THESIS

CHARACTERIZATION OF THE RESISTOME AND MICROBIOME OF RETAIL MEATS PROCESSED FROM CARCASSES OF CONVENTIONALLY AND NATURALLY RAISED CATTLE

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ABSTRACT

CHARACTERIZATION OF THE RESISTOME AND MICROBIOME OF RETAIL MEATS PROCESSED FROM CARCASSES OF CONVENTIONALLY AND NATURALLY RAISED CATTLE

Concern over human exposure to antimicrobial resistance (AMR) via consumption of meat products has raised questions about use of antimicrobial drugs in food-animal production. This concern has led to an increase in consumer demand for meat products from naturally-raised cattle, or those raised without use of antimicrobials. While previous studies have assessed AMR gene presence in cattle and throughout the beef supply chain, very little work has surveyed the resistome on retail meats available for consumer purchase. The objective of this study was to determine the extent of antimicrobial resistance and characterize the microbiome in retail ground beef products from naturally-raised (raised without antibiotics) and conventionally-raised cattle utilizing 16S rRNA and targeted shotgun metagenomic, high-throughput sequencing techniques.

Differing in packaging types and lean points, samples of ground beef derived from carcasses of cattle that were conventionally-raised (n = 50) or naturally-raised cattle (n = 50) were purchased from retail outlets in six major metropolitan cities throughout the United States. Samples were shipped to Colorado State University and processed following 48 hours of refrigeration at 4°C. Thirty-gram portions of each sample were removed and subjected to DNA extraction procedures via DNeasy PowerFecal Microbial Kit. Cell lysates were composited by production system and city before being subjected to paired-end 16s rRNA gene sequencing and targeted shotgun metagenomic sequencing using an enrichment system developed in our

laboratory. Microbiome analysis was performed from 16s data with QIIME2 v.2018.4 by utilizing many of the available plugins. Resistome analysis of enriched metagenomic data was performed using a modified AMRPlusPlus pipeline.

Microbiome alpha diversity analysis indicated that ground beef processed from conventionally-raised animals had a greater ($P < 0.05$) species richness than natural ground beef products. Microbiome composition differed ($P < 0.05$) between samples of differing production systems based on abundance weighted UniFrac distances. Additionally, when analyzed using unweighted UniFrac distances, microbial composition differed ($P < 0.05$) between samples from different cities. Differences in product packaging availability between cities may have caused these differences detected in microbiome composition, as well as environmental contamination or product handling in distribution.

Targeted shotgun sequencing yielded a total of 4.6 trillion reads across all 60 composite samples, with only 58 samples containing hits to AMR. Of these 58 samples, 10.1 million reads were assigned to: 520 groups, 101 mechanisms of resistance, and 22 classes of antibiotics. The three most abundant classes of resistance detected included tetracyclines (56% of assigned reads), multi-drug resistance (21% of reads), and beta-lactams (7% of reads). An analysis of similarity on samples ordinated using Euclidian distances suggested that the overall resistome differed ($P < 0.05$) by production system, likely driven by greater antimicrobial resistance group variation among conventional retail samples.

Results from this study profiled resistance and characterized microbial composition of retail beef products from two major production practices. While the results do not discredit concern over imprudent use of antibiotics in beef production, differing management techniques

in cattle production do not appear to have a direct impact on the resistome or microbiome of final retail products available to consumers.

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LITERATURE REVIEW

1.10 A Public Health Crisis

Bacterial resistance to antimicrobials is a "public health crisis" and "one of the biggest health challenges of our time".[1] Concern over antibiotic resistance has risen to such a height that the World Health Organization established a Global Action Plan on Antimicrobial Resistance, which outlines surveillance and optimization of antibiotic use for the future.[2] This is important because the overuse or misuse of antibiotics in human and veterinary medicine may select and allow for the proliferation of resistant bacterial populations within a host.

Antibiotic resistance is a phenomenon that has occurred for millennia[3]; however, evidence suggests that an increase in treatment failures of bacterial infections and a rise in multi-drug resistance (MDR) over the last fifty years may be linked to the extensive use of antibiotics in modern medicine.[4] As the discovery of new antibiotic treatments has slowed to a crawl since the 1940s and 1950s, multidrug resistant infections and treatment failure are a high concern amongst the medical and scientific communities. A multidrug resistant strain of bacteria is resistant to three or more classes of antibiotic treatment. While less common, but of significant concern, "pandrug" resistance (PDR) is defined as being resistant to all antimicrobial agents.[5]

1.11. History of Antibiotics

Compounds with antibiotic properties have been in use for centuries.[6] However, the modern antibiotic era began with the synthesis of salvarsan, a drug developed to treat syphilis at the start of the 20th century. A serendipitous event in 1928 by Alexander Fleming led to the

discovery of penicillin,[7] which quickly replaced salvarsan as the most widely used antibiotic worldwide. The twenty years following Fleming's achievement came to be known as the golden age of antibiotic discovery, as over half of the classes of antibiotics used today were discovered during this time.[8] This era began with isolation of streptomycin in 1944 from a soil organism, *Streptomyces griseus*, which quickly resulted in a worldwide effort to uncover other naturally occurring antibiotics.[9]

As the initial surge in antibiotic discovery from soil bacteria slowed, researchers began calling for new techniques to unearth novel antibiotic treatments capable of combating antibiotic resistant infections. Previously, antibiotic discoveries were made utilizing susceptibility testing originally employed by Fleming in 1928. While efficacy of those techniques cannot be disputed, such techniques were limited to culturable bacteria. In recent years, progress has been made in developing culture-independent techniques; one of which has led to discovery of a new antibiotic treatment used to combat resistant bacteria.[10,11] The successful utilization and development of culture-independent techniques, thus far, holds promise for future antibiotic discoveries.

1.12 Antibiotic Resistance

Discovery of antibiotics revolutionized medicine, saved countless lives, and is considered a major turning point in human history. Unfortunately, extensive use of such drugs in human and veterinary medicine has been accompanied with a rise in resistant strains of bacteria.[12] While penicillin resistance was observed *in vitro* years before its extensive use began in human medicine,[13] penicillin resistance did not draw much attention until penicillin resistant infections, combined with treatment failure, began occurring within the human populace.[14]

The most prevalent gram-negative pathogens, *Escherichia coli* and *Salmonella enterica,* cause a variety of diseases in both humans and animals. Multi-drug resistance was first detected in *E. coli* and *Salmonella* in the late 1950s and 1960s.[15] Within the last fifty years, a correlation between exposure of these pathogens to antibiotics and selection for antibiotic resistance has been observed.[12] Additionally, numerous accounts of other multi-drug resistant enteric pathogens have been isolated since the first detection of resistance in *E. coli* and *Salmonella*.[16,17,18]

As food production and distribution systems continue to evolve in their complexity, there is a need for continued antimicrobial resistance monitoring and surveillance. This is especially true due to the many antimicrobial mechanisms of resistance.

1.13. Antimicrobial Mechanisms of Resistance

Antibiotics are used in the treatment of bacterial infections and are deemed effective when able to successfully induce cell death or inhibit cellular growth of a target pathogen via inhibition of DNA synthesis, RNA synthesis, cell wall synthesis, or protein synthesis.[19] Resistant bacteria are those that have a mechanism for counteracting effects of antimicrobial agents. The biochemical mechanism of resistance utilized by bacteria often includes one or more of the following: antibiotic inactivation, target modification, and/or altered permeability.[20]

Antibiotic inactivation predominately affects beta-lactams and aminoglycosides due to enzymatic action via beta-lactamases and aminoglycoside-modifying enzymes, respectively. Beta-lactamases inactivate through hydrolysis of ester and amide bonds, which are molecular structures that make up penicillins, cephalosporins, monobactams, and carbapenems. Imprudent use of beta-lactams is thought to have contributed to emergence of extended spectrum beta-lactamases (ESBL).[21] ESBL producing bacteria exhibit co-resistance to multiple classes of

antibiotics, including third-generation cephalosporins, which can result in major therapeutic challenges and creates potential for important medical treatment failure. Extended spectrum beta-lactamase are typically identified in enteric pathogens - stressing the importance of efficient infection control systems within the agricultural industry.[22]

Additionally, aminoglycoside-modifying enzymes reduce affinity on the surface of bacteria to antimicrobial agents and impede binding of antimicrobials to the 30S subunit. Together, these biochemical mechanisms result in extended spectrum resistance to aminoglycosides and fluoroquinolones. Aminoglycoside-modifying enzymes have been identified in *Staphylococcus aureus* isolates and can exacerbate human *Staphyloccus aureus* infections.[23] In addition to antibiotic inactivation, target modification is another biochemical mechanism of resistance.

Alterations of antibiotic binding sites of the target location within a bacterial cell, or target modification, is a common mechanism of antimicrobial resistance. Occurring through spontaneous mutation or selection of existing genes, a minor alteration can confer resistance to antibiotics, depending on the site of the mutation. For example, alterations to the ribosomal subunit can render antibiotic treatments that target protein synthesis ineffective. Furthermore, alterations to the bacterial cell wall can affect antibiotics that target and disrupt cell wall synthesis, such as beta-lactams.[24] Similar to target site alteration, slight mutations in the bacterial genome can result in target protection or the synthesis of specialized proteins capable of binding to the active site and dislodging the antimicrobial compound. An example of this is tetracycline resistance, which is commonly achieved through a mechanism of action via the Tet(O) gene, as synthesized Tet(O) can bind directly to the 16S ribosomal subunit and directly dislodge bound

tetracycline.[25] In addition to target modification and antibiotic inactivation, some bacteria have developed resistance to antibiotics through changes in membrane permeability.[26]

For example, many antibiotics used to treat gram-negative bacterial infections target cell components located within the cytoplasm or cell-membrane, which alters the cell's permeability. To prove effective, an antimicrobial compound must permeate the outer cell wall and/or cytoplasmic membrane to reach its target. Alterations to porins, which are channels through which substances pass from outside the cell to the inside, can result in resistance to antibiotics. For example, any shift in the type of porins being expressed by a cell, change in porin expression levels, or impairment of porin expression, may prevent an antibiotic from reaching its target.

While limited permeability results in low-level resistance, it often is associated with presence of efflux pumps. Production of efflux pumps allows the cell to extrude toxic compounds from within the cell to the outside environment. Efflux pumps may be specific to one substrate or can be found on a range of structurally dissimilar substrates, which can often be associated with multi-drug resistance.[27] Efflux pumps can provide resistance against metals, biocides, and organic solvents, making them especially difficult to treat if present and over-expressed in human pathogens.[28]

Focusing on altered permeability, specifically the development of efflux pump inhibitors, may be the next step in the fight against antimicrobial resistance. As for now, there are only a few critically important antimicrobials, defined by the World Health Organization, that are essential in the fight against antimicrobial resistance.

1.14 Critically Important Antibiotics

The World Health Organization defines critically important antimicrobials as 1) a class of antibiotics that is the sole or one of limited therapies available to treat a harmful bacterial infection and 2) the class of antimicrobial that is used to treat a human infection that was either transmitted from a non-human source or acquired resistance genes from a non-human source.[29] Among the list of critically important antimicrobials outlined by the World Health Organization, cephalosporins (3rd, 4th, and 5th generation), glycopeptides, and macrolides, are classified as the highest priority classes of antibiotics.

Among the aforementioned critically important antimicrobials, third-generation cephalosporin resistance poses a serious threat to human health because of its role in treating food-borne enteric pathogens. Ceftriaxone, which is a cephalosporin, is commonly used to treat serious *Salmonella* and *E. coli* infections in humans. Evidence suggests that application of third-generation cephalosporins selects for cephalosporin resistant *Salmonella* and *E. coli* in animals. The U.S. Centers for Disease Control and Prevention (CDC) estimates that 1.2 million infections from non-typhoidal *Salmonella* and 265,000 infections from Shiga-toxin producing *E. coli* occur each year.[30] Additionally, the CDC estimates that 6,200 ceftriaxone resistant non-typhoidal *Salmonella* infections occur annually.[31] While these statistics are sobering, resistance to third-generation cephalosporins is only a small piece to the broader puzzle that makes up antimicrobial resistance. Glycopeptides also are critically important antimicrobial that are important to investigate.

