobial Resistance in Escherichia coli Isolates from Lettuce, Irrigation Water, and Soil. Appl Environ Microbiol. 2013 Nov;79(21):6677–83.

60. Durso LM, Miller DN, Wienhold BJ. Distribution and Quantification of Antibiotic Resistant Genes and Bacteria across Agricultural and Non-Agricultural Metagenomes. PLoS ONE. 2012 Nov 2;7(11):e48325.

61. Walsh F, Duffy B. The Culturable Soil Antibiotic Resistome: A Community of Multi-Drug Resistant Bacteria. PLoS ONE. 2013 Jun 12;8(6):e65567.

62. Brown MG, Balkwill DL. Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. Microb Ecol. 2009 Apr;57(3):484–93.

63. D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, et al. Antibiotic resistance is ancient. Nature. 2011 Sep 22;477(7365):457–61.

64. Allen HK, Moe LA, Rodbumrer J, Gaarder A, Handelsman J. Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. ISME J. 2009 Feb;3(2):243–51.

65. Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, et al. Antibiotic Resistance Is Prevalent in an Isolated Cave Microbiome. PLoS ONE. 2012 Apr 11;7(4):e34953.

66. Perron GG, Whyte L, Turnbaugh PJ, Goordial J, Hanage WP, Dantas G, et al. Functional Characterization of Bacteria Isolated from Ancient Arctic Soil Exposes Diverse Resistance

Mechanisms to Modern Antibiotics. PLoS ONE [Internet]. 2015 Mar 25 [cited 2015 Jun 18];10(3). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4373940/

67. D'Costa VM, McGrann KM, Hughes DW, Wright GD. Sampling the Antibiotic Resistome. Science. 2006 Jan 20;311(5759):374–7.

68. Nesme J, Cécillon S, Delmont TO, Monier J-M, Vogel TM, Simonet P. Large-scale metagenomic-based study of antibiotic resistance in the environment. Curr Biol CB. 2014 May 19;24(10):1096–100.

69. Li B, Yang Y, Ma L, Ju F, Guo F, Tiedje JM, et al. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. ISME J. 2015 Apr 28;

70. Segawa T, Takeuchi N, Rivera A, Yamada A, Yoshimura Y, Barcaza G, et al. Distribution of antibiotic resistance genes in glacier environments. Environ Microbiol Rep. 2013 Feb 1;5(1):127–34.

71. Alexander TW, Inglis GD, Yanke LJ, Topp E, Read RR, Reuter T, et al. Farm-to-fork characterization of Escherichia coli associated with feedlot cattle with a known history of antimicrobial use. Int J Food Microbiol. 2010 Jan 31;137(1):40–8.

72. Alexander TW, Yanke LJ, Topp E, Olson ME, Read RR, Morck DW, et al. Effect of Subtherapeutic Administration of Antibiotics on the Prevalence of Antibiotic-Resistant Escherichia coli Bacteria in Feedlot Cattle. Appl Environ Microbiol. 2008 Jul;74(14):4405–16.

73. Alexander TW, Reuter T, Sharma R, Yanke LJ, Topp E, McAllister TA. Longitudinal Characterization of Resistant Escherichia coli in Fecal Deposits from Cattle Fed Subtherapeutic Levels of Antimicrobials. Appl Environ Microbiol. 2009 Nov;75(22):7125–34.

74. Amachawadi R g., Scott H m., Aperce C, Vinasco J, Drouillard J s., Nagaraja T g. Effects of in-feed copper and tylosin supplementations on copper and antimicrobial resistance in faecal enterococci of feedlot cattle. J Appl Microbiol. 2015 Jun;118(6):1287–97.

75. Jacob ME, Fox JT, Narayanan SK, Drouillard JS, Renter DG, Nagaraja TG. Effects of feeding wet corn distillers grains with solubles with or without monensin and tylosin on the prevalence and antimicrobial susceptibilities of fecal foodborne pathogenic and commensal bacteria in feedlot cattle. J Anim Sci. 2008 May;86(5):1182–90.

76. Schmidt JW, Griffin D, Kuehn LA, Brichta-Harha DM. Influence of Therapeutic Ceftiofur Treatments of Feedlot Cattle on Fecal and Hide Prevalences of Commensal Escherichia cóli Resistant to Expanded-Spectrum Cephalosporins, and Molecular Characterization of Resistant Isolates. Appl Environ Microbiol. 2013 Apr;79(7):2273–83.

77. Alali WQ, Scott HM, Norby B, Gebreyes W, Loneragan GH. Quantification of the bla(CMY-2) in feces from beef feedlot cattle administered three different doses of

ceftiofur in a longitudinal controlled field trial. Foodborne Pathog Dis. 2009 Oct;6(8):917–24.

