Environmental and biological drivers of bacterial and antimicrobial resistance carriage by house flies (*Musca domestica* L.)

by

Victoria Lynne Pickens

B.S., Oklahoma State University, 2019

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2025

Abstract

House flies (*Musca domestica* L.) pose a significant risk to human and animal health worldwide due to their filth-associated lifecycle, mobility, and ubiquity in urban and rural environments. Frequent interactions with decaying microbe-rich organic matter used as developmental and nutritional resources for house flies results in the acquisition and movement of pathogenic and antimicrobial resistant (AMR) bacteria between environments visited for these reproductive and trophic needs. Confined cattle operations serve as an optimal environment to study fly-mediated pathogen and AMR acquisition, transmission and persistence due to the high antibiotic use, abundant bacteria sources and large house fly populations at these facilities. The first objective of this study was to assess biological and environmental drivers of bacterial abundance, as well as trends of AMR for suspected coliform (SC) bacteria carried by adult house flies in both dairy and beef cattle production facilities in Kansas. Cultured aerobic bacteria and SC were enumerated from field collected male and female flies from beef and dairy cattle operations and a subset of SC isolates were tested for susceptibility against five antibiotics to identify multi-drug resistant (MDR) bacteria. Female flies harbored more bacteria than male flies and abiotic factors such as ambient and soil temperatures correlated with both culturable bacteria and SC abundances in flies. The type of farm (beef or dairy) only correlated with SC abundance in flies. Antimicrobial resistant and MDR isolates were recovered from male and female flies from all beef and dairy operations, but SC isolates from beef flies had a notably wider range of AMR phenotypes than isolates from dairy flies. *Escherichia/Shigella sp.* was the most commonly identified taxa displaying AMR and MDR phenotypes. The second objective assessed biological and environmental drivers of Gram-negative bacteria abundance in house flies collected from beef cattle operations in Kansas and compared AMR Gram-negative bacteria isolated from house

flies to isolates cultured from environmental substrates (manure compost, manure patties, feed, and water). Gram-negative bacteria were enumerated from cultures of male and female house fly, manure patty, feed, and water samples, followed by the selection and identification of AMR isolates after antibiotic susceptibility testing. Female flies carried greater bacterial loads than males, and bacterial abundance in flies varied between locations over time. Correlations between AMR phenotypes of Gram-negative bacteria isolated from flies and environmental samples were dependent on the location, with fly isolates typically reflecting AMR phenotypes observed in environmental isolates from the same location. Because AMR and MDR *Escherichia coli* were commonly observed in house flies and all environmental sample types in this study, the final objective further explored the relationship between AMR house fly and environmental isolates (manure, feed, etc.) through comparative genomics. Whole genome sequencing and predictive phylogenetic tree reconstruction of assembled isolate genomes indicated that AMR *E. coli* isolated from flies were closely related to isolates from manure patties, feed, and water from the same location. Furthermore, 90% of the antimicrobial resistance genes (ARGs) identified across all environmental isolates were present in the genomes of fly isolates. Additionally, observed resistance genotypes and predicted ARG mobility overlapped between AMR *E. coli* from flies, manure patties, feed, and water. Overall, the findings of these studies not only emphasize the environmental and biological factors influencing the role of house flies carrying and potentially disseminating microbial threats to human and animal health, but also the potential of using adult house flies for surveillance of bacteria and AMR in cattle operations.

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Dedication

To my brother, Cullen, whose exuberant curiosity was a lasting reminder that science is a boundless playground for those who remain kids at heart.

Chapter 1 - House flies are a One Health issue for confined cattle

Introduction

Animal husbandry has seen many changes since the domestication of livestock approximately 11,000 years ago (Zeder 2008). Urbanization, industrialization, and advancements in transport and technology since the 1920s have resulted in animal production systems shifting significantly from an individual raising and selling a few animals towards intensive animal farming carried out by commercial entities. To increase efficiency and meet increasing consumer demand, many beef and dairy cattle productions systems have concentrated their animals into confined (sometimes also called concentrated) cattle operations (CCOs), which are an industry-specific form of animal feeding operations (AFOs). Used interchangeably with terms such as "feedyards", "feedlots", or "dairies", CCOs are generally categorized as AFOs. The United States Department of Agriculture (USDA) and Environmental Protection Agency (EPA) define AFOs as "operations where 1) animals have been, are, or will be stabled or confined and fed or maintained for a total of 45 days or more in any 12-month period, and 2) crops, vegetation, forage growth, or post-harvest residues are not sustained in the normal growing season over any portion of the lot or facility" (USDA and EPA 1999).

Cattle are the dominant contributors to global values for terrestrial farmed animals, particularly within livestock systems that incorporate AFOs (Schrobback et al. 2023). Among these systems, CCOs arguably face a more complex mosaic of challenges that may elevate risks to both human and animal health. The increased production scale of CCOs allows stakeholders to more efficiently meet consumer demands, but the high density and close proximity of animals facilitates the transmission of pathogens. The frequent influx and efflux of cattle for some systems, like beef cattle feedyards, can further heighten the risk of pathogen transmission by

introducing pathogens from other areas, as these cattle are often moved between multiple operations or states across the United States. Additionally, environmental and management-related stressors, such as extreme heat or handling procedures, can compromise cattle immune responses and increase disease susceptibility (Chen et al 2015). As a result, disease incidence and antibiotic use remain high in CCOs, despite more restrictive regulations in recent years, with food animal antibiotic consumption projected to continue increasing globally over the next decade (Tiseo et al. 2020). Compounding these risks, the large volumes of animal feed and waste generated in high-density food animal operations can serve as reservoirs for pathogenic microorganisms if not properly managed.

Pathogenic bacteria important to human and animal health have increasingly acquired antimicrobial resistance (AMR), creating a devastating global One Health problem. Mitigation strategies tackling AMR prioritize AFOs, as food animal production consumes approximately 70 percent of all medically important antibiotics in the United States (FDA-CVM 2024). Due to high risk for disease in confined cattle, as well as the high demand for quality and safe cattle products, cattle are the second-largest food animal consumer of antibiotics in the United States (FDA-CVM 2024). The United States National Strategy for Combating Antibiotic-Resistant Bacteria was announced in 2014 as an attempt to combat the growing threat of antibiotic resistance. Government agencies have been working collaboratively to tackle the issue of AMR through multiple routes, including the promotion of appropriate and responsible use of antimicrobials in both human and animal clinical settings, as well as food production systems. The use of medically important antibiotics solely for growth promotion in food animals has been banned in the United States since 2017, yet veterinarians are permitted to prescribe medically important antibiotics for animal health needs.

Use of antibiotics has been shown to select for the emergence of AMR in both human and animal pathogens harbored by cattle (Hoelzer et al. 2017). Additionally, resistance genes have been found in much of the environment within beef and dairy cattle operations, such as cattle manure, water, and feed (Rovira et al. 2019). Bacteria prevalent in these systems, like *Escherichia coli*, are prone to acquiring from or transferring resistance to other bacteria of the same or different species (Hunter et al. 1992). There are also cases where the transfer of a single plasmid transfers resistance genes to multiple antibiotic classes, like florfenicol and ceftiofur resistance in *E. coli* (Meunier et al. 2010). In instances where bacteria are being transported frequently throughout a system, this can become an increasing threat to human and animal health as many bacteria naturally possess antibiotic resistance mechanisms. Therefore, despite more conscious usage of antibiotics reduces selection for AMR bacteria, the exposure of bacteria to new environments and other bacteria could still promote AMR prevalence, even in the absence of direct use pressure.