Glycopeptides are typically administered to treat *Staphylococcus aureus* and *Campylobacter spp.* infections acquired from non-human sources. Glycopeptides, specifically vancomycin, have been the predominant form of treatment for methicillin-resistant

Staphylococcus aureus (MRSA) infections.[32] Emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) has heightened concern over the misuse of glycopeptides, especially in hospital settings where nosocomial transmission of vancomycin-resistant *enterococci* (VRE) and MRSA/VRSA is quite common.[33,34] While not as virulent as *Staphylococcus* or *E. coli*, *Enterococci* are of particular concern due to their previously observed role in transmission of AMR via horizontal gene transfer.[35] Contrastingly, macrolides, which are the third critically important antimicrobial listed by the World Health Organization, are heavily used in beef production as well as for treatment of enteric pathogens in humans.

Macrolides are a class of antibiotics with a broad-spectrum of activity since they work against a variety of gram-negative and gram-positive bacteria. The use of macrolides in food-animal production began in the 1960s, and since then, a total of seven different macrolides have been approved by the FDA for use in animal agriculture.[36] Tylosin, a macrolide approved for agricultural use, is an important feed additive used in beef production to reduce the prevalence of liver abscesses in fed cattle.[37] Greater than 70% of cattle in feedlots that contain more than 1000 head of cattle are administered Tylosin.[38] This use of macrolides in agriculture has been heavily scrutinized, with critics suggesting that administration of Tylosin to fed cattle increases the proportion of macrolide resistant bacteria.[39,40,41] This is a concern for human health, as well, since macrolides are typically used to treat *Campylobacter sp.* infections originating from non-human sources. As the leading bacterial foodborne pathogen worldwide, *Campylobacter sp.* infections that are resistant to antibiotics is alarming, especially for severe cases or those involving immune-compromised patients, since such cases require antibiotic use. While resistance to common macrolides, such as erythromycin, has historically been low, reports have

noted an increase in prevalence of resistance in recent years.[42,43] This suggests a need for further investigation of macrolide resistance in food products in coming years.

In addition to the critically important antimicrobials, the World Health Organization has also classified some antimicrobials as highly important. For example, the World Health Organization classifies tetracyclines as highly important antimicrobials. Tetracyclines are classified as highly important because they play an important role in beef cattle production and serve as a limited therapy for various bacterial infections in humans. Tetracyclines are administered to fed beef and dairy cattle as prophylactic treatment for Bovine Respiratory Disease and mastitis, as well as to reduce liver abscess prevalence and to treat foot rot. Chloretetracycline and oxytetracycline are naturally-occurring, first-generation tetracyclines most commonly administered for the aforementioned purposes, and remain the class of antimicrobials sold most frequently in the United States for food-producing animals.[44] Previous research has indicated that heavy use of tetracyclines in beef production leads to a modest increase in prevalence of tetracycline resistant bacteria.[45] However, prior work suggests that the proportion of resistant bacteria may return to normal following a short withdrawal period.[46] Although tetracyclines are not used to treat food-borne infections and are not classified as critically important, there remains a concern over the transmission of antimicrobial resistance genes conferring tetracycline resistance within the environment as a consequence of excessive use.[47] Such concerns have caused an increased focus on agricultural use of antibiotics for food-animal production.

1.15 Agricultural Use of Antibiotics

One area of focus when examining agricultural use of antibiotics for food-animal production is the high population density of modern livestock operations. Aggressive infection management strategies are required to maintain herd health, which often means utilization of antibiotics.[48] Since the 1940s, antibiotics have become critically important in improving prevention, control, and treatment of infectious diseases in animals.[49] Antibiotics also have been used in other ways in livestock operations.

Not long after discovery of antibiotics, use in livestock was observed as a means for improving animal health and as a consequence, improving feed efficiency.[50] Since then, over 100 different antibiotics, including beta-lactams, aminoglycosides, tetracyclines, amphenicols, macrolides, sulfonamides, fluoroquinolones, lincosamides, polypeptides, and polyenes have been used around the world for various reasons in livestock operations.[51] Most importantly, application of antibiotics has played a critical role in treatment and control of pathogens, including *E. coli*, *Salmonella*, and *Staphylococcus aureus*. Treatment and control of these particular pathogens is very important for animal and human health. However, administration of antibiotics in cattle varies depending on multiple factors.

For example, administration of antibiotics to cattle is dependent upon the desired outcome and illness being targeted for treatment. An individual animal exhibiting signs of clinical illness may be treated with antibiotics. However, metaphylactic treatment is the most common practice of antimicrobial application in maintaining a healthy herd. Metaphylactic treatment has been shown to reduce rates of Bovine Respiratory Disease (BRD) and liver abscesses, which are two of the most commonly observed illnesses in feedlot cattle.[52] Therefore, it is evident that antibiotics play a crucial role in livestock operations. So much so, that it is

estimated that 60% of the medically important drugs sold and distributed domestically are intended for use in food-producing animals.[36] Among domestically sold and distributed drugs, 80% of cephalosporins, 51% of aminoglycosides, and 49% of tetracyclines were intended to be used in cattle production.

Understanding antibiotic use in food-animal production and emergence of antibiotic resistant infections in humans is increasingly more challenging due to the numerous interactions occurring between animals, humans, and the environment. Despite this challenge, previous work has suggested that there is a link between antibiotic use in food-animal production and antibiotic resistant infections in humans.[48] As the concern regarding the emergence of antimicrobial resistance continues to increase, many regulatory agencies throughout the world have begun banning use of antibiotics intended as growth promoting agents in food-animal production.[53] This trend began with Sweden in 1986, when the country became the first nation to ban use and monitor withdrawal of growth promoting antibiotic use. In 1995, Denmark followed Sweden's decision, and by 2006, all of the European Union had outlawed use of antibiotics for growth promotion purposes in food-producing animals.[54] The United States also has taken action in regards to antibiotic use during food-animal production.

As of January 2017, the United States Food and Drug Administration (FDA) implemented GFI #233, the Veterinary Feed Directive Final Rule, which bans use of critically-important antibiotics as growth promoters via feed or water and requires that the administration of all antibiotics for prophylactic and metaphylactic use be prescribed by a veterinarian.[55] While this directive may reduce rates of misuse and overuse of drugs in the United States' livestock operations, there is still a growing need for extensive research on antimicrobial resistance, particularly in meat production.

1.16. Antimicrobial Resistance in Beef

Antimicrobial resistance is of great concern in commonly consumed foods because it results in 23,000 deaths, annually, due to compromised antibiotic ability to treat severe infections.[30] Furthermore, the CDC estimated that 410,000 antibiotic resistant food-borne infections occur per year just from *Salmonella* and *Campylobacter* transmission via food consumption. Use of antimicrobials in food-animal production has heightened concern over the presence of AMR in meat products, specifically. So much so that there has been an impact on consumer purchasing decisions, which has resulted in a slight shift in the demand for organic and naturally sourced food products, including meat.[56]

Despite this shift in demand patterns, there remains a lack of empirical evidence regarding the impact that differing beef production systems can have on presence of AMR in beef products. While AMR has been investigated at various stages throughout beef production, very little work has been performed on meat products at the retail level.[57] As of 2017, the United States Department of Agriculture – Economic Research Services estimated that the average person living within the U.S. consumes 25.8 kg of beef per year.[58] While the opportunity for consumer exposure seems likely, the current gap in the scientific literature creates challenges in quantifying the impact of exposure, infection, and treatment failure due to AMR bacteria through meat consumption. One specific challenges that exists is the current techniques used to investigate antimicrobial resistance in food-borne isolates.

1.20 Culture-Independent Techniques

Historically, antimicrobial resistance research was performed via culture-dependent techniques on food-borne pathogen isolates. Traditional methods typically involved disk diffusion tests, broth microdilution, or minimum inhibitory concentration testing.[59] Although

effective, these techniques are limited to culturable bacteria. Previous research suggests that less than 1% of the microbial world can be cultured in a lab, which means there is a need to move beyond culture-dependent techniques.[60] This would allow for more robust investigations of antimicrobial resistance among environmental samples.[60] Development and advancement of culture-independent techniques in recent years has opened doors to studying whole communities of microbes and how their interactions may impact human health.[61]

1.21. History of Culture-Independent Approaches for Biological Research

Culture-independent methods have their roots in the discovery of genetics, dating back to Gregor Mendel's Principle of Independent Assortment and Principle of Segregation, which Mendel developed during the 1860's. Theodor Boveri built upon Mendel's laws in the early 1900's when Boveri provided a mechanistic basis for the laws originally posited by Mendel. Boveri would go on to hypothesize about the relationship between genetic instability and cancer development following observations on haploid cells - many of which remain true to this day.[62] Nearly 40 years later, Oswald Avery, Colin McLeod, and Maclyn McCarty built off of Fredrick Griffith's principles by demonstrating the effect of bacterial transformation using virulent and non-virulent strains of *Pneumoccocus* to infect mice.[63] A few years later, Alfred Hershey and Martha Chase demonstrated that, when infected with a bacteriophage, DNA is inserted into a target cell rather than protein. These conclusions, in tandem with the work of Avery, McLeod, and McCarty, provided enough evidence to conclude that DNA was the genetic element of the cell.[64]

Working from the crystallographic data produced by Rosalind Franklin, James Watson and Francis Crick published their famous paper on the double-helix molecular structure of DNA

in 1953.[65] Their findings were paramount in advancing the field of molecular biology. A decade later, discovery of DNA's double-helix structure by Franklin, Watson, and Crick would prove instrumental in Fred Sanger's development of a means for sequencing whole nucleic acid sequences using radiolabeled particles.[66] Additionally, the discovery of thermostable DNA polymerase and its use in the development of the polymerase chain reaction (PCR) in 1988 provided geneticists with the ability to improve the concentrations of low-biomass samples for sequencing.[67] These advancements in sequencing technology propelled the genomics revolution forward. By the end of the 20th century, the National Institute of Health (NIH) was well on its way towards sequencing, identifying, and mapping the entirety of the human genome.[68]

Development of pyrosequencing heralded a new age of DNA sequencing technologies. While pyrosequencing and Sanger sequencing both required direct action via DNA polymerase in order for sequencing to occur, pyrosequencing can: use natural nucleotides, be observed in real-time, and increase sequencing yields by orders of magnitude.[69] The first high-throughput sequencing platform widely available to consumers was the 454, which utilized pyrosequencing techniques previously described.

The success of the 454 spurred further advancements in high-throughput sequencing technologies with the introduction of the Solexa method and the standard Genome Analyzer version GAIIx (later known as Illumina).[70] These machines utilized bridge-amplification, and while they produced very short reads compared to that of pyrosequencing, these machines were capable of generating paired-end sequence data. The benefits of increased accuracy and information from using paired-end reads quickly led to development of the Illumina HiSeq and MiSeq platforms.[71] While the HiSeq allowed for much greater read length and sequencing depth, the MiSeq still had long read lengths and the added benefit of faster turn-around at a lower

cost.[72] The greater accessibility and affordability of these sequencing platforms will continue to diversify and expand genomic research and application worldwide – much like it has since Antonie Philips van Leeuwenhoek first discovered bacteria in 1676.[73]

1.22. Metagenomics of Meat ("Meatagenomics")

Modern molecular techniques have opened the door to microbiological research not previously possible before the advent of DNA sequencing. Carl Woese first proposed the idea to utilize ribosomal RNA genes as molecular markers for phylogenetic classification during the late 1970s.[74] This idea, in conjunction with the development of Sanger sequencing and other techniques like PCR, had a remarkable impact on molecular biology and the characterization of microbial communities. Many improvements have been made since the first iterations of these technologies, which provides a means for scientists to explore the metagenome of a variety of ecological and environmental samples. The metagenome has been defined in a number of different ways, but generally encompasses "individual genome-level characterization of a community or its members, high-throughput gene-level studies of communities with methods borrowed from genomics or other 'omics' studies which are aimed at understanding trans-organismal behaviors and the biosphere at the genomic level".[75] Metagenomic research continues to shed light on the symbiotic relationship between the microbial communities and their environment. While metagenomics can be performed using a variety of methods - each specific to the research question being asked - 16S rRNA gene sequencing and shotgun sequencing are two popular methods utilized to characterize ecological samples.