78. Lowrance TC, Loneragan GH, Kunze DJ, Platt TM, Ives SE, Scott HM, et al. Changes in antimicrobial susceptibility in a population of Escherichia coli isolated from feedlot cattle administered ceftiofur crystalline-free acid. Am J Vet Res. 2007 May;68(5):501–7.

79. Kanwar N, Scott HM, Norby B, Loneragan GH, Vinasco J, McGowan M, et al. Effects of Ceftiofur and Chlortetracycline Treatment Strategies on Antimicrobial Susceptibility and on tet(A), tet(B), and blaCMY-2 Resistance Genes among E. coli Isolated from the Feces of Feedlot Cattle. PLoS ONE. 2013 Nov;8(11):1–13.

80. Zaheer R, Cook S, Klima C, Stanford K, Alexander T, Topp E, et al. Effect of subtherapeutic vs. therapeutic administration of macrolides on antimicrobial resistance in Mannheimia haemolytica and enterococci isolated from beef cattle. Antimicrob Resist Chemother. 2013;4:133.

81. Mirzaagha P, Louie M, Sharma R, Yanke LJ, Topp E, McAllister TA. Distribution and characterization of ampicillin-and tetracycline-resistant Escherichia coli from feedlot cattle fed subtherapeutic antimicrobials. BMC Microbiol. 2011 Jan;11(1):78–92.

82. Platt TM, Loneragan GH, Scott HM, Norby B, Thomson DU, Brown MS, et al. Antimicrobial susceptibility of enteric bacteria recovered from feedlot cattle administered chlortetracycline in feed. Am J Vet Res. 2008 Aug 1;69(8):988–96.

83. Harvey R, Funk J, Wittum TE, Hoet AE. A metagenomic approach for determining prevalence of tetracycline resistance genes in the fecal flora of conventionally raised feedlot steers and feedlot steers raised without antimicrobials. Am J Vet Res. 2009 Feb;70(2):198–202.

84. Chantziaras I, Boyen F, Callens B, Dewulf J. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. J Antimicrob Chemother. 2013 Nov 11;dkt443.

85. Alexander TW, Yanke JL, Reuter T, Topp E, Read RR, Selinger BL, et al. Longitudinal characterization of antimicrobial resistance genes in feces shed from cattle fed different subtherapeutic antibiotics. BMC Microbiol. 2011 Jan;11(1):19–30.

86. Alexander T w., Jin X, Li Q, Cook S, McAllister T a. Characterization of tetracycline resistance genes in Escherichia coli isolated from feedlot cattle administered therapeutic or subtherapeutic levels of tetracycline. Can J Microbiol. 2013 Apr;59(4):287–90.

87. Reinstein S, Fox JT, Shi X, Alam MJ, Renter DG, Nagaraja TG. Prevalence of Escherichia coli O157:H7 in organically and naturally raised beef cattle. Appl Environ Microbiol. 2009 Aug;75(16):5421–3.

88. LeJeune JT, Christie NP. Microbiological quality of ground beef from conventionally-reared cattle and “raised without antibiotics” label claims. J Food Prot. 2004 Jul;67(7):1433–7.

89. Johnson JR, Kuskowski MA, Smith K, O’Bryan TT, Tatini S. Antimicrobial-Resistant and Extraintestinal Pathogenic Escherichia coli in Retail Foods. J Infect Dis. 2005 4/1/2005;191(7):1040–9.

90. Ghosh S, LaPara TM. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J. 2007 May 24;1(3):191–203.

91. McKinney CW, Loftin KA, Meyer MT, Davis JG, Pruden A. tet and sul Antibiotic Resistance Genes in Livestock Lagoons of Various Operation Type, Configuration, and Antibiotic Occurrence. Environ Sci Technol. 2010 Aug 15;44(16):6102–9.

92. Kemper N. Veterinary antibiotics in the aquatic and terrestrial environment. Ecol Indic. 2008 Jan;8(1):1–13.

93. Wenguang Xiong, Yongxue Sun, Xueyao Ding, Mianzhi Wang, Zhenling Zeng. Selective pressure of antibiotics on ARGs and bacterial communities in manure-polluted freshwater-sediment microcosms. Front Microbiol. 2015 Mar;6:1–8.

94. Hua Fang, Huifang Wang, Lin Cai, Yunlong Yu. Prevalence of Antibiotic Resistance Genes and Bacterial Pathogens in Long-Term Manured Greenhouse Soils As Revealed by Metagenomic Survey. Environ Sci Technol. 2015 Jan 20;49(2):1095–104.

95. Xiong W, Sun Y, Ding X, Zhang Y, Zhong X, Liang W, et al. Responses of plasmid-mediated quinolone resistance genes and bacterial taxa to (fluoro)quinolones-containing manure in arable soil. Chemosphere. 2015 Jan 3;119:473–8.