CCOs face many challenges regarding human and animal health that are further exacerbated by the biology and behaviors of house flies (*Musca domestica* L.). Commonly a persistent pest of confined cattle and other livestock, house flies increase the risk of bacterial pathogens and AMR dissemination amongst a variety of environments. Adult house flies are known vectors of pathogenic and AMR bacteria that are capable of freely moving between both human and animal dwellings. Their dependence on bacteria as larvae and coprophagous behaviors result in frequent interactions with microbe-rich substrates where they acquire pathogenic and AMR bacteria (Nayduch and Burrus 2017). Furthermore, their facilitation of horizontal gene transfer within the alimentary canal could have serious implications for house flies as drivers of the development and dissemination of evolving AMR bacteria.

The Biology and Ecology of House Flies in Animal Production Systems

House flies pose a considerable risk to human and animal health through their biology, physiology, and behaviors, which encourage the acquisition and dissemination of bacteria. For over a century house flies have been designated as a "filth fly," a group of flies known to frequent unsanitary environments due to their use of excrement and decaying matter for nutrition and reproduction (Howard 1911). House flies are holometabolous insects that carry out their egg, larval, and pupal stages in microbe-rich substrates like accumulated plant materials, human and animal feces, and food waste (Cook et al. 2011). Their synanthropic and opportunistic nature makes house flies global pests of urban or rural settings alike and, as adults, they can move interchangeably between sanitary and unsanitary environments (Thomas and Skoda 1993, Lole 2005, Winpisinger et al. 2005). Additionally, their high fecundity, propensity for pesticide resistance (Scott et al. 2013), and rapid lifecycle enable house flies to easily reach pestiferous levels in areas where microbe-rich substrates are abundant, such as CCOs, and remain a persistent nuisance to animals, workers, and nearby residences (Geden et al. 2021).

The overall fecundity, lifespan, and length of time between developmental stages of house flies are dependent on resource quality and availability, temperature, and humidity. Female house flies have been observed to lay up to 100-150 eggs in a single gonotrophic cycle (West 1951), potentially laying anywhere from 700-900 eggs in her lifetime (West 1951; Fletcher et al. 1990). House flies are estimated to yield much lower egg clutches under field climate conditions, averaging only 40 or 94 eggs per single gonotrophic cycle in poultry and dairy cattle farm substrates, respectively (Lysyk 1991, Krafsur 1985). After hatching, microbes are a dietary essential for the survival and fitness of house fly larvae (Schmidtmann and Martin 1992, Zurek et al. 2000). Adult females have also been observed to directly consume these

substrates (Thomson et al. 2017), but whether microbe consumption from these substrates is necessary for adult females or simply opportunistic behavior during oviposition is yet to be determined. Under optimal conditions, laboratory-reared house flies have been observed to develop from eggs to adults as quickly as 6.92 days when held at 33°C (Larsen and Thomsen 1940), but fluctuating resources and climatic variability in the field are likely to delay house flies' larval development. Adult female flies survive up to 6 weeks under optimal laboratory conditions (Fletcher et al. 1990), but this lifespan is significantly lower in the wild where the provision of different food resources at cattle dairies were found to alter adult male and female longevity (Grimenstein et al. 2025). Additionally, mark-release-recapture studies estimate a much wider lifespan range dependent on the type of farming operation, for example 1-6 days on poultry farms (Lysyk and Axtell 1986, Lysyk 1991) versus 3-19 days on dairy cattle farms (Krafsur et al. 1985, Kristiansen and Skovmand 1985, Butler et al. 2013).

The dietary preferences of adult house flies differ by sex and sexual maturation, although resource availability can play a key role in feeding choices of flies. Neupane et al. (2023) found that, given the choice between different food resources, adult male house flies preferred the carbohydrate-rich diet (sugar) and less frequently fed on multiple foods, regardless of mating status. In contrast, female house flies more often consumed multiple foods and largely preferred protein and carbohydrate-rich food (milk), particularly when presumably mated, supporting prior evidence that females require protein or protein components for egg development (Spiller 1964). These patterns are also observed in CCOs, where female house flies are more often observed in multiple environments (animal mucous, feces, manure compost, feeds) in contrast to male flies that are predominately found on animal feed in storage or bunks (Nayduch et al. 2023). Given the choice between various livestock manures, adult female house flies will more frequently

select cattle manure for oviposition (Shah et al. 2016). Additionally, the overall survival and fitness of fly progeny reared in cattle manure is significantly higher than house fly larvae reared in other livestock manures (Khan et al. 2012). The selectivity of house flies due to fly sex or mating status is additionally believed to affect their consumption of microbe-rich resources within CCOs. When given the choice between cattle manure or a sucrose solution, female and male fly gut contents varied depending on both fly sex and the duration of exposure, with only female house flies containing manure after four hours, but only sucrose solution detected in the gut of either sex after 24 hours (Thomson et al. 2017). Overall, both male and female house flies are observed to frequent feed bunks in CCOs where there is an open supply of nutrient-rich feed throughout most of the day. Infestation of cattle feed bunks by house flies results in increased agitation behaviors exhibited by feeding cattle, increasing the amount of energy they expend and causing the animals to spend less time feeding (Zamudio 2022). These relationships could have significant implications for estimating risks associated with CCOs prolonging fly lifespans and providing a variety of food resources, as well as the competency of house flies as vectors for pathogenic or AMR bacteria, but further research is needed.

Due to their mobility, adult house flies can explore environments in search of resources containing sugars and proteins to acquire additional nutrients necessary for survival and reproduction (Goodman et al. 1968). Differences in the feeding preferences of males versus female flies at different mating stages are believed to not only influence attractant efficiency for traps, but also better inform risks associated with bacterial transmission by house flies (Thompson et al. 2017, Neupane et al. 2023). As pathogenic microorganisms can be shared between animals and humans, there are also concerns of house flies moving off-site into urban areas, where they could potentially disseminate zoonotic pathogens. House flies have been

reported to travel distances up to 20-30 km (Alam and Zurek 2004) however, house fly dispersal is believed to more often dependent on resource availability. House flies are less likely to disperse from dairy farms if they do not sanitize potential fly breeding sites (Pickens et al.1987) or will remain near feed bunks at stocker beef cattle facilities (Nayduch et al. 2023). Nevertheless, house flies have been observed to move offsite while carrying multiple bacterial pathogens, and so are a risk for the dissemination of pathogens or AMR bacteria to other areas.

House Fly Vector Potential: Mechanical and Biological Transmission of Pathogens

Because house flies are highly mobile and coprophagous, they can additionally pose a significant risk for the transmission of microorganisms both within and beyond animal production facilities. For some pathogens, such as *Campylobacter jejuni* and *C. coli,* house flies have even been found to be more prevalent carriers than other filth fly species (Hald et al. 2008). House flies acquire and transfer bacteria within environments largely by mechanical transmission, either through direct contact with their body surfaces or the regurgitation/defecation of bacteria from within their gut. Discrepancies exist between the level of risk associated with each route of transmission, particularly when considering the associations and biology of both the fly and bacteria in these instances, and further research is needed to better understand these interactions and their implications for human and animal health. The capacity of house flies to serve as vectors for various pathogens has been found to depend on several factors, including the bacterium's initial dosage, survivability against gut digestive or immune processes, competition with environmental or gut microbiomes, and proliferation either inside or outside of the house fly, or within the environment once transferred (Nayduch and Burrus 2017).

House fly external surfaces are considered the lowest-risk route for bacterial transmission. Bacteria typically contaminate house flies' body surfaces through direct contact with contaminated substrates, enabling bacterial cells to attach to their mouthparts, tarsal pads, setae, wings, and segmental grooves (Yap et al. 2008). Park et al. (2019) noted that a higher diversity of bacterial taxa was observed within the gut but remains consistent across geographic locations and habitat. In contrast, bacterial taxa on house fly surfaces are less diverse, but variable between geographic locations and habitat. The external surfaces of house flies may therefore not be a suitable environment for the prolonged survival or proliferation of acquired bacteria, either due to the biological needs of the bacteria or shedding of the bacteria by the fly during various activities (e.g., grooming, flight). As demonstrated by inoculating house flies with *Pseudomonas aeruginosa*, this can affect their ability to transmit pathogenic bacteria via their body surfaces at infectious dosages (Jacques et al. 2017). In contrast, higher loads of *Helicobacter pylori* were recovered for house fly body surfaces than the gut (Magdy et al. 2023), though whether they could deliver an infectious dose via contact with their body surface was not determined.