1.23. Metagenomics Used in Research

Before emergence of genomic sequencing capabilities, microbial species and communities were explored with the exclusive use of traditional, cultural methods. Metagenomic methods, including 16S rRNA gene sequencing and shotgun sequencing, can be used in conjunction with culture methods. However, they are often utilized as standalone techniques. Often referred to as amplicon sequencing, 16S rRNA gene sequencing utilizes a hypervariable, highly conserved region within the 16S ribosomal RNA of a bacterial cell to identify an isolate or characterize an entire microbial community within a given ecological niche. DNA from samples is typically isolated via DNA extraction kits, and the V4 region of the 16S rRNA gene is amplified using specific primers and PCR. The V4 region is typically used in evaluation of phylogeny of bacterial communities, as studies comparing the nine variable regions making up the 16S gene have indicated the V4 region is the most reliable in representing the full length of 16S rRNA gene in downstream phylogenetic analysis.[76] Shotgun sequencing uses a different approach following DNA isolation. Rather than amplifying a specific gene region, the DNA is sheared into small fragments, which are then sequenced. Amplicon sequencing has many advantages over shotgun sequencing when characterizing the microbiome of a given ecological sample. Nevertheless, shotgun sequencing has many other complementary advantages that cannot be ignored, such as its ability to sequence genomic regions outside of the 16s gene.[77]

Microbiome research utilizing amplicon sequencing was popularized following inception of the NIH's Human Microbiome Project (HMP) - a follow-up to the Human Genome Project performed during the late 1990s and early 2000s. The HMP explored the relationship between humans and the many microbial niches within and on the surface of the human body.[78] In addition, the Human Microbiome Project spurred many studies that focus on the importance of

the human gut microbiome and the impact it has on human health, specifically gastrointestinal health. One such study utilized 16S rRNA gene sequencing to explore effects of a fecal microbiota transplant from a healthy donor to an individual infected with *Clostridium difficile*. Results from the 16S analysis indicated that the fecal transplant altered gut microbiome enough to rid the individual of infection without the dramatic effects seen following antibiotic use.[79] Fecal microbiota transplants have since become widely accepted amongst the medical community, as it eliminates the need for antibiotics for a common gastrointestinal condition.

Similar to amplicon sequencing, shotgun sequencing can characterize microbial communities residing within a given ecological sample. Rather than amplifying a specific region of the genome and aligning sequenced reads to a reference database, reads produced from shotgun sequencing can be assembled *de novo*. This method has led to discovery of new bacteria and characterization of specific prokaryotic genetic elements.[80,81] In addition to studying the phylogenetic classification of microbial communities, shotgun sequencing has been extensively utilized in characterizing other important genetic elements of bacteria. In addition, shotgun sequencing has been instrumental in providing insight into the pathogenicity of virulent bacteria. before availability of next-generation sequencing, little was known about virulent, genetic elements of bacteria that lead to human infection. Modern genomic sequencing capabilities have provided new tools to explore mechanisms of pathogenicity. For example, high-throughput sequencing has been used to compare genomes of pathogenic *Escherichia coli* O157:H7 to non-pathogenic *Escherichia coli* K12, leading to identification of *E. coli* O157:H7 pathogenicity islands (O-islands) that code for production of shiga toxins, along with other proteins that aid in infection of a host.[82]

In addition to virulence factors, shotgun sequencing has been used extensively in antimicrobial resistance research. As antimicrobial resistance continues to remain a top public health concern among global health agencies, such as the World Health Organization and the United States Centers for Disease Control and Prevention, increased attention to research investigating mechanisms and transmission of antimicrobial resistance has occurred. To date, antimicrobial resistance has been explored and characterized in numerous ecological niches, including (but not limited to) ocean water,[83] soil microbes,[84] and the human gut.[85] Studies also have investigated changes in antimicrobial resistance throughout an entire production system, such as starting at entrance into a feedlot and concluding at carcass fabrication following slaughter.[57] The diversity of antimicrobial resistance research occurring speaks to the impact that shotgun sequencing has had on the scientific community. While exploratory research will continue as scientists seek to better understand antimicrobial resistance, development of new antibiotics will be paramount in combating emergence of antimicrobial resistant bacterial infections. Previously, Song and others compared genomes of various pathogens previously sequenced using shotgun sequencing to identify more than 200 genes essential to growth among gram-positive bacteria.[86] Similarly, 27 genes essential to growth were found in *E. coli*.[87] Identification of such genes sheds light on potential mechanisms of resistance, while also providing targets to focus future antibiotic development efforts, especially when utilizing next-generation sequencing and bioinformatic techniques.

1.24. Bioinformatic Techniques

Next-generation sequencing has transcended the field of human genomics and embedded itself in various realms of biological study. Study design, DNA extraction procedures, and

bioinformatic methods evolved in order to address the multitude of differing scientific objectives. DNA and RNA sequencing technologies have allowed for the exploration and characterization of the molecular and microbial world with unbound potential.

When designing a metagenomic study, factors such as cost, sample size, and sample collection must be carefully considered. Due to the current cost of next-generation sequencing, sample size of metagenomic research tends to be smaller than studies utilizing traditional culture methods. Additionally, contamination of any bacteria or bacterial DNA can severely affect results from a metagenomic study, especially when working with low biomass samples. Extreme care should be taken when collecting samples and processing samples in a lab to ensure that a clean environment and equipment are used because DNA has been found to reside ubiquitously within lab settings and DNA extraction kits.[88] Further, it is good practice to collect extra samples since having to return to a location to gather more samples may result in temporal differences in downstream analyses.[89] Once collected, samples should be frozen at -80 degrees Celsius as this has been found to best preserve DNA.[90] It can also be beneficial to freeze multiple aliquots of a single sample, allowing for a single freeze-thaw cycle to occur in the case of re-extraction. While freezing helps to preserve DNA, every freeze-thaw cycle a sample goes through can have implications on rare species of the sample microbiota.[91] Maintaining freeze-thaw consistency across all samples should be accounted for when designing a metagenomic study to reduce bias during processing and DNA extraction.

Following extraction, the DNA must be collected and readied into a form that is compatible for sequencing, a process referred to as library preparation. Simply, this process requires addition of sequencing adapters that differ depending on the sequencing platform being used. For 16S sequencing, library preparation also requires addition of primers that can anneal to

a specified region of the 16S rRNA gene of the DNA, which then can be amplified using limited cycles of PCR. Libraries can be prepared as paired-end or single-end. Paired-end involves an overlap at the ends of reads, which typically results in higher quality, full-length reads.

Sequencing genomic material generates large data files requiring computationally intensive operations to analyze. This need has given rise to the interdisciplinary field of bioinformatics. Bioinformatics has been previously defined as "conceptualizing biology in terms of macromolecules (in the sense of physical chemistry), and then applying 'informatics' techniques (derived from disciplines such as applied maths, computer science, and statistics) to understand and organize the information associated with these molecules, on a large-scale".[92] Shotgun metagenomic data can be analyzed through *de novo* or reference-based assembly. Reference-based assembly utilizes a reference genome, and with the use of an alignment tool, sequenced reads are aligned to the reference genome allowing for further downstream analysis. Reference-based assembly is faster and requires less computational power than *de novo* assembly, which assembles sequenced reads one at a time to construct a longer, contiguous set of overlapping reads known as "contigs". Despite the challenges *de novo* assembly presents, it can be useful when samples drastically differ from the reference genome.[93]

Once assembled, shotgun metagenomic data can be managed in a variety of ways, including binning and gene annotation. Binning attempts to group similar reads together based on some characteristic or criteria of interest. This method is commonly utilized in microbiome research to group reads of similar taxonomic hierarchies together via comparison to a reference.[94] Annotation of metagenomic data provides further advantages to binning and can be used to identify genes of interest, such as antimicrobial resistance genes. Gene annotation

algorithms classify predicted genes into groups based on homology searches of well-characterized, available annotated data.[95]

Due to bias inherent to high-throughput sequencing, normalization of data is typically required before sound conclusions can be drawn from metagenomic data. Normalization adjusts for variation in sequencing depth introduced by inconsistencies of sequencing runs.[96] In the past, a Poisson distribution was used to correct for sequencing depth variation in the data. However, it was found to be too restrictive - predicting smaller variation than what is present in real data. This results in a heightened false-discovery rate among large genomic data sets. Later methods were developed that utilized a negative-binomial distribution instead.[97] Since development of these methods, many novel normalization techniques have been proposed and utilized in metagenomic research. Rarefaction, or the process of bootstrapping samples without replacement at a determined threshold, has been shown to more clearly cluster samples based on biological origin than other normalization techniques.[98] Built off the total sum scaling (TSS) technique, cumulative sum scaling (CSS) is another commonly used normalization technique that addresses challenges in assessing differential abundance of species.[99] Also an extension of the quantile normalization approach, CSS has improved marker gene survey data analysis.

Shotgun metagenomic methods are a potential tool that will continue to be utilized in a growing number of scientific and medical fields. As metagenomic methods continue to develop and downstream bioinformatic tools for data analysis advance, use of high-throughput sequencing will prove to be an invaluable tool in addressing complex issues as the scientific community turns more attention to the many interactions occurring at the microbial and molecular level.

CHAPTER 2

CHARACTERIZATION OF THE RESISTOME AND MICROBIOME OF RETAIL MEATS PROCESSED FROM CARCASSES OF CONVENTIONALLY AND NATURALLY RAISED CATTLE

Summary

The objective of this study was to characterize the resistome and microbiome of retail ground beef products derived from carcasses of cattle that were managed in two different production systems. Retail samples of ground beef were purchased from six major metropolitan cities throughout the United States. Samples consisted of ground beef products processed from conventionally-raised (N = 300, n = 50 / city) and naturally-raised (N = 299, n = 50 / city) cattle. The DNA was isolated from each sample and composited into groups containing products of like production systems and packaging (n = 30). Once DNA was isolated and composited, libraries were prepared on each sample before being subjected to 16S rRNA amplicon sequencing and targeted-shotgun sequencing. Raw sequence files were imported into QIIME2, the microbiome of all samples was characterized and evaluated using a variety of bioinformatic tools. Reads generated via targeted-shotgun sequencing were run through a modified AMRPlusPlus bioinformatic pipeline that included trimming low quality reads, removing Bovine DNA, assembling contigs, and classifying contigs to Hidden Markhov Models (HMM) trained on MEGARes AMR database to generate a count matrix of hits to classes and mechanisms of antimicrobial resistance. Counts were normalized and subjected to various downstream statistical analyses that allowed for characterization of the retail ground beef resistome and a comparison between differing beef production systems.

Microbiome analysis indicated all retail beef samples were dominated by Firmicutes and Proteobacteria, accounting for more than 90% of all assigned exact sequence variants. Samples from different production systems did not differ in overall microbial composition. However, differences were detected ($P < 0.05$) in samples collected from different cities, which was perhaps a result of variation in packaging of available products from city to city, or perhaps due to differences in handling. Evaluation of sample resistomes indicated that all samples were dominated by resistance to tetracyclines, followed by multi-drug resistance via efflux pumps, and beta-lactam resistance. An analysis of similarity performed on AMR counts ordinated on a non-metric multidimensional scale using Euclidian distances suggested that the overall resistome of samples of ground beef derived from cattle finished via differing production system differed ($P < 0.05$). Additionally, resistome composition of retail beef products collected from different cities differed ($P < 0.05$). Further, retail samples collected from Seattle had a greater number of hits ($P < 0.05$) to antimicrobial resistance than samples from any other city. These results established patterns of ecological resistance among retail ground beef samples processed from two different production systems commonly utilized in the United States.