96. Heuer H, Smalla K. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ Microbiol. 2007 Mar;9(3):657–66.

97. Joy SR, Li X, Snow DD, Gilley JE, Woodbury B, Bartelt-Hunt SL. Fate of antimicrobials and antimicrobial resistance genes in simulated swine manure storage. Sci Total Environ. 2014 May 15;481:69–74.

98. Zhang Y, Boyd SA, Teppen BJ, Tiedje JM, Li H. Organic acids enhance bioavailability of tetracycline in water to Escherichia coli for uptake and expression of antibiotic resistance. Water Res. 2014 Nov 15;65:98–106.

99. Zhang Y, Zhang C, Parker DB, Snow DD, Zhou Z, Li X. Occurrence of antimicrobials and antimicrobial resistance genes in beef cattle storage ponds and swine treatment lagoons. Sci Total Environ. 2013 Oct 1;463–464:631–8.

100. Zhou Z, Raskin L, Zilles JL. Effects of Swine Manure on Macrolide, Lincosamide, and Streptogramin B Antimicrobial Resistance in Soils. Appl Environ Microbiol. 2010 Apr 1;76(7):2218–24.

101. Zhu Y-G, Johnson TA, Su J-Q, Qiao M, Guo G-X, Stedtfeld RD, et al. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl Acad Sci. 2013 Feb 26;110(9):3435–40.

102. Peak N, Knapp CW, Yang RK, Hanfelt MM, Smith MS, Aga DS, et al. Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. Environ Microbiol. 2007 Jan;9(1):143–51.

103. Udikovic-Kolic N, Wichmann F, Broderick NA, Handelsman J. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. Proc Natl Acad Sci. 2014 Oct 21;111(42):15202–7.

104. Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G. The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. Science. 2012 Aug 31;337(6098):1107–11.

105. Forsberg KJ, Patel S, Gibson MK, Lauber CL, Knight R, Fierer N, et al. Bacterial phylogeny structures soil resistomes across habitats. Nature. 2014 May 29;509(7502):612–6.

106. Knapp CW, Dolfing J, Ehlert P a. I, Graham DW. Evidence of Increasing Antibiotic Resistance Gene Abundances in Archived Soils since 1940. Environ Sci Technol. 2010 Jan 15;44(2):580–7.

107. Singer RS, Williams-Nguyen J. Human health impacts of antibiotic use in agriculture: A push for improved causal inference. Curr Opin Microbiol. 2014 Jun;19:1–8.

108. Centers for Disease Control and Prevention. Active Bacterial Core Surveillance (ABCs) Report Emerging Infections Program Network Methicillin-Resistant Staphylococcus aureus, 2012 [Internet]. Centers for Disease Control and Prevention; 2012 [cited 2015 Jun 22]. Available from: http://www.cdc.gov/abcs/reports-findings/survreports/mrsa12.pdf

109. Klare I, Badstübner D, Konstabel C, Böhme G, Claus H, Witte W. Decreased incidence of VanA-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. Microb Drug Resist Larchmt N. 1999;5(1):45–52.

110. Bogaard AE van den, Bruinsma N, Stobberingh EE. The effect of banning avoparcin on VRE carriage in The Netherlands. J Antimicrob Chemother. 2000 Jul 1;46(1):146–8.

111. Vincent C, Boerlin P, Daignault D, Dozois CM, Dutil L, Galanakis C, et al. Food Reservoir for Escherichia coli Causing Urinary Tract Infections. Emerg Infect Dis. 2010 Jan;16(1):88–95.

112. Bergeron CR, Prussing C, Dutil L, Boerlin P, Daignault D, Reid-Smith RJ, et al. Chicken as Reservoir for Extraintestinal Pathogenic Escherichia coli in Humans, Canada. Emerg Infect Dis. 2012 Mar;18(3):415–21.

113. Mather AE, Reid SWJ, Maskell DJ, Parkhill J, Fookes MC, Harris SR, et al. Distinguishable Epidemics of Multidrug-Resistant Salmonella Typhimurium DT104 in Different Hosts. Science. 2013 Sep 27;341(6153):1514–7.

114. Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, et al. In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci. 2012 Jan 31;109(5):1691–6.

115. Beaber JW, Hochhut B, Waldor MK. SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature. 2004 Jan 1;427(6969):72–4.

116. Guerin E, Cambray G, Sanchez-Alberola N, Campoy S, Erill I, Da Re S, et al. The SOS response controls integron recombination. Science. 2009 May 22;324(5930):1034.

117. Hu Y, Yang X, Qin J, Lu N, Cheng G, Wu N, et al. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. Nat Commun. 2013;4:2151.

118. Forslund K, Sunagawa S, Coelho LP, Bork P. Metagenomic insights into the human gut resistome and the forces that shape it. BioEssays. 2014 Mar 1;36(3):316–29.