Regurgitation and defecation, collectively known as "spotting," by house flies represent higher-risk pathways for bacterial transmission. Eight different bacterial pathogens have been demonstrated as recoverable from fly regurgitation and defecation spots: *Aeromonas hydrophila* (McGaughey and Nayduch 2009), *Campylobacter jejuni* (Gill et al. 2016), *Corynebacterium pseudotuberculosis* (Braverman et al. 1999), *E. coli* O157:H7 (Sasaki et al. 2000), *Pseudomonas aeruginosa* (Joyner et al. 2013), *Salmonella typhimurium* (Chifanzwa 2011, Nayduch et al. 2018), *Salmonella schottmulleri* and *Shigella dysenteriae* (Hawley et al. 1951), *Staphylococcus aureus* (Nayduch et al. 2013). Fly spots have been observed to be suitable environments

conducive to pathogen survival and proliferation, even on the surfaces of leafy greens (Wasala et al. 2013 Directly feeding *Pseudomonas aeruginosa* (Joyner et al. 2013), *E. coli* O157:H7 (Fleming et al. 2014), *Staphylococcus aureus* (Nayduch et al. 2013), and *Salmonella* Typhimurium (Nayduch et al. 2018) to house flies demonstrated acquisition, survival, and excretion of pathogenic bacteria. Alarmingly, some studies even found evidence of these bacteria proliferating within the fly gut (Joyner et al. 2013, Nayduch et al. 2018), emphasizing the potential of house flies as facultative biological vectors as well as mechanical vectors. Another potential route of infection includes direct ingestion of flies by animals, which can deliver infectious dosages directly to animals (Shane et al. 1984). Primarily, this route of transmission is only a concern for CCOs if fly carcasses are consumed by cattle during ingestion of water or feed, and studies have yet to be conducted that evaluate the frequency at which this occurs. Nonetheless, direct transmission of *E. coli* O157:H7 by house flies to confined cattle has been demonstrated (Ahmad et al. 2007), though the route of transmission was unclear and warrants further investigation.

The Role of House Flies in the Dissemination and Evolution of AMR

The rising prevalence of AMR bacterial pathogens is a concern for human and animal health globally. In 2019, AMR infections resulted in approximately 4.95 million human deaths worldwide (Murray et al. 2022). Furthermore, AMR infections cost the United States $4.6 billion in 2017 (Nelson et al. 2021), making them a significant burden to the healthcare system as well as a major threat to human health. For livestock, AMR additionally threatens food security through lowered production outputs and higher costs to both prevent and treat infections with AMR pathogens, as well as handle downstream complications resulting from infection. Numerous laboratory studies have incriminated house flies as carriers of AMR bacterial

pathogens of both human and animal health importance (Nayduch and Burrus 2017), including *E. coli* (Buma et al. 1999), *Salmonella enterica* (Wang et al. 2011, Xu et al. 2018, Alam et al. 2020), and *Campylobacter spp.* (Ommi et al. 2016). However, these studies are highly artificial and cannot capture the complexity of AMR dissemination in field settings.

Current research is limited regarding the demonstration of house flies' ability to acquire and transmit AMR bacteria in field settings, particularly for CCOs. House flies can acquire AMR bacteria from environmental sources, as demonstrated by feeding flies cattle manure contaminated with AMR *E. coli* or *Salmonella* Typhimurium (Thomson et al. 2017). Shared genotypes of AMR *E. coli* were recovered from house flies and swine or poultry manure collected the same locations, suggesting acquisition, and spreading of these AMR bacteria between house flies and manure (Sola-Gines et al. 2015, Usui et al. 2015). Macovei et al. (2008) even demonstrated that field-collected adult house flies from beef cattle operations successfully contaminated ready-to-eat food with AMR *Enterococcus faecalis*. These findings have serious implications for the transmission of AMR bacteria by house flies offsite to surrounding residential or urban areas.

In addition to being capable mechanical and biological vectors of bacterial pathogens, house flies can enhance the horizontal transfer of AMR genes within their gut as adults. House flies can simultaneously harbor many species of bacteria and there are bacterial species, such as *E. coli*, which readily acquire and transfer resistance genes in the environment (Hunter et al. 1992), as well as survive within the gut of house flies (Wasala et al. 2013, Fleming et al. 2014, Thomson et al. 2017). The horizontal transfer of plasmid-encoded antibiotic resistance has already been demonstrated between donor and recipient bacteria (Petridis et al. 2006, Akhtar et al. 2009, Fukuda et al. 2016), as well as off target commensal bacteria within the house flies' gut

(Fukuda et al. 2016, Gan et al. 2024). Consequently, it is believed house flies are potentially facilitating the survival, creation, evolution, and dispersal of new pathogenic and AMR bacteria (Petridis et al. 2006, Onwugamba et al. 2018).

Summary

Facilities rearing and housing large number of cattle produce copious manure and other resources, which support the proliferation of both house flies and bacteria. House flies are known to both acquire and transmit pathogenic and AMR bacteria and can even facilitate the transfer of AMR genes between bacteria within their gut after acquiring them (Petridis et al. 2006, Akhtar et al. 2009, Fukuda et al. 2016, Gan et al. 2024). Additionally, the movement of house flies between substrates for different functional needs presents the opportunity for microbes of different microenvironments within CCOs to interact and exchange AMR genes. Antimicrobials provide an efficient and cost-effective means of treating bacterial pathogens relevant to medical and veterinary health. Antimicrobial use pressures microorganisms to rapidly develop and spread AMR, eventually leading to the development of AMR pathogens. As the second largest animal production consumer of antibiotics (FDA-CVM 2021), cattle operations are environments where AMR is developing and are therefore optimal for the surveillance and mitigation of emerging AMR. This thesis aims to address the following knowledge gaps: 1) understand which factors influence the bacterial abundance and AMR prevalence carried by house flies in CCOs, 2) determine whether trends of AMR bacteria in house flies correlate with AMR bacteria in environments at CCOs, and 3) genomically characterize AMR bacteria from house flies versus environmental substrates at CCOs. Furthermore, this research will emphasize the importance of proper house fly management as a means for mitigating AMR, protecting both animal and human health.

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Chapter 2 - Bacterial abundance and antimicrobial resistance prevalence carried by adult house flies (Diptera: Muscidae) at Kansas dairy and beef cattle operations

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Abstract

House flies (*Musca domestica* L.) are filth-breeding pests of urban and rural environments around the world. Frequenting microbe-rich substrates for nutritional and reproductive needs, house flies pose a risk to human and animal health through their carriage and transmission of pathogenic and antimicrobial resistant bacteria (AMR). Adult house flies were collected from Kansas beef and dairy cattle operations to assess factors influencing bacterial abundance and AMR incidence flies. Aerobic culturable bacteria and suspected coliforms (SC) were enumerated from fly homogenate cultured on nonselective (tryptic soy agar) and selective (violet red bile agar VRBA) media, respectively. Unique morphotypes of SC isolates were screened for tetracycline resistance and tested for resistance to 4 additional antibiotics to identify multi-drug resistant (MDR) isolates. Female house flies carried greater abundances of both culturable bacteria and SC than male flies. Abiotic factors such as ambient and soil temperatures correlated with culturable bacteria and SC abundances in flies, but farm type correlated only with SC abundance and trends of resistance phenotypes observed in SC isolates. Male and female flies from both farm types carried one or more AMR and MDR SC isolates (73.02% AMR and

31.09% MDR). The majority of AMR and MDR bacteria were *Escherichia/Shigella sp.*, which possessed the widest range of phenotypic resistance variability found in our study. Our results further emphasize the role house flies play in harboring bacteria of risk to human and animal health and identified factors of potential use for the development of strategies to mitigate house fly transmission of bacterial pathogens and AMR within confined cattle operations.