Introduction

Global demand for animal protein is rising at an unprecedented rate.[100] Modern food-animal production systems commonly utilize antimicrobials to satisfy a need for improved production efficiency to meet demand.[101] Prophylactic use of antimicrobials in livestock production is thought to be associated with increased antimicrobial resistance in foodborne pathogens,[102] potentially leading to treatment failure in human infections. Traditionally, feed additive forms of tetracyclines and macrolide antibiotics have been used to improve herd health

and as a consequence, production efficiency of beef cattle. Implementation of the U.S. Food and Drug Administration's Veterinary Feed Directive: Final Rule prohibited sub-therapeutic use of medically important antibiotics in January 2017, which includes oxytetracycline, chloretetracycline, and tylosin.[55] In 2015, the United States beef industry produced 23.7 billion pounds of beef, of which 2.4 billion was exported to nations around the world.[103,104] The sheer volume of production and export of beef highlights the importance of regulations seeking to reduce imprudent use of antibiotics. While the link between antibiotic use in food-animal production and human treatment failure has not been established, many studies unequivocally support the notion that overuse and misuse of antibiotics in food production impacts human health.[105]

In recent years, a relationship has been observed between increasing consumer demand for meats produced from antibiotic-free livestock. In fact, 40% of U.S. shoppers would like their local meat retailer to stock more antibiotic-free meats.[106] Despite this, there remains a lack of empirical evidence in the literature regarding how differing production systems may impact prevalence of AMR in beef. Traditionally, AMR has been studied using culture-dependent susceptibility testing; however, much of the microbiome harbored within food producing animals is unculturable bacteria.[60] To overcome this, metagenomic methods, including shotgun and 16S rRNA gene sequencing, can be utilized to comprehensively study and characterize antimicrobial resistance and microbial composition on an ecological basis.

Targeted shotgun metagenomics is an emerging technology that allows for identification and detection of resistance genes to metals, biocides, and antibiotics that are found within all resident bacteria within an ecological niche.[107] Previous work has employed targeted-shotgun metagenomics to look at presence of AMR throughout various stages of beef production.

However, little work has been performed on retail meat products available for consumer purchase.[57] This gap in the data hinders an accurate assessment of the impact that antimicrobial use in beef production may have human health. Thus, the purpose of this study was to characterize and determine the extent of AMR in retail ground beef products processed from conventionally-raised cattle and cattle raised without antibiotics (naturally-raised).

Materials and Methods

Sampling of Ground Beef

Samples of ground beef were purchased at retail outlets from six major metropolitan cities throughout the United States over 10 months: Fort Collins, CO; Seattle, WA; Atlanta, GA; San Francisco, CA; Dallas, TX; and New York City, NY. These cities were selected to represent as much of the U.S. ground beef consumer population as possible (Supplementary 1). Fifty ground beef samples derived from carcasses of either conventionally-raised (N = 300; n = 50 / city) or naturally-raised cattle (N = 299; n = 50 / city) were collected, except for New York, where only 49 natural ground beef samples were acquired. Samples were purchased from retail cases and meat counters, consisting of chub, tray-overwrap, tray-lidded, and vacuum-sealed packaging types (Supplementary 2, Supplementary 3). Samples ranged in lean point from 73% to 96%. Retailers were visited within each city (Average of 13 stores / city) and one of each type of ground beef product was purchased until all samples were collected. USDA Organic Labels or "Never Ever" label claims were used to differentiate products of differing production systems (Conventional vs. Natural). Samples were placed on ice and shipped overnight to Colorado State University (Fort Collins, CO) for further processing.

Sample Processing and DNA Isolation

Upon arrival at Colorado State University, samples were refrigerated and processed within 48 h. A 30 g portion of each sample was aseptically removed from packaging and placed into separate WhirlPak bags for DNA isolation. One hundred ml of phosphate-buffered saline (PBS; GE Healthcare Life Sciences, Logan, UT) was added to each sample, and the mixture was massaged by hand for 30 s to create a homogenate. Three 15 ml aliquots per homogenized sample were transferred to separate 50 ml conical tubes and centrifuged at 4°C, 10,000 × *g,* (Sorvall Legend X1R centrifuge, Thermo Scientific, Waltham, MA) for 10 m. The supernatant was poured off, and pellets were stored at -80°C until DNA isolation.

Pellets were thawed to 4°C before DNA isolation. As part of the DNA isolation process, Powerbead solution (Qiagen, Hilden, Germany) was added to each conical based on weight -- for every 0.5g, 750 µL of solution was added up to 2,250 µL. Samples were vortexed until homogenized, then combined into one conical for each composite group. Samples were composited together based on similarities in packaging type, lean point, and production system (e.g., 10 samples of conventionally-raised ground beef from chub packaging with a lean point ranging from 80-85% were grouped together). An aliquot of 950 µL was used for DNA isolation using the DNeasy PowerFecal Microbial Kit (Qiagen, Hilden, Germany) and extracted according to manufacturer's instructions.

Library Preparation and Sequencing

16S Sequencing. Aliquots of DNA from each composite sample were shipped to Novogene Corporation (Beijing, China) for 16S rRNA library preparation and sequencing. The V4 region of the 16S subunit was amplified with the 515/806R primer set. Paired-end

sequencing (2 x 250 bp) was conducted on an Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA) platform.

Shotgun Sequencing. Shotgun libraries were prepared using the SureSelectXT-HS Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library (Agilent Technologies) with 'MEGaRICH', a custom-designed bait set specific to AMR genes, following the protocols described by Noyes and others.[107] Samples were shipped to the University of Colorado-Denver Genomics and Microarray Core Facility (Denver, CO) where paired-end (2 x 150 bp) sequencing of the libraries was performed on an Illumina NovaSeq 6000 System (Illumina, Inc., San Diego, CA) with a target of 100 million paired-end reads per sample.

16S Bioinformatics and Statistics

Demultiplexed samples were obtained from Novogene and processed with QIIME2 v. 2018.4.[109] Files were imported into QIIME2 with the paired-end option, where all reads were truncated at 225 base pairs at the 5' end. Sequences were denoised, merged, and exact sequence variants (ESV) were modeled using DADA2.[110] Phylogenetic trees were generated using FastTree2[111] and taxonomic classification was assigned using a pretrained Naïve Bayes classifier trained on the Greengenes database.[112] Samples were filtered to exclude mitochondria and chloroplast before being rarefied at 40,276 reads, maintaining all 60 samples.

Alpha diversity was measured using Faith's phylogenetic diversity[113] and beta diversity was assessed using weighted and unweighted UniFrac distances.[114] Alpha diversity was compared between treatments using a Kruskal-Wallis pairwise comparison. Beta diversity was assessed between treatment and sampling location using a PERMANOVA pairwise comparison[115]; these differences were visualized with EMPeror.[116] An analysis of composition of microbiomes[117] was performed to measure differential abundance of phyla between samples of

differing production systems and city. Significance for all microbiome analysis was determined at $\alpha = 0.05$, with a false discovery rate correction performed when appropriate.

Targeted Shotgun Metagenomic bioinformatics and Statistics

Demultiplexed FASTQ files were received from the University of Colorado-Denver Genomics and Microarray Core Facility. Samples were processed using the AMRPlusPlus Pipeline[118] with modifications. Samples first underwent quality control via Trimmomatic to remove low quality reads[119] before being filtered to remove bovine DNA via the Burrows-Wheeler Alignment tool.[120] Duplicate reads were removed via BBTools' deduplication script.[121] Samples were converted from FASTQ to FASTA format, and IDBA-UD was used to assemble contigs.[122] Once assembled, HMMERv3.1 was used to classify contigs to hidden Markov models (HMM) trained on the MEGARes AMR gene database for identification of AMR genes within samples.[123] Raw reads were realigned to contigs and classified as HMMs to generate a count matrix of antimicrobial resistance. Each HMM was aggregated into a class and mechanism of AMR. Genes with wild-type potential, as defined by the AMRplusplus pipeline, were removed from the analysis. Genes that were considered 'wild-type' and needed SNP confirmation, as defined by Lakin and others,[117] were removed from the analysis.

Antimicrobial resistance count data were normalized via cumulative sum scaling using the default value.[124] Samples were ordinated on a non-metric multi-dimentional scale (NMDS) by production system and city using Euclidian distances via the MetaMDS function from Vegan in R version 3.4.2.[125] Separation of ordinated groups was evaluated via analysis of similarity (ANOSIM).[126] Log fold changes in abundance of AMR genes were assessed by modeling the distribution of AMR counts via a Zero-Inflated Gaussian model. This was performed via the meg_fitZig() function in the metagenomeSeq Bioconductor package in R. Adjustments were

made for multiple comparisons using Benjamini-Hochberg procedure.[127] A Wilcoxon rank sum, non-parametric test was performed on rarefied Inverse Simpson's Index values to compare alpha diversity (number of unique AMR groups present and measure of how evenly the groups are distributed within a sample) among samples originating from differing cattle production systems. Statistical significance was determined at $\alpha = 0.05$.

Results

16S Microbiome

In total, reads were assigned to 62 phyla of bacteria across 60 samples of ground beef. Samples were dominated by Firmicutes and Proteobacteria regardless of production system or city (Figure 1). Firmicutes accounted for 51% and 46% of assigned Exact Sequence Variants (ESV) for conventional and natural ground beef samples, respectively. Furthermore, proteobacteria accounted for 42% of ESVs from conventional samples and 50% of ESVs assigned from natural ground beef samples. The third most abundant phylum was classified as unassigned bacteria that was further classified as belonging to a prokaryotic representative genome when aligned to the National Center for Biotechnology Information's nucleotide database via BLASTN 2.8.1. Of the remaining 59 classified phyla, all had a relative abundance of less than 1 percent across samples of differing production systems.

Alpha diversity, as measured by Faith's phylogenetic diversity metric, indicated that samples from carcasses of cattle raised conventionally had more ($P < 0.05$) species richness than ground beef samples from carcasses of cattle raised naturally. Beta diversity measured via Weighted UniFrac distances, which weights distances by relative abundance of phyla, suggested that microbiomes of ground beef samples differed ($P < 0.05$) by production systems of origin

(Figure 2). Analysis by product packaging indicated that samples packaging in tray-overwrap were statistically different in microbial composition compared to vacuum packaged retail products ($P = 0.001$) (Figure 3). Additionally, differences were detected between microbial composition of samples collected in New York and Fort Collins, and New York and San Francisco ($P = 0.013$ and $P = 0.010$, respectively). An analysis with Unweighted UniFrac, which only considers presence/absence of microbial communities, suggested that samples differed ($P < 0.05$) by city, but did not differ ($P > 0.05$) among production systems.

Analysis of composition of microbiomes (ANCOM) suggested that there were no phyla of bacteria that were differentially abundant between samples derived from carcasses of cattle originating from differing production systems. A comparison between the six cities from which samples were collected indicated that eight phyla (Acidobacteria, Actinobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Firmicutes, Gemmatimonadetes, and Nitrospirae) were differentially abundant (Figure 4). The "W" statistic, indicative of the number of hypotheses that were successfully rejected, ranged from 8-10 for all phyla. Gemmatimonadetes had a W statistic of 10 and a high F-statistic of 41.365, suggesting a large change in abundance between cities for this phylum of bacteria. The remaining phyla had comparable W statistics to Gemmatimonadetes, and the low F-statistics suggested that the change in abundance across cities for these phyla was not large. The difference in presence and absence of these rare phyla determined to differentially abundant may be contributing to the differences observed in beta-diversity of samples from differing cities.