119. Wichmann F, Udikovic-Kolic N, Andrew S, Handelsman J. Diverse Antibiotic Resistance Genes in Dairy Cow Manure. mBio. 2014 May 1;5(2):e01017–13.

120. Durso LM, Harhay GP, Bono JL, Smith TPL. Virulence-associated and antibiotic resistance genes of microbial populations in cattle feces analyzed using a metagenomic approach. J Microbiol Methods. 2011 Feb;84(2):278–82.

121. Hodgins D, Shewen P. Pasteurella and Mannheimia spp. infections. Infectious disease of livestock. 2nd ed. Cape Town, South Africa: Oxford University Press; 2004. p. 1672–6.

122. Griffin D. Economic impact associated with respiratory disease in beef cattle. Vet Clin North Am Food Anim Pract. 1997 Nov;13(3):367–77.

123. Fulton RW, Cook BJ, Step DL, Confer AW, Saliki JT, Payton ME, et al. Evaluation of health status of calves and the impact on feedlot performance: assessment of a retained ownership program for postweaning calves. Can J Vet Res. 2002 Jul;66(3):173–80.

124. Fulton RW, Blood KS, Panciera RJ, Payton ME, Ridpath JF, Confer AW, et al. Lung pathology and infectious agents in fatal feedlot pneumonias and relationship with mortality, disease onset, and treatments. J Vet Diagn Investig Off Publ Am Assoc Vet Lab Diagn Inc. 2009 Jul;21(4):464–77.

125. Booker CW, Abutarbush SM, Morley PS, Jim GK, Pittman TJ, Schunicht OC, et al. Microbiological and histopathological findings in cases of fatal bovine respiratory disease of feedlot cattle in Western Canada. Can Vet J Rev Vét Can. 2008 May;49(5):473–81.

126. Lubbers BV, Hanzlicek GA. Antimicrobial multidrug resistance and coresistance patterns of Mannheimia haemolytica isolated from bovine respiratory disease cases--a three-year (2009-2011) retrospective analysis. J Vet Diagn Investig Off Publ Am Assoc Vet Lab Diagn Inc. 2013 May;25(3):413–7.

127. Benedict KM, Gow SP, Checkley S, Booker CW, McAllister TA, Morley PS. Methodological comparisons for antimicrobial resistance surveillance in feedlot cattle. BMC Vet Res. 2013 Oct 21;9(1):216.

128. Alexander TW, Cook SR, Yanke LJ, Booker CW, Morley PS, Read RR, et al. A multiplex polymerase chain reaction assay for the identification of Mannheimia haemolytica, Mannheimia glucosida and Mannheimia ruminalis. Vet Microbiol. 2008 Jul 27;130(1-2):165–75.

129. Booker CW, Abutarbush SM, Schunicht OC, Jim GK, Perrett T, Wildman BK, et al. Evaluation of the efficacy of tulathromycin as a metaphylactic antimicrobial in feedlot calves. Vet Ther Res Appl Vet Med. 2007;8(3):183–200.

130. Nutrient Requirements of Beef Cattle, 8th Revised Edition. 8 edition. National Academies Press; 2015. 400 p.

131. Agresti A, Coull BA. Approximate is Better than "Exact" for Interval Estimation of Binomial Proportions. Am Stat. 1998 May 1;52(2):119–26.

132. Carey V, Zeger SL, Diggle P. Modelling multivariate binary data with alternating logistic regressions. Biometrika. 1993 Sep 1;80(3):517–26.

133. Dohoo I, Martin W, Stryhn H. Veterinary Epidemiologic Research. 2nd edition. Charlottetown, CA: VER Inc.; 2009. 865 p.

134. Ward TI. Antimicrobial resistance patterns of selected bacterial pathogens of feedlot cattle [microform].

135. Baggott D, Casartelli A, Fraisse F, Manavella C, Marteau R, Rehbein S, et al. Demonstration of the metaphylactic use of gamithromycin against bacterial pathogens associated with bovine respiratory disease in a multicentre farm trial. Vet Rec. 2011 Mar 5;168(9):241–241.

136. Catry B, Duchateau L, Van de Ven J, Laevens H, Opsomer G, Haesebrouck F, et al. Efficacy of metaphylactic florfenicol therapy during natural outbreaks of bovine respiratory disease. J Vet Pharmacol Ther. 2008 Oct;31(5):479–87.

137. Ribble CS, Shoukri MM, Meek AH, Wayne Martin S. Clustering of fatal fibrinous pneumonia (shipping fever) in feedlot calves within transport truck and feedlot pen groups. Prev Vet Med. 1994 Dec;21(3):251–61.

138. Katsuda K, Kohmoto M, Mikami O. Relationship between serotype and the antimicrobial susceptibility of Mannheimia haemolytica isolates collected between 1991 and 2010. Res Vet Sci. 2013 Apr;94(2):205–8.