Introduction

House flies are worldwide pests of humans and animals that opportunistically utilize a variety of microbe-rich substrates for nutritional and developmental needs. Over 200 different species of microbes have been identified in wild house fly surveys (Nayduch and Burrus 2017), and a single fly can carry up to 100 different potential pathogens (Greenberg 1973). House fly interactions with microbe-rich substrates begin immediately after hatching as larvae require microbes in their diet to develop properly (Schmidtmann and Martin 1992, Zurek et al. 2000). Microbe-fly interactions continue into adulthood with house flies frequently exploring microbe-rich substrates for nutrition and, in the case of females, oviposition (Shah et al. 2016). Environments which produce copious amounts of microbe-rich substrates, like manure produced from confined cattle operations, are both conducive to house fly proliferation and rich sources of microbes for fly acquisition during oviposition. The persistent quest for oviposition sites may drive females to have more frequent exposure to microbe-rich substrates, resulting in greater bacterial loads than male flies (Neupane et al. 2020).

Antimicrobial resistance (AMR) in bacterial pathogens is an increasing concern for livestock producers, especially those keeping animals in confined spaces such as dairies and feedlot operations. As one of the largest consumers of antibiotics important for both human and animal health (FDA-CVM 2021), cattle industries are at risk of losing a cost-effective tool for

combatting common bacterial pathogens that impact animal wellness and production yields. Surveillance of reservoirs of AMR bacteria and antimicrobial resistance genes (ARGs) within these operations is important for developing management strategies to efficiently mitigate the development and spread of AMR. Researchers have identified numerous AMR bacteria and pathogens in environmental sources associated with livestock production, such as cattle manure, feed, and water (Guo et al. 2021, Ma et al. 2021). Despite current efforts to manage these substrates and limit antibiotic usage in livestock, AMR remains a rising threat to human and animal health (Naghavi et al. 2024). Therefore, methods to monitor AMR incidence, emergence, and prevalence are still urgently needed to mitigate this threat.

Insects, particularly house flies, are believed to bridge AMR bacteria between environmental sources in livestock production and, as such, could help assess AMR prevalence in these facilities (Zurek and Gosh 2014, Nayduch and Burrus 2017, Onwugamba et al. 2018, Nayduch et al. 2023). Although house flies frequently interact with microbe-rich substrates within the environment and actively proliferate within large-scale cattle operations, information on their role in the prevalence and transmission of AMR bacteria is limited. Flies have been shown to carry and transmit pathogenic and AMR bacteria, including from cattle manure to fresh produce, in laboratory assays (Talley et al. 2009, Thomson et al. 2021). Additionally, AMR bacteria have been isolated from house flies collected at livestock operations, with a single fly capable of carrying multiple species of AMR bacteria (Chakrabarti et al. 2010, Onwugamba et al. 2018, Neupane et al. 2020, Geden et al. 2021). However, prior studies have focused on house fly carriage of limited AMR bacterial species of interest, particularly pathogens. Many non-pathogenic bacteria, including flora such as coliforms, can serve as rich sources of ARGs that can be horizontally transferred to other bacterial species (D'Costa et al. 2006, Wellington et al.

2013). Biotic and abiotic drivers affecting the house fly's carriage and dissemination of AMR bacteria, particularly non-pathogens, in livestock production settings is unknown. Using a culture-based approach, we evaluated the overall abundance of bacteria and the prevalence of suspected coliforms, including those with AMR, carried by house flies in confined beef and dairy cattle production environments. Additionally, biotic and abiotic factors, such as fly sex, farm type, location, collection date, and climate, were investigated to assess their potential influence on bacteria and AMR prevalence and abundance in house flies.

Materials and Methods

Sample Collection

Adult house flies (*Musca domestica* L.) were collected from a dairy and a beef feedlot cattle operation in each of 3 counties in northeastern Kansas, USA (Riley, Marion, and Washington). Beef and dairy operations in each county were within 25 km of each other, and facility capacity, total number of animals present, feed type, and manure management strategies were recorded for each site during each visit. Flies were sampled from all sites on the same collection date every other week beginning in mid-August and ending in early October of 2019. Historical weather data for climate variables measured hourly by the Manhattan, Rock Springs, and Washington (Kansas Mesonet 2021) weather stations were recorded for beef and dairy sites within Riley, Marion, and Washington counties, respectively (Appendix Table A.1).

Using disinfected sweep nets soaked overnight in 10% bleach, house flies were collected randomly from animal feed bunks in front of 2 to 3 pens at each site and placed into a sterile petri dish on ice for immobilization. Six male and 6 female flies per facility and collection date (n = 352 total flies) were placed individually into sterile 1.5 ml microcentrifuge tubes using gloves and forceps. Forceps were disinfected by immersion in 70% ethanol between samplings.

Tubes containing flies remained on ice during transport to the laboratory and were processed within 8 h of collection.

Bacterial Culture and Enumeration

In the laboratory, each fly was homogenized with a sterile plastic pestle in 500 µl of sterile phosphate-buffered saline (PBS; 1.37 M NaCl, 27 mM KCl, 101.4 mM NaH2PO4, 17.6 KH2PO4, pH 7.4; Fisher Scientific, Pittsburg, PA, USA). After homogenization, an additional 500 µl of PBS was added and the sample was briefly vortexed. Homogenate was 10-fold serially diluted and 100 µl of each dilution was spread-plated in duplicate on tryptic soy agar (TSA; Becton Dickinson and Company, Sparks, MD, USA) for culturable aerobic bacteria enumeration, and in duplicate on violet-red bile agar (VRBA; Becton Dickinson and Company, Sparks, MD, USA) for coliform enumeration. Culture plates were incubated at 37 °C for 18 to 24 h, after which colony forming units (CFUs) were enumerated for all plated dilutions on TSA and VRBA. Only pink or red CFUs indicating selection of lactose-fermenting Gram-negative bacteria were counted on VRBA (hereafter "suspected coliforms (SC)").

Selection, Susceptibility Testing, and Identification of Suspected Coliform Morphotypes

Suspected coliforms (observed lactose fermentation) with differing morphologies (hereafter "SC isolates" representing SC morphotypes used for AMR testing) from each collection date were picked from VRBA cultures with a sterile inoculating loop, quadrant streaked for isolation onto a new VRBA plate and incubated at 37 °C for 24 h. Prior to storage, a single colony from each SC morphotype was quadrant streaked onto TSA and incubated at 37 °C for 24 h. Subsequently, a colony from TSA was selected and used to inoculate 5 ml tryptic soy broth (TSB; Becton Dickinson and Company, Sparks, MD, USA), which was incubated at 100

rpm at 37 °C for 4 to 6 h. Cultures were cryo-preserved as 25% glycerol stocks and stored at −80 °C until resuscitation for downstream identification and AMR susceptibility testing.

To initially screen SC isolates for suspected tetracycline resistance, glycerol stocks of SC isolates were partially thawed at room temperature before adding 100 µl to 5 ml of TSB and incubating for 6 to 8 h at 37 °C and 100 rpm. A loop of culture was streaked onto VRBA and incubated for 18 to 24 h at 37 °C. Isolated colonies from each sample were subcultured onto TSA and incubated at 37 °C for another 24 h to confirm purity. An isolated colony was streaked onto a quadrant of Mueller-Hinton (MH; Becton Dickinson and Company, Sparks, MD, USA) agar supplemented with tetracycline (16 µg/ml) which is the CLSI MIC breakpoint concentration for resistance in Enterobacterales (CDC 2019). SC isolates were considered potentially tetracycline-resistant if growth was observed after incubation for 24 to 48 h at 37 °C and selected for later disk diffusion susceptibility testing. Potentially tetracycline-resistant SC isolates were subcultured onto TSA before creating a new glycerol culture stock.