Shotgun Metagenomics Results

In total, 4.6T reads were sequenced across 60 composited ground beef samples (average 77.5M reads; range 7.7M to 190M reads). Trimming of low quality base pairs resulted in an

average removal of 0.7% of reads (range: 0.1% to 6.4% reads removed). Alignment to the bovine genome (off-target host DNA) allowed removal of an average of 98.6% of reads per sample (range: 92.8% to 99.8%).

Of the 60 ground beef samples sequenced, 58 had hits to contigs (10.1M total reads; average 173k; range 89 to 5.7M) aligned to HMM models trained on the MEGARes database. Of these 58 samples, reads were assigned to a total of 520 groups, 101 mechanisms of resistance, and 22 classes of antibiotics. A total of 28 and 30 samples contained hits to AMR for samples from carcasses of cattle raised conventionally vs. naturally, respectively.

Samples originating from carcasses of cattle produced under differing systems did not differ ($P > 0.05$) in abundance of normalized AMR counts. Resistance to tetracyclines was the most abundant class of resistance among all composite ground beef samples, accounting for 56% of total hits and 47% and 61% of samples from carcasses of cattle raised conventionally vs. naturally, respectively (Figure 5). Across all samples, tetracycline resistance was a result of ribosomal protection proteins (65% of hits to tetracycline), major facilitator superfamily (MFS) efflux pumps (34%), transcriptional repressors (0.5%), and inactivation enzymes (0.3%). Multi-drug resistance (MDR) was the second most abundant class of resistance detected, accounting for 21% of total hits; 24% of hits in samples from carcasses of cattle produced conventionally vs. 18% of hits in samples from carcasses of cattle raised naturally. Multi-drug efflux pumps were responsible for 82% of hits to MDR, followed by MDR regulators (17%) and mutant porin proteins (0.7%). Remaining classes of resistance were as follows, in decreasing order of AMR counts: beta-lactams (7%); macrolides, lincosamides, and streptogrammins (MLS) (6%); glycopeptides (3%); aminoglycosides (2%); and cationic antimicrobial peptides (2%). Beta-lactam resistance was predominately a result of penicillin binding protein (41%), followed by

beta-lactamases class A (31%), class B (12%), class C (8%), and class D (5%). Mutant porin proteins and beta-lactamase regulators accounted for less than 4% of beta-lactamase resistance. Furthermore, MLS resistance was a result of a variety of efflux pumps (71%), ATP-binding cassette transporters (15%), 23S rRNA methyltransferases (9%), and nucleotidyltransferases (5%). Fifteen additional gene classes of AMR were detected but accounted for less than 5% of total hits to AMR genes.

An analysis of similarity (ANOSIM) performed on samples ordinated on a non-metric multidimensional scale using Euclidian distances at the class of resistance level of annotation suggested that the overall resistome differed ($P = 0.028$) between production systems. Despite this, the magnitude of difference was small (ANOSIM R statistic = 0.063). When ordinated at the mechanism of AMR level of annotation, differences were no longer detected in resistome composition between production systems ($P = 0.122$) (Figure 6). Further investigation into the difference between classes of resistance in samples of differing production systems via a Zero-Inflated Gaussian model suggested that conventional ground beef samples contained more ($P = 0.001$) tunicamycin resistance than natural ground beef, however it was only detected in nine samples at an average expression of 0.62 Log. Commonly used in experimental biology, tunicamycin resistance is likely occurring in ground beef as a result of natural evolution of an ancestral resistance gene. Furthermore, hits to tetracycline AMR did not differ ($P = 0.086$) by production system of origin. The greater variation observed in ordinated samples among the conventional retail beef samples is due to a greater variation at the AMR group level (Table 1). A Wilcoxon rank-based, non-parametric test performed on rarefied Inverse Simpson's Index values indicated that AMR alpha diversity did not differ at the class ($P = 0.775$) or mechanism ($P = 0.882$) levels of AMR (Figure 7).

Analyzing ground beef samples by city provided an alternative view of resistance patterns in retail ground beef samples. In terms of relative abundance, tetracycline resistance was the most abundant class of antimicrobial resistance among samples collected from Atlanta, New York, Seattle, and San Francisco, and the second most abundant class of resistance detected in samples from Fort Collins and Dallas (Figure 8). Among samples collected from Fort Collins and Dallas, MDR was the most abundant class of AMR. Further, MDR remained the second most abundant class of AMR in samples collected from Atlanta, New York, Seattle, and San Francisco. Overall, ground beef collected from Seattle contained more ($P < 0.001$) hits to AMR than samples collected from the five other major cities. Analysis of similarity performed on ordinated data by city suggested that resistome composition differed ($P < 0.001$) for both class and mechanism of AMR (Figure 9).

Discussion

Evaluation of the resistome of retail ground beef via shotgun metagenomics established clear AMR patterns. Tetracycline resistance was the most common antimicrobial class of resistance detected across all production backgrounds. While culture-independent investigation of AMR genetics in ground beef has not been explored before in the literature, consistent results when culture-dependent, antibiotic susceptibility testing of bacteria isolated from the surface of ground beef were reported.[128,129] Ground beef microbiome community composition in this study were dominated by Firmicutes and Proteobacteria. This is typical of ground beef as these phyla contain the major spoilage bacteria that tend to increase in relative abundance over the course of a product's shelf-life. Previous studies that profiled antimicrobial resistant bacterial taxa in the phyla Firmicutes and Proteobacteria isolated from various ecological sites found them to contain

greater abundance of tetracycline resistance, MDR efflux pumps, and sulfonamide resistance compared to other classes of ressitance.[130,131] Considering the unrelated nature of these sites (Wastewater sludge and deep terrestrial subsurface) and the similarity in resistance profiles present to those characterized in this study, tetracycline and MDR efflux pumps may be ubiquitous in ground-beef products with or without administration of antibiotics during cattle production.

Despite the differences in antimicrobial use throughout differing cattle production systems, resistance patterns of the microbial communities in ground beef derived from carcasses of naturally-raised versus conventionally-raised cattle were marginally different. These results were surprising considering the common, prophylactic use of macrolides in conventional beef production. A previous study has suggested that a link may exist between antibiotic use in food-animal production and the increasing number of AMR infections in humans.[132] While results of this present study did not discredit the severity and concern of overuse and misuse of antibiotics in agriculture, these production practices did not appear to have a direct impact on the resistome and microbiome of retail meats available to consumers. As increasing amounts of resources are allocated towards AMR research, these results suggested that targeting a single production practice may not be feasible in reducing human exposure to AMR via meat consumption.

Although production system did not substantially impact the microbiome and resistome of ground beef in this study, the city in which samples were collected played a significant role. It is likely that there are some contamination consequences of unique, rare taxa occurring in the retail meats. However, due to differences in consumer product demand from city to city, it is difficult to draw a sound conclusion on the major driving factor of this phenomenon. For example, ground beef samples from carcasses of cattle that were conventionally-raised come in a

variety of packaging types (i.e., chub, tray-overwrap, vacuum-sealed, tray-lidded) while ground beef derived from naturally-raised cattle are sold almost exclusively as vacuum-sealed products. Additionally, chub packaged ground beef could not be found in the thirteen retailers visited in New York City. Due to these limitations, the effects of packaging could not be accurately assessed. Nevertheless, due to differences created from manipulating product packaging (e.g., creating varied amounts of anaerobic atmosphere via vacuum or modified atmosphere packaging), it is likely that packaging differences had a large impact on the microbiome, and as a consequence, the resistome of the ground beef. Previous work that compared microbial differences in beef packaged in aerobic and anaerobic conditions using pyrosequencing technology supported this claim.[133]

The lack of a well characterized, definitive link between antimicrobial use in food-animal production and presence of AMR in retail meats presents a challenge in determining the impact antimicrobial use in agriculture is having on human AMR exposure and subsequent treatment failure. However, results presented here indicated that possible sub-therapeutic antimicrobial use in food-animal agriculture does not have a profound effect on the retail meat resistome. This study was limited in its lack of ability to characterize, using metadata, pre-harvest antimicrobial use in cattle from which products were derived. While we are confident that the natural ground beef products came from cattle that had never received antibiotic treatment (i.e., certificated products), there is a chance that a given conventional sample could have come from an animal also raised without antibiotics but was processed and sold as conventionally-raised beef. Considering that organic and natural meat products retain less than 5% of the retail market, it is likely that beef sampled as conventional product was in fact processed from trimmings derived from beef animals raised conventionally.

While clear patterns of resistance were characterized in ground beef, phenotypic expression cannot be inferred from these data. This study was the first to utilize a targeted shotgun metagenomics approach to characterize antimicrobial resistance in retail meats. Because of this, it was difficult to contextualize these results with respect to other research studies. As we continue to explore transmission and human health risks posed through use of antimicrobials during livestock production, further research profiling antimicrobial resistance throughout the entire meat production and consumption process - from feedlot entry to potential treatment failure in humans - should be considered. While this study specifically targeted United States beef production, these approaches to AMR investigation can easily be applied to other species of retail meat and expanded to evaluate entire meat production systems. This study highlights the utility of a targeted shotgun metagenomic approach to investigating AMR in meat and provides a unique look at AMR exposure in retail products that can be used to inform future agricultural and public health regulatory agencies.

Table 1: Differential abundance of antibiotic resistance classes between composite ground beef samples from carcasses of cattle raised conventionally vs. naturally analyzed via Zero-Inflated Gaussian model on Log2 Normalized count data. Positive values indicate greater abundance of resistance in conventional samples while negative values indicate greater counts of resistance in natural samples.

Class	Log2 Fold Change
Tunicamycin	2.686115001
Tetracyclines	-1.251806805
Mycobacterium tuberculosis-specific Drug	1.112170229
Multi-drug resistance	-0.446142428
MLS	-0.491187798
Cationic antimicrobial peptides	0.557158806
Rifampin	0.436263863
Metronidazole	-0.574404896
Thiostrepton	0.43883238
Sulfonamides	0.238045287
Fusidic acid	0.36197791
Aminocoumarins	0.227588553
Trimethoprim	0.128634895
betalactams	0.091152264
Glycopeptides	-0.122402114
Aminoglycosides	-0.022585187
Phenicol	-0.04817035
Bacitracin	-0.1117917
Fluoroquinolones	-0.067964756
Elfamycins	0.095860374
Fosfomycin	0.04688083
Lipopeptides	0.150724212