139. Klima CL, Alexander TW, Hendrick S, McAllister TA. Characterization of Mannheimia haemolytica isolated from feedlot cattle that were healthy or treated for bovine respiratory disease. Can J Vet Res Rev Can Rech Vét. 2014 Jan;78(1):38–45.

140. Catry B, Haesebrouck F, Vliegher SD, Feyen B, Vanrobaeys M, Opsomer G, et al. Variability in acquired resistance of Pasteurella and Mannheimia isolates from the nasopharynx of calves, with particular reference to different herd types. Microb Drug Resist Larchmt N. 2005;11(4):387–94.

141. Esaki H, Asai T, Kojima A, Ishihara K, Morioka A, Tamura Y, et al. Antimicrobial susceptibility of Mannheimia haemolytica isolates from cattle in Japan from 2001 to 2002. J Vet Med Sci Jpn Soc Vet Sci. 2005 Jan;67(1):75–7.

142. Rérat M, Albini S, Jaquier V, Hüssy D. Bovine respiratory disease: efficacy of different prophylactic treatments in veal calves and antimicrobial resistance of isolated Pasteurellaceae. Prev Vet Med. 2012 Mar 1;103(4):265–73.

143. Wettstein K, Frey J. Vergleich der antimikrobiellen Resistenz von ausgewählten Infektionserregern des Respirationstraktes bei verschiedenen Tierarten. Schweiz Arch Für Tierheilkd. 2004 Sep 1;146(9):417–22.

144. Lamm CG, Love BC, Krehbiel CR, Johnson NJ, Step DL. Comparison of antemortem antimicrobial treatment regimens to antimicrobial susceptibility patterns of postmortem lung isolates from feedlot cattle with bronchopneumonia. J Vet Diagn Investig Off Publ Am Assoc Vet Lab Diagn Inc. 2012 Mar;24(2):277–82.

145. Smith R, Coast J. The true cost of antimicrobial resistance. BMJ. 2013;346:f1493.

146. Commissioner O of the. Testimony - Antibiotic Resistance and the Use of Antibiotics in Animal Agriculture [Internet]. [cited 2015 Jul 22]. Available from: http://www.fda.gov/NewsEvents/Testimony/ucm219015.htm

147. Alban L, Nielsen EO, Dahl J. A human health risk assessment for macrolide-resistant Campylobacter associated with the use of macrolides in Danish pig production. Prev Vet Med. 2008 Feb 1;83(2):115–29.

148. Cox LA, Popken DA. Quantifying potential human health impacts of animal antibiotic use: enrofloxacin and macrolides in chickens. Risk Anal Off Publ Soc Risk Anal. 2006 Feb;26(1):135–46.

149. Alali WQ, Scott HM, Christian KL, Fajt VR, Harvey RB, Lawhorn DB. Relationship between level of antibiotic use and resistance among Escherichia coli isolates from integrated multi-site cohorts of humans and swine. Prev Vet Med. 2009 Aug 1;90(3-4):160–7.

150. Benedict KM, Gow SP, Reid-Smith RJ, Booker CW, McAllister TA, Morley PS. Latent class comparison of test accuracy when evaluating antimicrobial susceptibility using disk diffusion and broth microdilution to test Escherichia coli and Mannheimia haemolytica isolates recovered from beef feedlot cattle. Epidemiol Infect. 2014 Nov;142(11):2314–25.

151. Performance Standards for antimicrobial disk and diluation susceptibility tests for bacteria isolated from animals, 4th edition: approved standard, M31-A4. Clinical and Laboratory Standards Institute; 2013.

152. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria, second edition: approved guidelines, M45-A2. Clinical and Laboratory Standards Institute; 2010.

153. Performance standards for antimicrobial susceptibility testing, 21st informational supplement, M100-S18. Clinical and Laboratory Standards Institute; 2008.

154. Performance standards for antimicrobial susceptibility testing, 21st information supplement, M100-S21. Clinical and Laboratory Standards Institute; 2011.

155. Performance standards for antimicrobial susceptibility testing, 22nd informational supplement, M100-S22. Clinical and Laboratory Standards Institute; 2012.

156. Benedict KM, Gow SP, Reid-Smith RJ, Booker CW, Morley PS. Metrics for quantifying antimicrobial use in beef feedlots. Can Vet J. 2012 Aug;53(8):841–8.

157. Zeger SL, Liang KY, Albert PS. Models for longitudinal data: a generalized estimating equation approach. Biometrics. 1988 Dec;44(4):1049–60.

158. Wolfinger R. Laplace's Approximation for Nonlinear Mixed Models. Biometrika. 1993;80(4):791–5.

159. Greven S, Kneib T. On the behaviour of marginal and conditional AIC in linear mixed models. Biometrika. 2010 Jul 27;asq042.