SC isolates which grew on tetracycline-infused MH agar were later resuscitated for disk diffusion to confirm tetracycline resistance via CLSI standards and investigate susceptibility to 4 other antibiotics of veterinary importance using the Kirby-Bauer disk diffusion method (Hudziki 2009). In preparation for antibiotic susceptibility testing, glycerol stocks were cultured onto VRBA, followed by streaking for isolation onto TSA. Isolated colonies from TSA were added to sterile saline and compared with a 0.5 Remel McFarland Equivalence Turbidity Standard (Remel, Lenexa, KS, United States) to achieve desired inoculum turbidity. Saline inoculums were spread onto a MH (Remel, Lenexa, KS) plate before adding a disk of each of the 5 following antibiotics: 16 µg tetracycline (Becton Dickinson and Company, Sparks, MD), 30 µg florfenicol (Nufluor; VetLab, Palmetto Bay, FL, USA), 5 µg enrofloxacin (Baytril; VetLab,

Palmetto Bay, FL, USA), 10 µg ampicillin (VetLab, Palmetto Bay, FL, USA), and 30 µg ceftiofur (Naxcel; VetLab, Palmetto Bay, FL, USA). Plates were incubated at 37 °C for 18 h and the zones of inhibition measured (mm) for each antibiotic. Zones were interpreted for susceptibility utilizing the 2020 Clinical and Laboratory Standards Institute (CLSI) VET01S Enterobacterales zone diameter breakpoints (CLSI 2020).

Identification of Antimicrobial Resistant Suspected Coliform Isolates

Single- or multi-drug resistant SC isolates were identified to genus via 16S rRNA Sanger sequencing. Isolates were resuscitated from 50 µl of glycerol stock onto VRBA and TSA as outlined above for tetracycline resistance screening methods. An isolated colony was picked from the TSA with a sterile toothpick and added to 50 µl of nuclease free water inside a 0.2-ml 96-well plate. The plate was boiled at 100 °C for 10 to 15 min in a thermal cycler (Bio-Rad T100 Thermal Cycler, Life Science, Hercules, CA, USA). Tubes were centrifuged for 3 to 5 min at 13,000 RPM to pellet cell debris, after which 20 µl of supernatant was stored as DNA template. Using universal primer pair 8F and 806R targeting the 16S rRNA gene (Relman et al. 1992, Turner et al. 1999), PCR reaction mixtures were prepared and 16S rRNA gene amplification performed as described previously (Neupane et al. 2019). PCR products were sent for Sanger sequencing by the University of Arizona Genetics Core (Tucson, AZ, USA). Bases were called and reads assigned quality scores by analyzing the chromatographs with *phred* (Ewing and Green 1998) and CodonCode Aligner v.10.0 (CodonCode Corporation, www. codoncode.com) using the standard default parameters. Quality trimming of reads was performed using *fastp* v.0.22.0 (Chen et al. 2018) with default sliding window of 4 and front mean and cuttail mean quality scores of 25. Trimmed reads were then assigned to the genus level with ≥80% confidence threshold using Ribosomal Database Project (RDP) Classifier (Wang et al. 2007). Any remaining

SC isolates with reads which were unassignable for failure to meet the above standards were instead identified using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) at the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS, USA). Open access to the repository hosting our workflow code for classifying Sanger sequences of 16srRNA gene is available online (https://github.com/vlpickens04/Sanger_Phred_Code).

Statistical Analysis

Serial dilution plates with CFUs in the range of 30 to 300 CFUs were enumerated and included in the quantitative data. Number of CFUs were averaged between plated dilutions and replicates of fly samples (n = 176 house flies/farm type; n = 352 total flies). For some samples, no dilutions yielded colony growth within the relevant range for either plated replicate, with colonies either too numerous to count (TNTC; > 300 CFUs) or too few to count (TFTC; < 20 CFUs). In these cases, samples underwent the following adjustments. Samples where dilutions skipped from TNTC down to TFTC were assigned 301 CFUs for the highest dilution at which TNTC was recorded. Samples with dilutions that jumped from TFTC to no growth were assigned 29 CFUs for the highest dilution with TFTC assigned. Only samples which yielded no growth for all plated dilutions were assigned 0 CFUs so that samples were not misrepresented as flies without any culturable bacteria. For all samples, CFUs from each replicate were averaged for cultures on TSA and VRBA and log10 transformed before statistical analysis.

All statistical analyses were performed in JMP Pro Statistical Software (JMP, Version 16.2, SAS Institute Inc., Cary, NC, USA). Log-transformed CFUs were analyzed using the linear regression and ANOVA approaches in the Standard Least Squares Personality of Fit Model to initially investigate the effect of fly sex, farm type, county, collection date, and their interactions

on culturable bacteria and SC abundances in house flies. Pairwise post hoc comparisons of least squares means estimates were calculated for the comparisons of fly sex across farm types and locations (farm type × county) on bacterial abundances, as well as the comparisons of collection date and the interaction of fly sex and collection date using the Tukey honestly significant difference (HSD) test.

The effect of collection date on culturable bacteria and SC abundances carried by house flies and their possible associations with climate variables were additionally explored. First, an initial variable reduction was performed by deriving principal components of the measured climate variables and bacterial enumerations via the principal component analysis (PCA), multivariate methods platform in JMP 16.2. Original variables observed in PCA to exhibit large loading values in the first 2 principal components were reserved and used as regressors in backwards regression via the least-squares means platform in JMP. Further variable reduction occurred in backwards regression step with removal of variables where $P \leq 0.05$, resulting in a final set of variables for prediction model exploration. Collection date and county were excluded from the regression model to reduce redundancy, as the same weather data was used for sites within the same county on the same collection date and were therefore confounding variables to the model. Fixed effects of fly sex, farm type, and their interactions were combined with selected continuous climate variables and used to predict culturable bacterial and SC abundances of house flies.

Contingency Analysis (Fit Y by X platform, JMP 16.2) was used to further explore the associations between SC abundance and antimicrobial resistance of SC isolates from house flies. Log transformed SC abundance data for each fly was assigned a category based upon the ranges of SC abundance observed for house flies in a zero-inflated Poisson distribution (1 = 0.00 to

0.50, 2 = 0.51 to 2.90, 3=3.00 to 3.99, 4=4.00 to 4.99, 5=5.00 to 5.99, 6 = ≥ 6.00; in Log10(CFU + 1) per fly). Flies possessing SC abundance in category 1 were removed to eliminate confounding interpretations for the dependence of SC isolate resistance profiles in house flies since this category was created for the outlying flies carrying 0 or too few CFUs for proper quantification (which were assigned a CFU value of 29), and no SC isolates were taken from these fly samples to assess AMR despite bacterial growth on culture plates. House flies from which SC isolates were tested for antimicrobial susceptibility were assigned to 2 different categories: (i) the number of antibiotics to which SC isolates the fly carried were resistant and (ii) the type of antibiotics to which SC isolates carried by the fly were resistant. To investigate the association between mean abundance and AMR characteristics of SC carried by house flies, the relative frequency of house flies carrying each category of SC abundance and SC isolates belonging to the number and type of antibiotic resistance categories was discerned by the Fisher's Exact Test. For all statistical tests, $P \leq 0.05$ was considered significant.