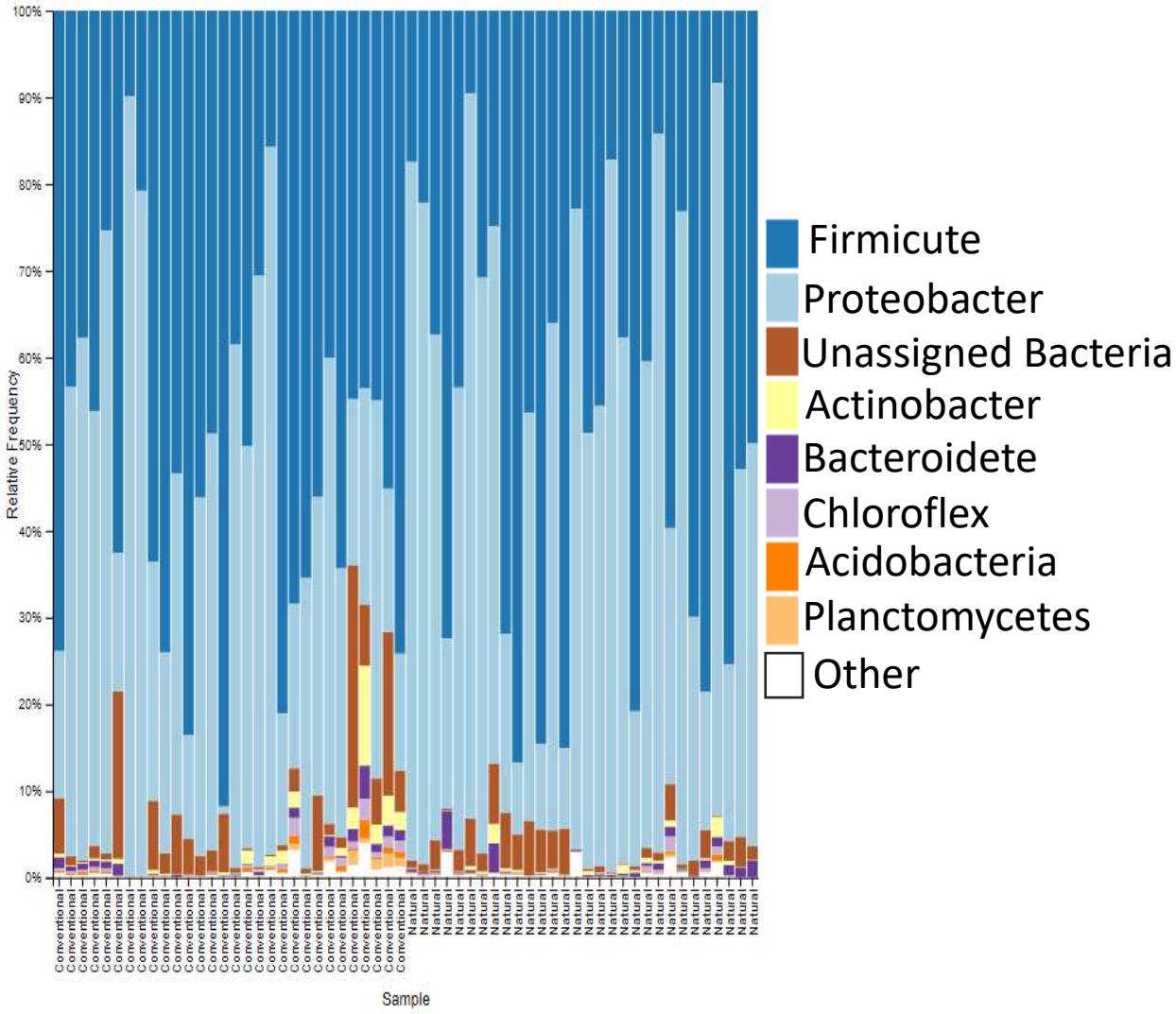

Figure 1: Relative abundance of the top nine most abundant phyla present in retail ground beef samples derived from carcasses of conventionally-raised and naturally-raised cattle. Phyla grouped into the "other" category accounted for no more than 5% of assigned exact sequence variants per sample.

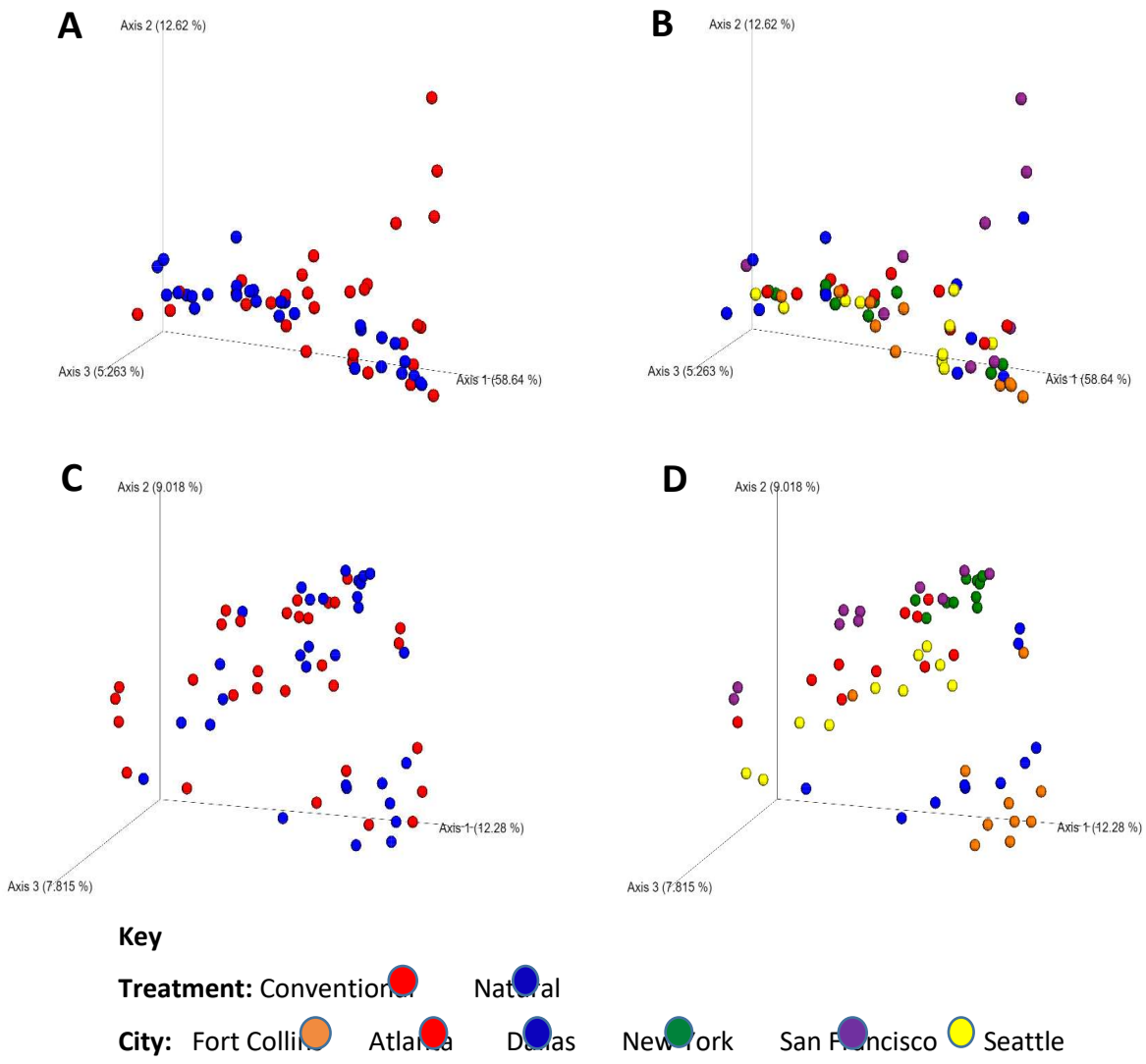

Figure 2: Weighted (A and B) and Unweighted (C and D) UniFrac distances of samples colored by treatment (A and C) and sampling location (B and D). Pairwise PERMANOVA tests suggest differences ($P < 0.05$) in microbiomes of samples from conventionally-raised and naturally-raised cattle via weighted UniFrac and between different cities via unweighted UniFrac.

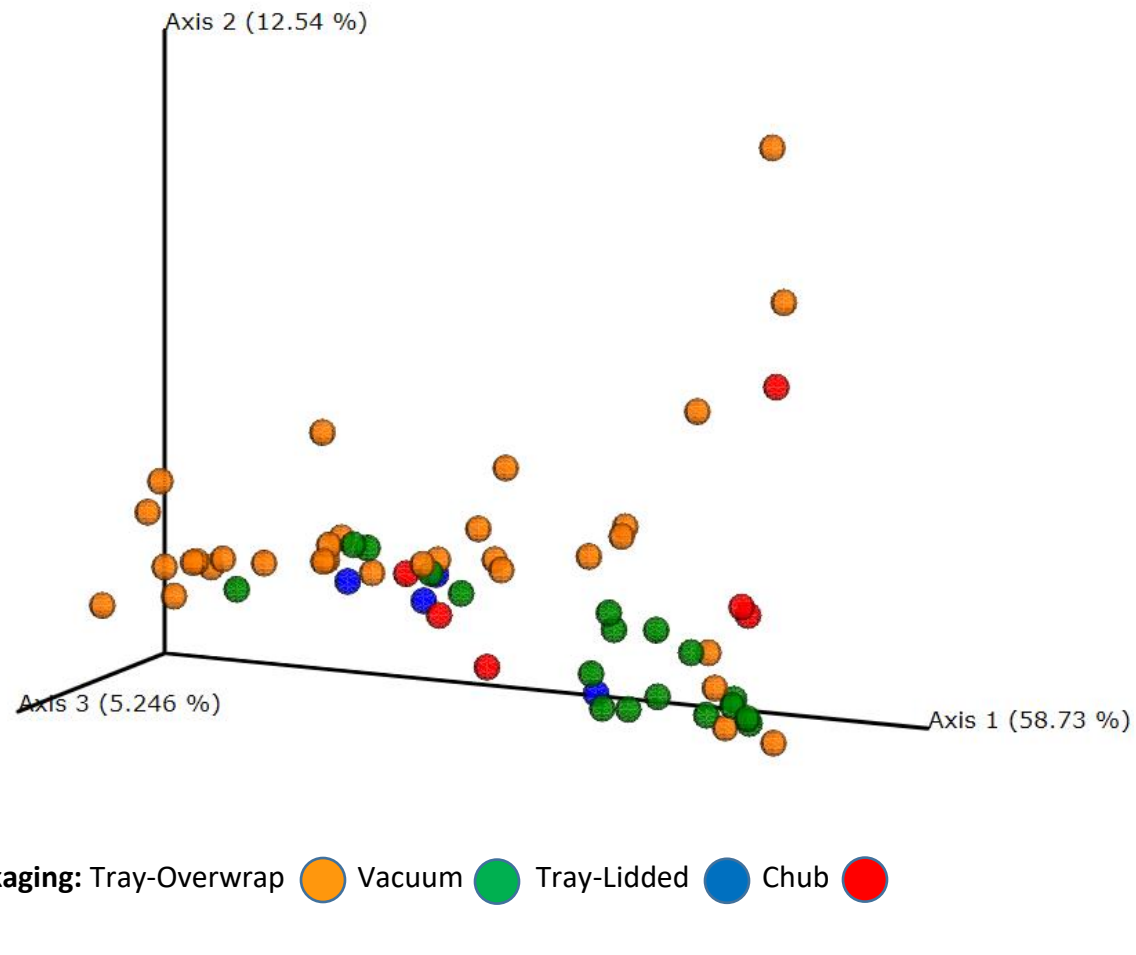

Figure 3: Principle Coordinates Analysis Plot of retail ground beef samples ordinated via Weighted UniFrac distances. Samples are colored by product packaging. Retail products collected in tray-overwrap packaging differed ($P = 0.001$) compared to vacuum sealed products.