160. Dohoo I, Stryhn H. Simulation Studies on the Effects of Clustering. Proc 11th Int Symp Vet Epidemiol Econ Cairn Aust. 2006;505.

161. Hill AB. The Environment and Disease: Association or Causation? Proc R Soc Med. 1965 May;58(5):295–300.

162. McEachran AD, Blackwell BR, Hanson JD, Wooten KJ, Mayer GD, Cox SB, et al. Research. Antibiotics, Bacteria, and Antibiotic Resistance Genes: Aerial Transport from

Cattle Feed Yards via Particulate Matter. Environ Health Perspect. 2015 Apr;123(4):337–43.

163. Wenguang Xiong, Yongxue Sun, Xueyao Ding, Mianzhi Wang, Zhenling Zeng. Selective pressure of antibiotics on ARGs and bacterial communities in manure-polluted freshwater-sediment microcosms. Front Microbiol. 2015 Mar;6:1–8.

164. Hua Fang, Huifang Wang, Lin Cai, Yunlong Yu. Prevalence of Antibiotic Resistance Genes and Bacterial Pathogens in Long-Term Manured Greenhouse Soils As Revealed by Metagenomic Survey. Environ Sci Technol. 2015 Jan 20;49(2):1095–104.

165. Kyselková M, Jirout J, Chroňáková A, Vrchotová N, Bradley R, Schmitt H, et al. Cow excrements enhance the occurrence of tetracycline resistance genes in soil regardless of their oxytetracycline content. Chemosphere. 2013 Nov;93(10):2413–8.

166. Cytryn E. The soil resistome: the anthropogenic, the native, and the unknown. Soil Biol Biochem. 2013;63:18–23.

167. Gow SP, Waldner CL. Antimicrobial drug use and reason for treatment in 203 western Canadian cow–calf herds during calving season. Prev Vet Med. 2009 Jul;90(1/2):55–65.

168. World Health Organization. Critically Important Antimicrobials for Human Medicine, 3rd revision [Internet]. World Health Organization; 2011 [cited 2015 Jul 25]. Available from: http://apps.who.int/iris/bitstream/10665/77376/1/9789241504485_eng.pdf

169. USDA. Dairy 2007, Part V: Changes in Dairy Cattle Health and Management Practices in the United States, 1996-2007 [Internet]. USDA Animal and Plant Health Inspection Service; 2009 [cited 2015 Jun 20]. Available from: http://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy07/Dairy07_dr_PartV_rev.pdf

170. Herrick JB, Haynes R, Heringa S, Brooks JM, Sobota LT. Coselection for resistance to multiple late-generation human therapeutic antibiotics encoded on tetracycline resistance plasmids captured from uncultivated stream and soil bacteria. J Appl Microbiol. 2014 Aug;117(2):380–9.

171. Tremblay C-L, Letellier A, Quessy S, Daignault D, Archambault M. Antibiotic-resistant Enterococcus faecalis in abattoir pigs and plasmid colocalization and cotransfer of tet(M) and erm(B) genes. J Food Prot. 2012 Sep;75(9):1595–602.

172. Gow SP, Waldner CL, Harel J, Boerlin P. Associations between Antimicrobial Resistance Genes in Fecal Generic Escherichia coli Isolates from Cow-Calf Herds in Western Canada. Appl Environ Microbiol. 2008 Jun 15;74(12):3658–66.

173. Smorawinska M, Szuplewska M, Zaleski P, Wawrzyniak P, Maj A, Pluciennicza k A, et al. Mobilizable narrow host range plasmids as natural suicide vectors enabling horizontal gene transfer among distantly related bacterial species. FEMS Microbiol Lett. 2012 Jan;326(1):76–82.

174. Stecher B, Denzler R, Maier L, Bernet F, Sanders MJ, Pickard DJ, et al. Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. Proc Natl Acad Sci U S A. 2012 Jan 24;109(4):1269–74.

175. Doucet-Populaire F, Trieu-Cuot P, Dosbaa I, Andremont A, Courvalin P. Inducible transfer of conjugative transposon Tn1545 from Enterococcus faecalis to Listeria monocytogenes in the digestive tracts of gnotobiotic mice. Antimicrob Agents Chemother. 1991 Jan;35(1):185–7.

176. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina Sequence Data. Bioinformatics. 2014 Apr 1;btu170.

177. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics. 2009 Jul 15;25(14):1754–60.

178. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinforma Oxf Engl. 2012 Dec 1;28(23):3150–2.

179. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, et al. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother. 2014;58(1):212–20.

180. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012 Nov;67(11):2640–4.

181. McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, et al. The Comprehensive Antibiotic Resistance Database. Antimicrob Agents Chemother. 2013 Jul 1;57(7):3348–57.