Results

Factors Affecting Culturable Bacteria and Suspected Coliform Abundances in House Flies

Culturable bacteria and SC were enumerated from 352 adult house flies for this study (n = 176 house flies/farm type). Irrespective of farm type, collection date, county, or fly sex, culturable bacterial CFUs ranged from 3.35 × 103 to 3.01 × 107 CFUs/fly (mean ± SEM: 1.57 ± 0.20 × 106 CFUs/fly) and SC CFUs ranged from 0 to 3.01 × 107 CFUs/fly (mean ± SEM: 1.91 ± 0.25 × 105 CFUs/fly). Overall, female house flies carried significantly greater abundances of both culturable bacteria (mean ± SEM: 2.38 ± 0.34 × 106; F = 83.48, df=1, P<0.0001) and SC (mean ± SEM: 3.05 ± 0.47×105; F = 52.34, df = 1, P < 0.0001) than male flies (culturable

bacteria mean ± SEM: 7.84 ± 2.05 × 105; SC mean ± SEM: 7.84 ± 1.64 × 104). No observable growth of SC CFUs was recorded for 14 house flies, of which 11 were male house flies. Comparisons of culturable bacteria and SC abundances in male and female flies from each location are summarized in Appendix Table A.2. An overall effect of county and collection date was also observed on abundances of both culturable bacteria (county: $F = 17.45$, df = 2, $P < 0.0001$; date: $F = 11.20$, df = 4, $P < 0.0001$) and SC (county: $F = 9.27$, df = 2, $P = 0.0001$; date: $F = 17.46$, df = 4, $P < 0.0001$) in house flies. Farm type did not significantly affect culturable bacterial abundance in house flies ($F = 0.64$, df = 1, $P = 0.42$) but SC abundance was significantly greater in flies from beef operations than flies from dairies ($F = 5.92$, df = 1, $P = 0.02$).

The interaction between collection date and county significantly affected the mean abundances of both culturable bacteria and SC in house flies (culturable: $F = 4.00$, df = 8, $P = 0.0002$; SC: $F = 2.39$, df = 8, $P = 0.02$) and the interaction between collection date and location (farm type × county) also had a significant effect (culturable: $F=4.64$, df=8, $P<0.0001$; SC: $F=3.75$, df=8, $P=0.0003$). The abundance of culturable bacteria recovered in house flies also was significantly affected by interactions between collection date and fly sex ($F=7.19$, df=4, $P<0.0001$), county and fly sex ($F=3.32$, df = 2, $P = 0.04$), and county, fly sex, and location (farm type × county) ($F = 2.77$, df = 8, $P = 0.006$). In contrast, only the interaction of collection date, county, and fly sex ($F = 2.17$, df = 8, $P = 0.04$) significantly affected SC abundance of house flies. No overall location effect (farm type × county) was observed for culturable bacteria ($F=1.95$, df=2, $P=0.14$) or SC ($F=0.80$, df=2, $P=0.45$) abundance.

The interaction between farm type and fly sex did not significantly influence the abundance of culturable bacteria ($F = 2.85$, df=1, $P=0.09$) or SC ($F=3.65$, df=1, $P=0.06$) in

house flies. While mean SC abundance was significantly greater in male house flies from beef versus dairy sites, mean culturable bacteria abundance in males did not differ between farm types (Figure 2.1 A and C).

Female house fly mean culturable bacteria and SC abundances did not significantly differ between beef or dairy farms but were greater than male flies both within and between farm types (Figure 2.1 A and C). Within each location, the culturable bacteria and SC abundances in female house flies was not always significantly greater than male flies (Figure 2.1 B and D). Within collection date, abundances of culturable bacteria and SC in house flies overall (i.e. irrespective of sex) were significantly lower in flies collected on later dates, when lower average ambient and soil temperatures were observed (Figure 2.2 A and C). However, within fly sex, significant differences in culturable bacteria and SC abundances did not always follow this same trend across the collection dates (Figure 2.2 B and D). Female house flies generally carried greater culturable bacteria and SC abundances than male flies within collection dates, although these differences were not always statistically significant (Figure 2.2 B and D). Female house flies carried significantly more culturable bacteria than male flies on 3 of the 5 collection dates (Aug 13, Aug 27, and Sep 24) and greater abundance of SC on 2 of the 5 collection dates (Aug 13 and Sep 24).

Environmental Predictors for Mean Bacterial Abundances Carried by House Flies

Following reduction via PCA and backwards regression to remove insignificant variables ($P > 0.05$; Appendix Figure A.1), a least square fit of the multivariate prediction generated models for predicting the mean abundances of culturable bacteria and SC in house flies using the parameters outlined in Table 2.1. Separate models were generated for predicting the abundance of culturable bacteria and SC in house flies based upon the listed parameters.

The equation for the best-fitting regression model to predict the mean abundance of culturable bacteria in house flies at beef and dairy operations is as follows:

$$Ycb = -0.66 - 0.25Xs + 0.26Xa + 0.07Xh + 0.01Xp \pm 0.35Xf \quad (1)$$

Where *Ycb* is the predicted mean Log10(CFU + 1) of culturable bacteria per fly,

- *Xs* is the average soil temperature of the collection date
- *Xa* is the average ambient temperature of the collection date
- *Xh* is the average humidity of the 3 d prior
- *Xp* is the average precipitation (cm) for the 3 d prior
- *Xf* is the condition for adding or subtracting 0.35 based upon the

  fly sex

  - if "female" + 0.35
  - if "male"—0.35

Based on the model, a positive relationship was predicted between culturable bacteria abundance, average ambient temperatures of the collection date, and average humidity and precipitation of the 3 d prior to collection (Equation 1). However, greater abundances of culturable bacteria were negatively associated with higher average soil temperatures on the date of collection. Additionally, culturable bacteria abundance for female flies are predicted to be greater than those of male house flies under the same conditions.

In contrast, the equation for the best-fitting regression model to predict the mean abundance of SC in house flies at beef and dairy operations is as follows:

$$Ycol = -1.44 + 0.29Xa + 1.65Xa + 0.09Xh - 0.38Xw + 0.04Xp \pm 0.41Xf \pm 0.41Xt. \quad (2)$$

Where *Ycol* is the predicted mean Log10(CFU + 1) of SC per fly,

- *Xa* is the average ambient temperature of the collection date

- *Xd* is the number of days with precipitation of the 3 days prior
- *Xh* is the average humidity of the 3 days prior
- *Xw* is the average soil temperature of the week prior
- *Xp* is the average precipitation (cm) for the 3 days prior
- *Xf* is the condition for adding or subtracting 0.41 based upon the

  fly sex

  - if "female" + 0.41
  - if "male"—0.41
- *Xt* is the condition for adding or subtracting 0.14 based upon the location farm type
  - if "dairy" + 0.14
  - if "beef"—0.14

A negative relationship was predicted between SC abundance and average soil temperatures in the week prior to collection (Equation 2). Alternatively, higher average ambient temperatures on the date of collection, higher average humidity and precipitation for the 3 d prior to collection, and more days with precipitation in the 3 d prior were predicted to increase culturable bacteria carried by house flies. The model predicted greater SC abundance in female house flies than male flies and greater SC abundance in flies from dairies than those from beef operations.

Notably, fly sex had the strongest influence in predicting mean abundances of both culturable bacteria and SC in house flies, followed by average ambient temperatures on the collection date (Table 2.1). However, ANOVA indicated a lack of fitness for the prediction profiler models of both the mean culturable bacteria ($F = 3.0904$, df=24, $P<0.0001$) and SC

(F=1.7371, df=52, P=0.0025) for flies, suggesting other potential coefficients unmeasured in this study exist that could improve accuracy of future models.

Antimicrobial Susceptibility and Identities of Suspected Coliform Morphotype Isolates

A total of 661 SC isolates were selected from the VRBA cultures of adult house flies collected at beef (n = 412 isolates) and dairy (n = 249 isolates) operations (Appendix Table A.3). These SC isolates were selected from 215 of the 352 house flies collected in this study and ranged from 1 to 15 SC isolates per fly. Following the agar-based tetracycline resistance screening, 260 beef SC isolates and 115 dairy SC isolates were suspected to be tetracycline resistant and underwent disk susceptibility testing. Despite growing on tetracycline-infused agar, 50.1% (n = 188) SC isolates suspected to be tetracycline-resistant were not resistant to tetracycline via Kirby Bauer disk testing. In total, 132 (31.8%) beef SC isolates and 55 (22.1%) dairy SC isolates were tetracycline resistant. However, 94 (25.1%) of the SC isolates found susceptible to tetracycline were instead resistant to ampicillin, 2 SC isolates were dual-resistant to florfenicol and ampicillin, and one SC isolate was dual-resistant to florfenicol and ceftiofur.