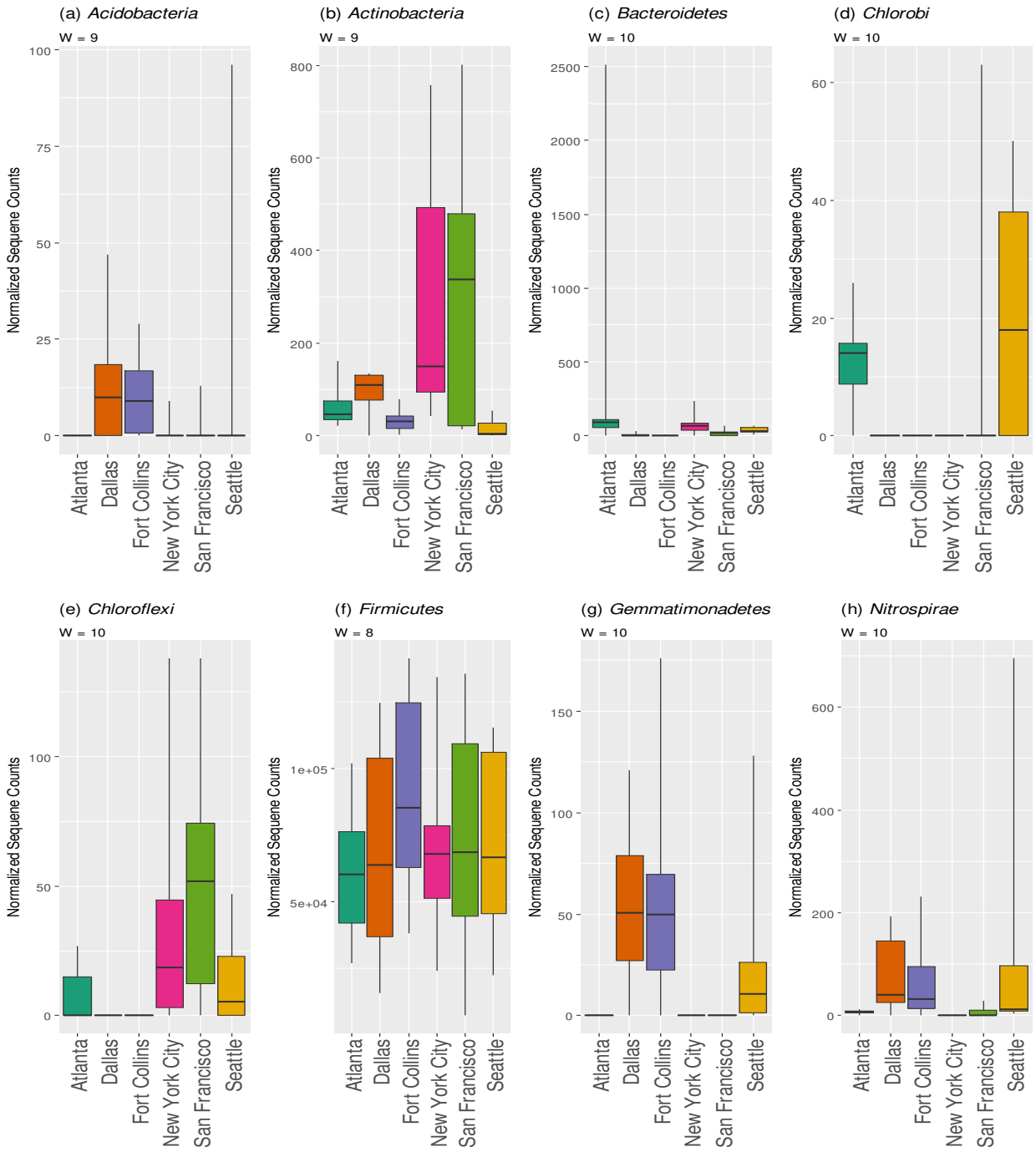

Figure 4: Rarefied counts of bacterial phyla found to be differentially abundant across the six sampling locations. Pairwise comparisons were performed on 11 phyla and 8 were differentially abundant from at least one phylum of bacteria. The "W" statistic indicates the number of pairwise comparison tests that were successfully rejected for a given phylum.

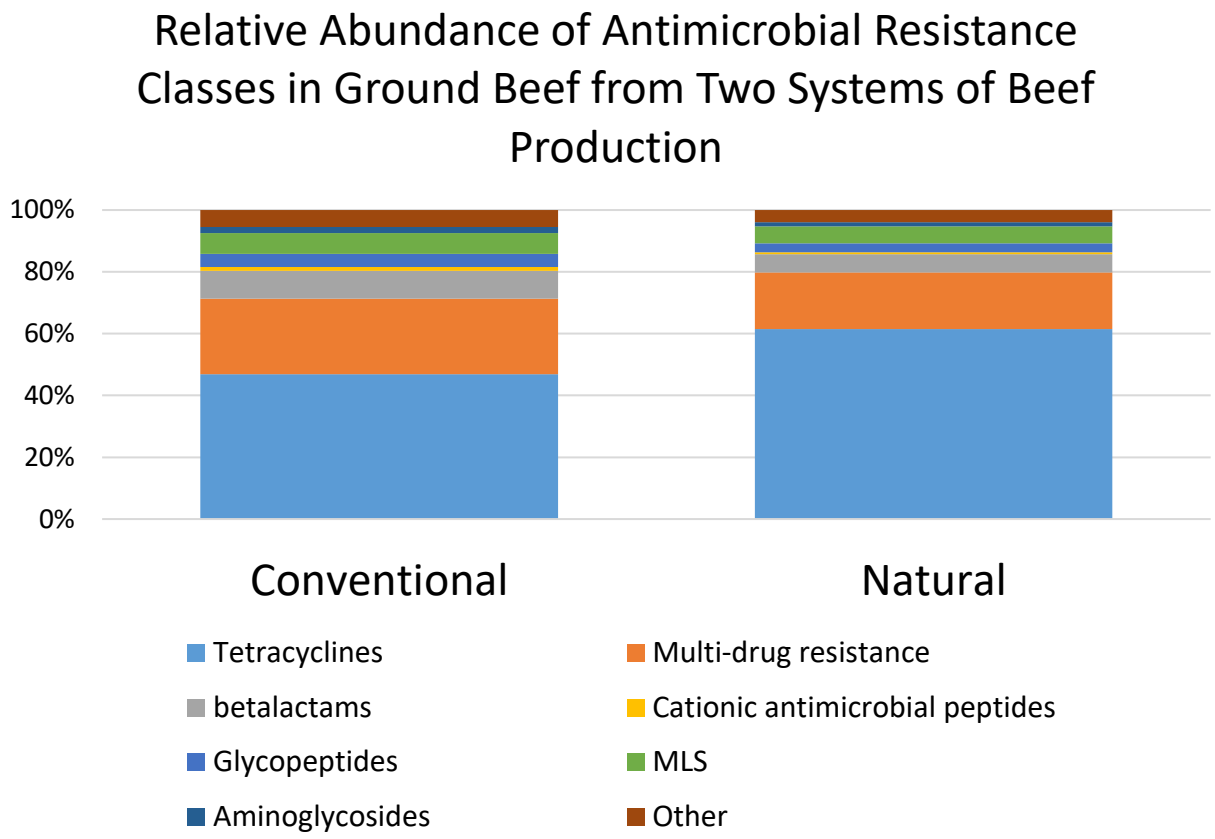

Figure 5: Relative abundance of antimicrobial resistance classes present in composite ground beef samples from conventionally-raised and naturally-raised cattle. Classes of antibiotics categorized as "other" accounted for less than 5% of total hits present in samples from both production systems. A Zero-inflated Gaussian model performed on Log2 normalized counts indicates that conventional samples contained more ($P = 0.001$) Tunicamycin resistance than natural ground beef samples.

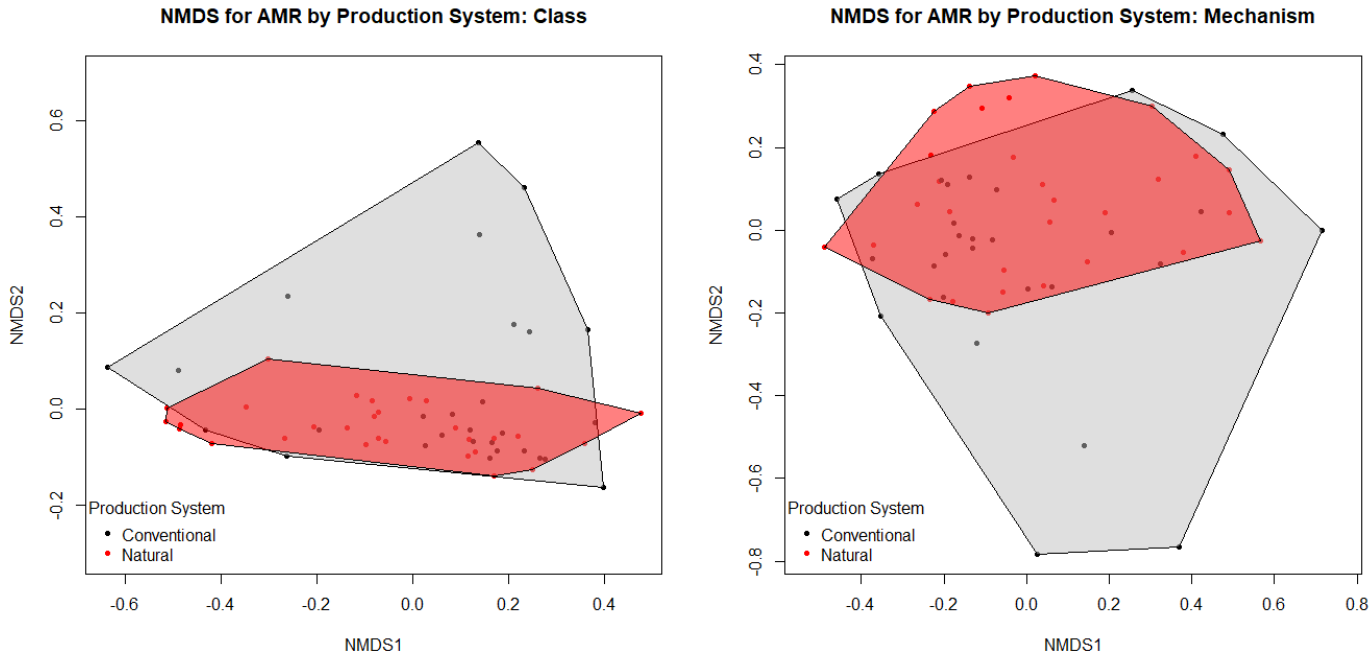

Figure 6: Non-metric Multidimensional Scaling plot of antimicrobial resistance detected in retail ground beef samples processed from conventionally-raised and naturally-raised cattle. Samples ordinated closely together have resistomes that are more similar than those ordinated further apart. Analysis by antimicrobial class indicated that samples differ ($P = 0.029$) between production systems. Differences were not detected ($P = 0.137$) when AMR was analyzed at the antimicrobial mechanism level of annotation.

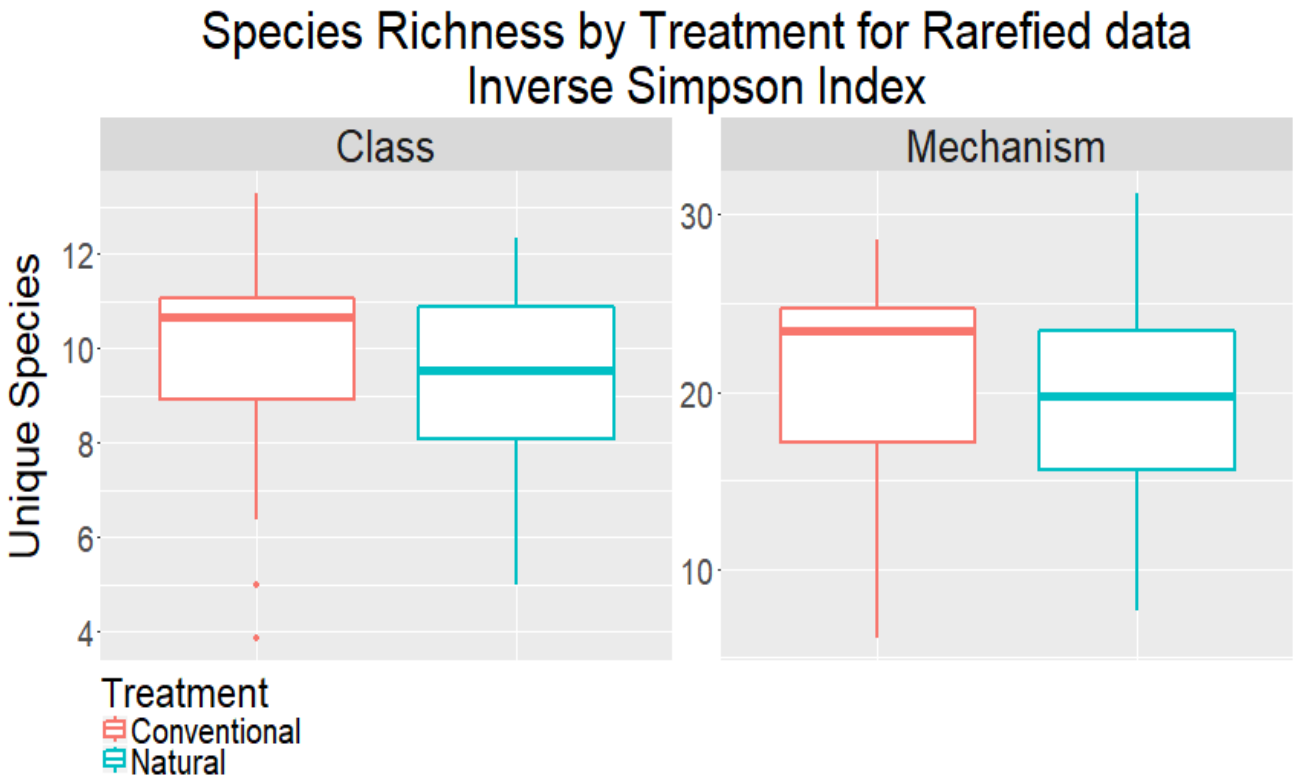

Figure 7: Alpha diversity of ground beef samples processed from conventionally-raised and naturally-raised cattle. A Wilcoxon rank-based, non-parametric test indicated samples of ground beef processed from conventionally-raised and naturally-raised cattle did not differ in alpha diversity when compared at the class ($P < 0.775$) and mechanism ($P < 0.882$) levels of resistance.