182. Paulson JN, Stine OC, Bravo HC, Pop M. Differential abundance analysis for microbial marker-gene surveys. Nat Methods. 2013 Dec;10(12):1200–2.

183. McMurdie PJ, Holmes S. Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. PLoS Comput Biol. 2014 Apr 3;10(4):e1003531.

184. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015 Jan 20;gkv007.

185. Legendre P, Gallagher ED. Ecologically meaningful transformations for ordination of species data. Oecologia. 2001 Oct 1;129(2):271–80.

186. Clarke KR. Non-parametric multivariate analyses of changes in community structure. Aust J Ecol. 1993 Mar 1;18(1):117–43.

187. Schmitt H, Stoob K, Hamscher G, Smit E, Seinen W. Tetracyclines and tetracycline resistance in agricultural soils: microcosm and field studies. Microb Ecol. 2006 Apr;51(3):267–76.

188. Tian B, Fadhil NH, Powell JE, Kwong WK, Moran NA. Long-term exposure to antibiotics has caused accumulation of resistance determinants in the gut microbiota of honeybees. mBio. 2012;3(6).

189. Zhang X-X, Zhang T, Fang HHP. Antibiotic resistance genes in water environment. Appl Microbiol Biotechnol. 2009 Jan 8;82(3):397–414.

190. Barlow RS, Fegan N, Gobius KS. Integron-containing bacteria in faeces of cattle from different production systems at slaughter. J Appl Microbiol. 2009 Aug;107(2):540–5.

191. Oikonomou G, Teixeira AGV, Foditsch C, Bicalho ML, Machado VS, Bicalho RC. Fecal Microbial Diversity in Pre-Weaned Dairy Calves as Described by Pyrosequencing of Metagenomic 16S rDNA. Associations of Faecalibacterium Species with Health and Growth. PLoS ONE. 2013 Apr;8(4):1–11.

192. Klein-Jöbstl D, Schornsteiner E, Mann E, Wagner M, Drillich M, Schmitz-Esser S. Pyrosequencing reveals diverse fecal microbiota in Simmental calves during early development. Front Microbiol. 2014 Nov;5:1–8.

193. Fouhy F, Ogilvie LA, Jones BV, Ross RP, Ryan AC, Dempsey EM, et al. Identification of Aminoglycoside and β-Lactam Resistance Genes from within an Infant Gut Functional Metagenomic Library. PLoS ONE. 2014 Sep;9(9):1–10.

194. Berge A c. b., Atwill E r., Sischo W m. Animal and farm influences on the dynamics of antibiotic resistance in faecal Escherichia coli in young dairy calves. Prev Vet Med. 2005 Jun 10;69(1/2):25–38.

195. Pereira RV, Siler JD, Ng JC, Davis MA, Grohn YT, Warnick LD. Effect of on-farm use of antimicrobial drugs on resistance in fecal Escherichia coli of preweaned dairy calves. J Dairy Sci. 2014 Dec;97(12):7644–54.

196. Pereira RVV, Santos TMA, Bicalho ML, Caixeta LS, Machado VS, Bicalho RC. Antimicrobial resistance and prevalence of virulence factor genes in fecal Escherichia coli of Holstein calves fed milk with and without antimicrobials. J Dairy Sci. 2011 Sep;94(9):4556–65.

197. B. Berge AC, Moore DA, Sischo WM. Field Trial Evaluating the Influence of Prophylactic and Therapeutic Antimicrobial Administration on Antimicrobial Resistance of Fecal Escherichia coli in Dairy Calves. Appl Environ Microbiol. 2006 Jun;72(6):3872–8.

198. USDA. Dairy 2007, Part V: Changes in Dairy Cattle Health and Management Practices in the United States, 1996-2007 [Internet]. USDA Animal and Plant Health Inspection Service; 2009 [cited 2015 Jun 20]. Available from:

http://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy07/Dairy07_dr_PartV_rev.pdf

199. Mitchell SM, Ullman JL, Teel AL, Watts RJ. pH and temperature effects on the hydrolysis of three β-lactam antibiotics: Ampicillin, cefalotin and cefoxitin. Sci Total Environ. 2014 Jan 1;466–467:547–55.

200. Peak N, Knapp CW, Yang RK, Hanfelt MM, Smith MS, Aga DS, et al. Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. Environ Microbiol. 2007 Jan;9(1):143–51.

201. Ham JM, DeSutter TM. Toward site-specific design standards for animal-waste lagoons: protecting ground water quality. J Environ Qual. 2000;29(6):1721–32.

202. Fernández Fuentes MÁ, Ortega Morente E, Abriouel H, Pérez Pulido R, Gálvez A. Antimicrobial resistance determinants in antibiotic and biocide-resistant gram-negative bacteria from organic foods. Food Control. 2014 Mar;37:9–14.