Of 661 SC isolates selected from house flies, 43.0% (n = 284/661) were resistant to one or more antibiotics. Of these 284 AMR SC isolates, 218 (76.8%) SC isolates were single-drug resistant to either tetracycline (n = 124; 56.9%) or ampicillin (n = 94; 43.1%) (Appendix Table A.4). Single-resistant SC isolates were largely *Escherichia/Shigella* (n = 99) and *Klebsiella* (n = 50) species, with 96.0% (n = 95/99) of *Escherichia/Shigella* isolates possessing tetracycline resistance and 98.0% (n = 49/50) of *Klebsiella* isolates possessing ampicillin resistance. The AMR phenotype and genera of single-resistant SC isolates were variable between beef and dairy operations (Figure 2.3), and individual sites within the same farm type (Appendix Table A.4).

Tetracycline-resistant SC isolates cultured from house flies collected at both beef and dairy operations included genera, such as *Citrobacter, Escherichia/Shigella, Kluyvera,* and *Providencia.* Tetracycline-resistant SC isolates from dairy house flies additionally included *Enterobacter* and beef house flies additionally had tetracycline-resistant *Klebsiella, Leclercia, Proteus, Pseudescherichia, Serratia,* and *Siccibacter* isolates. Ampicillin-resistant SC isolates from house flies at both farm types included *Escherichia/Shigella, Klebsiella, Kluyvera,* and *Serratia.* Additionally, *Citrobacter, Morganella,* and *Pseudocitrobacter* represented ampicillin-resistant isolates from dairy house flies and *Enterobacter, Kosakonia, Pantoea,* and *Raoultella* represented ampicillin-resistant isolates from beef house flies.

Sixty-six SC isolates were resistant to two or more antimicrobials and characterized as multi-drug resistant (MDR), including 8.0% (*n* = 20/249) of SC isolates from flies from dairy operations and 11.2% (*n* = 46/412) SC isolates from flies from beef operations. Bacterial genera and MDR phenotypes of SC isolates from flies were variable between sites within the same farm type (Appendix Table A.5). Of the 187 tetracycline-resistant SC isolates from house f lies, 64 (34.2%) SC isolates had additional resistance to florfenicol, enrofloxacin, ceftiofur, or ampicillin. Two MDR SC isolates did not have tetracycline resistance via Kirby Bauer testing but were dual resistant to florfenicol and ceftiofur or ampicillin. Dual resistance to tetracycline and ampicillin was the most common MDR phenotype for SC isolates from flies at both beef and dairy operations (*n* = 33/66; 50%) and had the widest variety of bacterial genera associated with this phenotype (Figure 2.4): *Enterobacter* (1), *Escherichia*/*Shigella* (7), *Klebsiella* (10), *Kluyvera* (1), *Morganella* (4), *Proteus* (2), *Providencia* (2), *Raoultella* (2), *Serratia* (4). Triple resistance to tetracycline, florfenicol, and ampicillin was the next most common MDR phenotype (n = 18/66; 27.3%), although this phenotype was only observed in the genera *Escherichia*/*Shigella* and

Pseudescherichia. Additionally, *Escherichia*/*Shigella* were associated with the widest variety of MDR phenotypes (Figure 2.4). SC isolates from flies collected at beef operations had a wider variety of MDR phenotypes than SC isolates from flies collected at dairies (Figure 2.4). Enrofloxacin resistance was only observed in 2 SC isolates recovered from 2 different female house flies at the Marion beef operation, one of which was a *Pseudomonas* isolate that was resistant to all 5 antibiotics.

Prevalence of House Flies Carrying AMR Isolates

No particular category for the mean abundance of SC CFUs enumerated from a fly was found to significantly increase the probability of whether SC isolates from a fly were resistant to one ($P = 0.6536$) or multiple ($P = 0.4493$) antibiotics. Overall, 73.02% ($n = 157/215$) of adult house flies from which SC isolates were susceptibility tested carried at least one AMR SC isolate and 26.05% ($n = 56/215$) carried at least one MDR SC isolate. In one case, as many as 9 different AMR SC isolates were recovered from a single male fly collected at a beef operation. No apparent difference between male or female house flies carrying AMR and MDR SC isolates at these operations was observed, but a larger proportion of house flies collected at beef operations carried AMR (77.31%) and MDR (31.09%) SC isolates than house flies from dairy operations (67.71% AMR; 19.57% MDR) (Figure 2.5).

Discussion

This study investigated potential biotic and abiotic factors affecting the abundances of culturable aerobic and suspected coliform (SC) bacteria carried by house flies in Kansas beef and dairy confined cattle operations. Similar to prior findings (Neupane et al. 2020), female house flies in this study overall had significantly greater abundances of culturable aerobic and SC bacteria than male flies. Female flies also carried significantly greater abundances of culturable

aerobic and SC bacteria within farm types, although this trend was not always significant within location or collection date. While female house flies are on average larger than male flies, body size is not considered correlated to the mean abundances of bacteria carried by house flies (Neupane et al. 2020). Rather, the frequent interaction of female house flies with microbe-rich substrates to seek out potential oviposition sites (Thomson et al. 2017), as well as their nutritional needs required for egg development (Hanski 1987) and feeding preferences (Neupane et al. 2023), are believed to influence their trophic activities and subsequent tendency to carry greater mean abundances of bacteria than male flies.

Coliforms are highly abundant in animal manure, which is frequented by female flies both for oviposition and as a food source (Thomson et al. 2017). Considering that manure accessibility and management practices often differ between beef and dairy facilities, we therefore predicted farm type would most likely affect SC abundance in female flies. However, farm type significantly influenced SC abundance in only male house flies, with male flies from dairy sites carrying significantly lower SC abundance compared to male flies collected from beef sites. Some male flies from 2 of the 3 dairy sites (Marion and Washington) did not have any detectable SC. Interestingly, SC abundance in male flies collected from Riley dairy were similar to male flies collected from the beef facilities. This is likely because the Riley dairy is located close to the Riley beef site (less than 600 m), and shares site management and manure practices. The abundance of SC among female flies from dairy and beef locations did not differ, indicating that female flies may persistently interact with manure, potentially for oviposition and feeding, irrespective of the different manure accessibility or management practices associated with these 2 types of cattle operations. Alternatively, males may only have ephemeral, infrequent

interactions with manure compared to females who inspect manure frequently for oviposition purposes.

Further, in contrast to female flies, which have a higher tendency to feed on a variety of food resources, male flies prefer sugar-rich foods when presented with a variety of food resources (Neupane et al. 2023). Therefore, male flies within cattle operations may interact with sources of SC other than manure that differ in availability, consistency, or management resulting in variable SC levels. Cattle feed is one such substrate where the formulation can differ substantially between operations to best meet desired end animal product (volume and quality of milk vs. weight gained as muscle and fat). These feed ingredients not only vary in attractiveness to house flies but also in their ability to support coliform growth. For example, fresh steam-flaked corn, a carbohydrate-rich cattle feed ingredient, has previously been identified as a site for coliform contamination by house flies within a beef cattle feedlot (Ghosh and Zurek 2015). The combination of these factors could influence SC accumulation by male flies at beef compared dairy sites. Our study only sampled 6 sites in a relatively small geographic area, and future studies should further investigate the sex-specific difference in SC acquisition and carriage in by house flies when feeding on different food sources across a variety of locations.