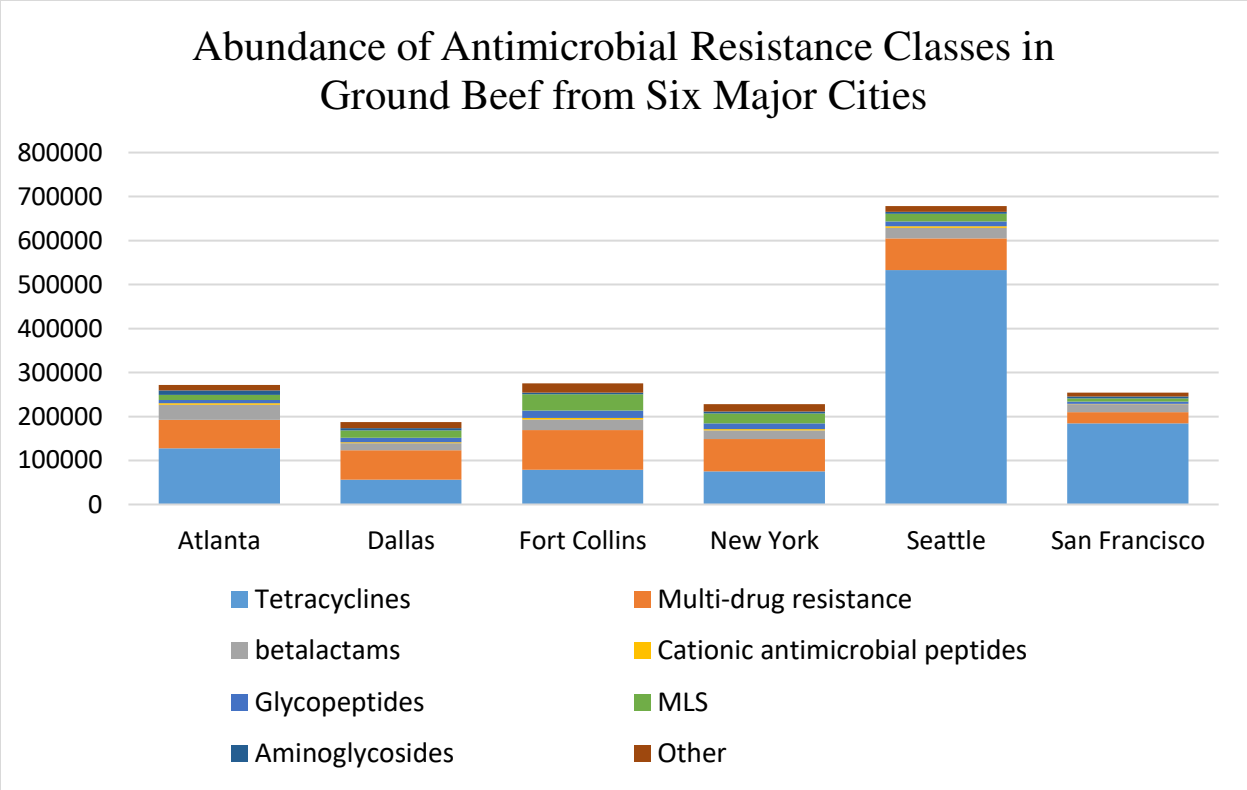

Figure 8: Abundance of antimicrobial resistance classes present in composite ground beef samples collected from six major metropolitan areas throughout the United States (Atlanta, GA; Dallas, TX; Fort Collins, CO; New York City, NY; Seattle, WA; and San Francisco, CA). Classes of antibiotics categorized as "other" accounted for less than 5% of total hits present in samples from all six cities. Samples collected from Seattle, WA contained more hits ($P < 0.001$) to resistance classes than samples from other cities.

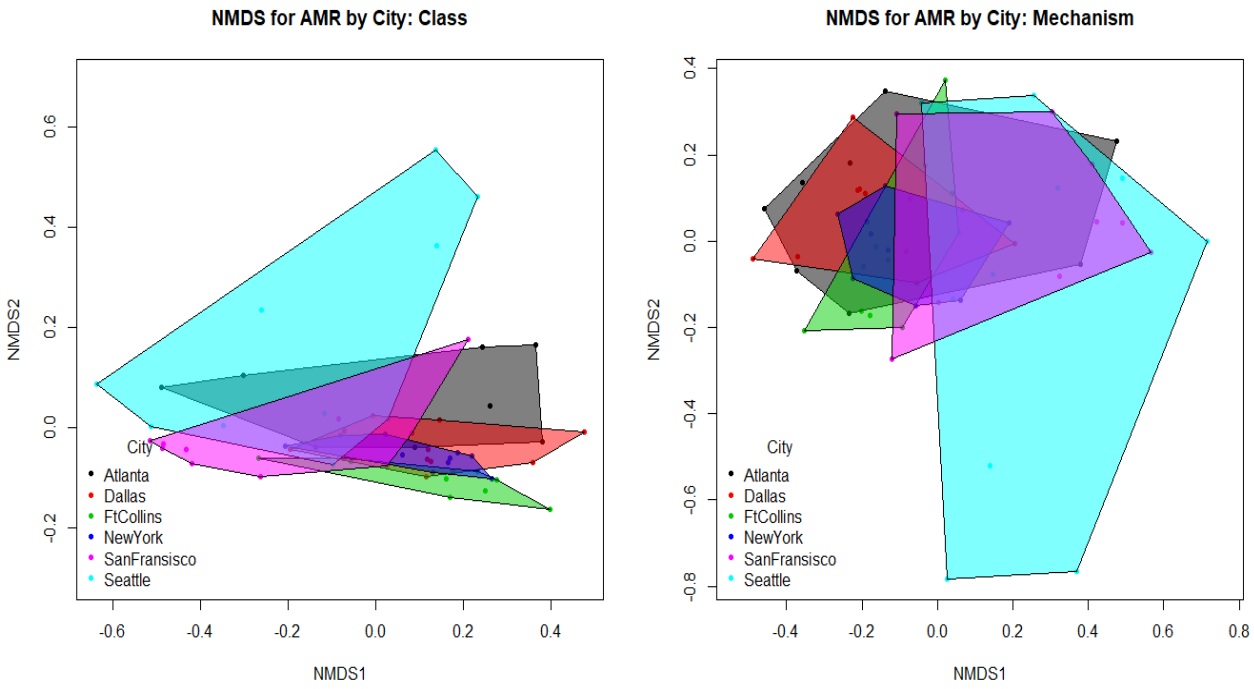

Figure 9: Non-metric Multidimensional Scaling plot of antimicrobial resistance detected in retail ground beef samples collected from six major U.S. metropolitan cities (Atlanta, GA; Dallas, TX; Fort Collins, CO; New York City, NY; San Francisco, CA; Seattle, WA). Samples ordinated closely together have resistomes that are more similar than those ordinated further apart. Resistome of samples collected from different cities differed ($P = 0.001$) for both class and mechanism of antibiotic resistance.

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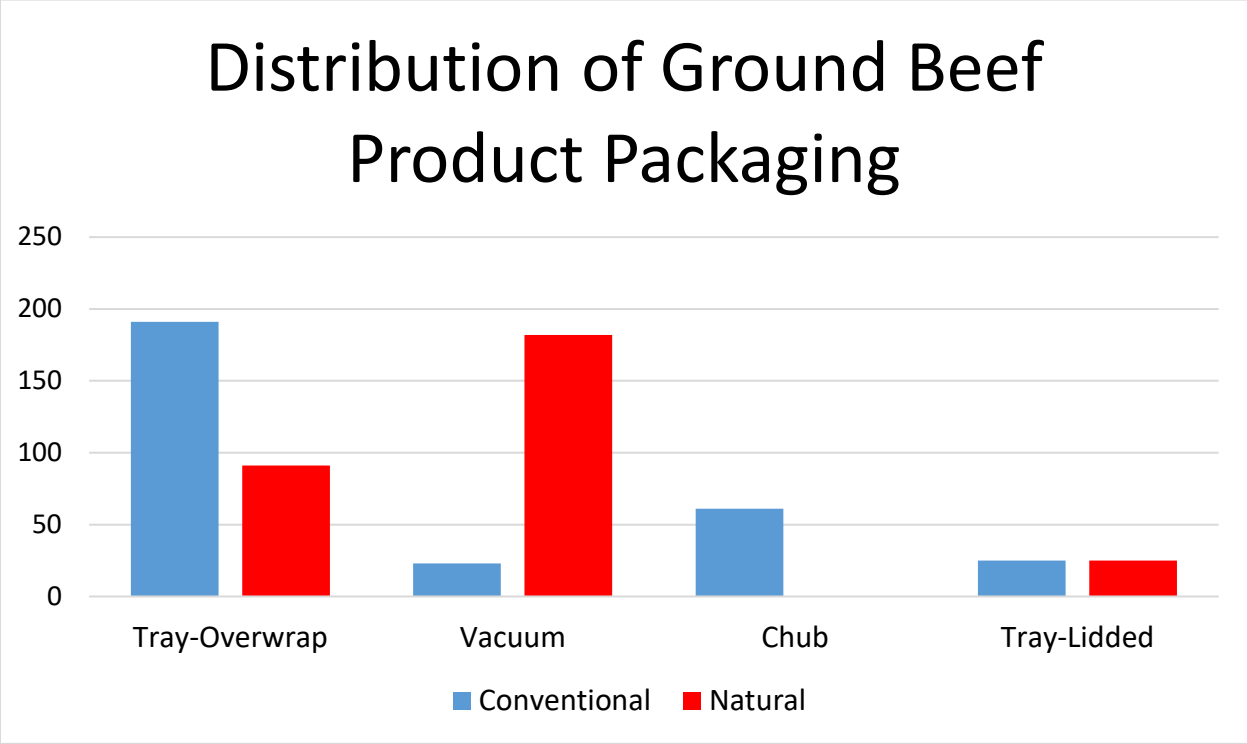
APPENDIX

Supplementary 1: Map of sampling locations and meat processors from which products were sourced. Samples were collected from Fort Collins, CO; Dallas, TX; Atlanta, GA; San Francisco, CA; Seattle, WA; and New York City, NY (Black stars). Establishment numbers were used to trace products back to processing location (grey dots). Samples collected from Seattle are not represented in this map due to loss of meta-data. Products sourced from 22 states were represented from the five cities represented.



Supplementary 2: Number of retail meat products collected within each production system by type of product packaging.

Supplementary 3: Product packaging types collecting throughout project duration: tray-overwrap (A), vacuum (B), chub (C), and tray-lidded (D).