203. Durso LM, Wells JE, Harhay GP, Rice WC, Kuehn L, Bono JL, et al. Comparison of bacterial communities in faeces of beef cattle fed diets containing corn and wet distillers' grain with solubles. Lett Appl Microbiol. 2012 Aug;55(2):109–14.

204. Shanks OC, Kelty CA, Archibeque S, Jenkins M, Newton RJ, McLellan SL, et al. Community Structures of Fecal Bacteria in Cattle from Different Animal Feeding Operations. Appl Environ Microbiol. 2011 May 1;77(9):2992–3001.

205. Daniel CR, Cross AJ, Koebnick C, Sinha R. Trends in meat consumption in the United States. Public Health Nutr. 2011 Apr;14(4):575–83.

206. Antibiotic Resistance from the Farm to the Table [Internet]. The Centers for Disease Control and Prevention; 2014 [cited 2015 Jul 27]. Available from: http://www.cdc.gov/foodsafety/from-farm-to-table.html

207. National Antimicrobial Resistance Monitoring System – Enteric Bacteria (NARMS): 2011 Executive Repor. U.S. Department of Health and Human Services, Food and Drug Administration; 2013.

208. Rolain J-M. Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. Front Microbiol. 2013 Jun 24;4:173–173.

209. Sheikh AA, Checkley S, Avery B, Chalmers G, Bohaychuk V, Boerlin P, et al. Antimicrobial resistance and resistance genes in Escherichia coli isolated from retail meat purchased in Alberta, Canada. Foodborne Pathog Dis. 2012 Jul;9(7):625–31.

210. Landers TF, Cohen B, Wittum TE, Larson EL. A Review of Antibiotic Use in Food Animals: Perspective, Policy, and Potential. Public Health Rep. 2012;127(1):4–22.

211. See Materials and Methods.

212. Legendre P, Gallagher ED. Ecologically meaningful transformations for ordination of species data. Oecologia. 2001 Oct;129(2):271–80.

213. Umu ÖCO, Frank JA, Fangel JU, Oostindjer M, da Silva CS, Bolhuis EJ, et al. Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations. Microbiome. 2015;3:16.

214. Mach N, Berri M, Estellé J, Levenez F, Lemonnier G, Denis C, et al. Early-life establishment of the swine gut microbiome and impact on host phenotypes. Environ Microbiol Rep. 2015 Jun 1;7(3):554–69.

215. Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ, et al. Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. Proc Natl Acad Sci U S A. 2012 Sep 18;109(38):15485–90.

216. Higgins PG, Pérez-Llarena FJ, Zander E, Fernández A, Bou G, Seifert H. OXA-235, a novel class D β-lactamase involved in resistance to carbapenems in Acinetobacter baumannii. Antimicrob Agents Chemother. 2013 May;57(5):2121–6.

217. Jung Y-H, Shin ES, Kim O, Yoo JS, Lee KM, Yoo JI, et al. Characterization of Two Newly Identified Genes, vgaD and vatG, Conferring Resistance to Streptogramin A in Enterococcus faecium. Antimicrob Agents Chemother. 2010 Nov;54(11):4744–9.

218. Cheng G, Hu Y, Yin Y, Yang X, Xiang C, Wang B, et al. Functional screening of antibiotic resistance genes from human gut microbiota reveals a novel gene fusion. FEMS Microbiol Lett. 2012 Nov;336(1):11–6.

219. Greig JD. The efficacy of interventions applied during primary processing on contamination of beef carcasses with Escherichia coli: A systematic review-meta-analysis of the published research. Food Control. 2012;27(2):385–97.

220. Wood DE, Salzberg SL. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 2014;15(3):R46.

221. McMurdie PJ, Holmes S. Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. PLoS Comput Biol. 2014 Apr 3;10(4):e1003531.

222. Smith DP, Peay KG. Sequence Depth, Not PCR Replication, Improves Ecological Inference from Next Generation DNA Sequencing. PLoS ONE. 2014 Feb 28;9(2):e90234.

223. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3:Article3.

224. Martínez JL, Coque TM, Baquero F. What is a resistance gene? Ranking risk in resistomes. Nat Rev Microbiol. 2015 Feb;13(2):116–23.

225. Tsai YL, Olson BH. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl Environ Microbiol. 1992 Jul 1;58(7):2292–5.

226. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. vegan: Community Ecology Package [Internet]. 2014. Available from: http://CRAN.R-project.org/package=vegan

227. Nesme J, Simonet P. The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environ Microbiol. 2015 Apr 1;17(4):913–30.

228. Ding T, Schloss PD. Dynamics and associations of microbial community types across the human body. Nature. 2014 May 15;509(7500):357–60.

229. Ma B, Forney LJ, Ravel J. Vaginal Microbiome: Rethinking Health and Disease. Annu Rev Microbiol. 2012;66(1):371–89.