Date of collection significantly influenced culturable bacteria and SC abundances in both male and female flies, in contrast to previous studies (Neupane et al. 2020). In the previous study, flies were only sampled over a 1.5-month period mid-summer, whereas in the current study, flies were collected over a 2-mo period from summer into early fall. A shorter time frame within a single season may have more stable climate conditions that preclude meaningful impacts of collection date and associated climatic variables on bacterial abundances. In the current study, significant differences in bacterial abundances in flies over time were only

observed later in our collection period when climate variables such as average daily ambient and soil temperatures were decreasing. This may be a result of increased interactions between house flies and environmental sources, as activities of adult flies in dairy cattle operations have been observed to increase with higher temperatures and flies may be therefore acquiring more bacteria (Zahn and Gerry 2020). Additionally, temperature is a known factor in bacterial propagation and the succession of bacterial communities within soil and other environments (Ratkowsky et al. 1982, Zhang et al. 2005) and impacts diversity and richness of ARGs within substrates that house flies frequent in confined cattle operations (Wang et al. 2022). Our findings suggest that local ambient and soil temperature and humidity may assist in predicting bacterial abundances in house flies at confined cattle operations and could be used to identify conditions during which house fly management may be more critical for mitigating pathogen dissemination. Sobur et al. (2022) found evidence of higher seasonal temperatures resulting in higher occurrences of *S. aureus* and MRSA isolates cultured from house flies in hospital-associated areas, as well as the isolates' resistance to individual and multiple antibiotics tested. A knowledge gap remains regarding the relationship between temperature or other climate factors and the ability of house flies to transmit pathogens and AMR bacteria.

Of the 215 flies from which SC isolates were tested for resistance to antibiotics in this study, 157 (73%) carried one or more AMR isolates, and one fly carried as many as 9 different AMR isolates. Since our methods did not test the AMR of every morphotype cultured from each fly, the proportion of house flies carrying AMR bacteria may be higher than our data reflects. Although our subjective method of selecting distinct morphotypes of suspected coliforms from house fly cultures limits a quantitative estimation of AMR bacteria carried by house flies, our results still provide strong evidence that most house flies in confined cattle operations are

carrying AMR bacteria and therefore are contributing to AMR persistence and spread in these environments. We found that the prevalence of AMR/MDR SC was similar between male and female house flies, which is further supported by the lack of correlation between the mean abundance of SC and carriage of AMR or MDR SC isolates by house flies. However, it is worth noting that the lack of SC was observed more often in male than female flies. Nevertheless, while female flies carried overall greater abundances of bacteria and may present the highest risk as either reservoirs or transmitters of AMR bacteria, AMR/MDR SC bacteria were found in both male and female flies from all locations.

A higher proportion of isolates from house flies collected from beef operations were tetracycline-resistant than those isolated from flies at dairies. Additionally, MDR isolates from beef operations possessed a wider variety of MDR phenotypes to the antibiotics tested than those from dairy operations. House flies from beef operations carried bacteria with resistance to florfenicol, enrofloxacin, and ceftiofur at higher rates than flies from dairies likely due to restrictive usage of these antimicrobial drugs in dairy production. Florfenicol and enrofloxacin are not labeled for use in female dairy cattle > 20 mo (FDA-HHS 2023), and therefore are unlikely to be administered. The prohibition of extralabel use of enrofloxacin and ceftiofur also may contribute to lower prevalence of resistance to these drugs in bacteria from both beef and dairy cattle systems (FDA-HHS 1997, 2012). In addition, feedlots source cattle from multiple locations while dairies usually operate as a closed herd with animals bred onsite. The constant influx of animals with differing treatment histories and geographic origins may also contribute to feedlots showing a wider variety of MDR phenotypes. Interestingly, bacterial abundances and AMR profiles carried by house flies from the Riley dairy and beef facilities were more similar to each other than our other sites of the same farm type. We believe the close proximity of the Riley

beef and dairy sites to each other (< 0.5 km) and other livestock nearby (swine, equine, and poultry teaching and research facilities) contributed to this phenomenon and may indicate cross contamination and widespread fly movement among these livestock facilities, as they are well within observed flight distances of house flies (Geden et al. 2021).

AMR SC isolates cultured from house flies in this study belonged to 16 different bacterial genera. Importantly, we identified 6 genera (*Kosakonia, Morganella, Pseudocitrobacter, Pseudescherichia, Raoultella, Siccibacter*) from which AMR isolates had not previously been reported from house flies collected in food or animal facilities (Nayduch et al. 2023). Of the 12 genera assigned to MDR SC isolates cultured from house flies, we further identified MDR *Morganella sp., Pseudescherichia sp.,* and *Raoultella sp.* from flies in both beef and dairy facilities. However, *Escherichia/Shigella sp.* were the most common AMR and MDR SC isolates cultured from house flies, were carried most frequently by house flies, and carried the widest variety of AMR profiles to the 5 antibiotics tested. Members of this taxonomic group include foodborne pathogens such as *Escherichia coli*, which commonly possess MDR genes that can be horizontally transferred (Hunter et al. 1992, Rovira et al. 2019), including within the gut of house flies (Petridis et al. 2014). Numerous studies have also identified culturable AMR/MDR *Escherichia/Shigella sp.* carried by house flies collected from other livestock facilities, including swine (Literak et al. 2009, Cervelin et al. 2018, Fukuda et al. 2018, Wadaskar et al. 2021), equine (Dolejska et al 2011), poultry (Poudel et al. 2019, Akter et al. 2020, Wadaskar et al. 2021), broiler (Solà-Ginés et al. 2015, Fukuda et al. 2018, Poudel et al. 2019), buffalo (Wadaskar et al. 2021), sheep (Wadaskar et al. 2021), and goat (Wadaskar et al. 2021). Consequentially, *Escherichia/Shigella sp.* may pose the greatest risk for the transmission and prevalence of AMR and MDR bacteria in house flies at cattle operations.

There are a few caveats important to consider when interpreting the results of our study. First, non-coliforms were selected during our attempts to select coliforms and included genera, such as *Pseudomonas*, *Serratia*, *Providencia*, *Kluyvera*, and *Proteus*, indicating difficulty in visual selection of coliforms from VRBA and likely overestimation of true coliforms in CFU estimates as well. However, this is why we used the term "suspected coliforms" throughout our study. Nonetheless, a number of the SC isolates belong to non-coliform genera that contain species that are pathogenic and/or known for their affinity to carry and transfer MDR to other bacteria (ie *Pseudomonas*), and still provide valuable data on fly carriage of taxa that can serve as a source of AMR for other bacterial pathogens within cattle operations. Second, we used CLSI guidelines for Enterobacterales on all isolates to help interpret disk susceptibility results. A few isolates were later identified as belonging to taxonomic groups outside of Enterobacterales. CLSI has interpretation guidelines for only a few other Gram-negative bacteria (*Pseudomonas aeruginosa, Bordetella bronchiseptica, Mannheimia haemolytica, Pasteurella multocida, Actinobacillus pleuropneumoniae, Histophilus somni*), and we did not have species-level identification to later correct resistance interpretations of *Pseudomonas sp*. isolates. We ultimately retained AMR interpretations for non-Enterobacterales to provide data on bacteria from flies that may likely be contributing to AMR and to help select AMR isolates for downstream analysis. We are currently performing whole genome sequencing of MDR isolates from this study and this will provide additional genetic evidence for suspected AMR in these isolates. Finally, because identifying a morphotype is subject to the experimenter's discretion, we likely underreported AMR/MDR phenotypes carried by flies. Current work by our group incorporates metagenomic analyses of whole house fly homogenates which can detect both pathogens and AMR genes carried in or on house flies. The combination of the culture-based

work we present here, and these molecular analyses will provide a more comprehensive picture of the role flies play in the carriage and movement of pathogens and AMR in cattle operations.

Together, the results of this study further emphasize the threat house flies pose to both animal and human health. House fly sex, farm type, and climate can significantly affect the abundance of bacteria and prevalence of AMR carried by house flies and therefore can be used as predictors for risk modeling. These parameters also should be considered for integrated AMR management and mitigation practices in confined animal feeding operations such as beef feedlots and dairies.

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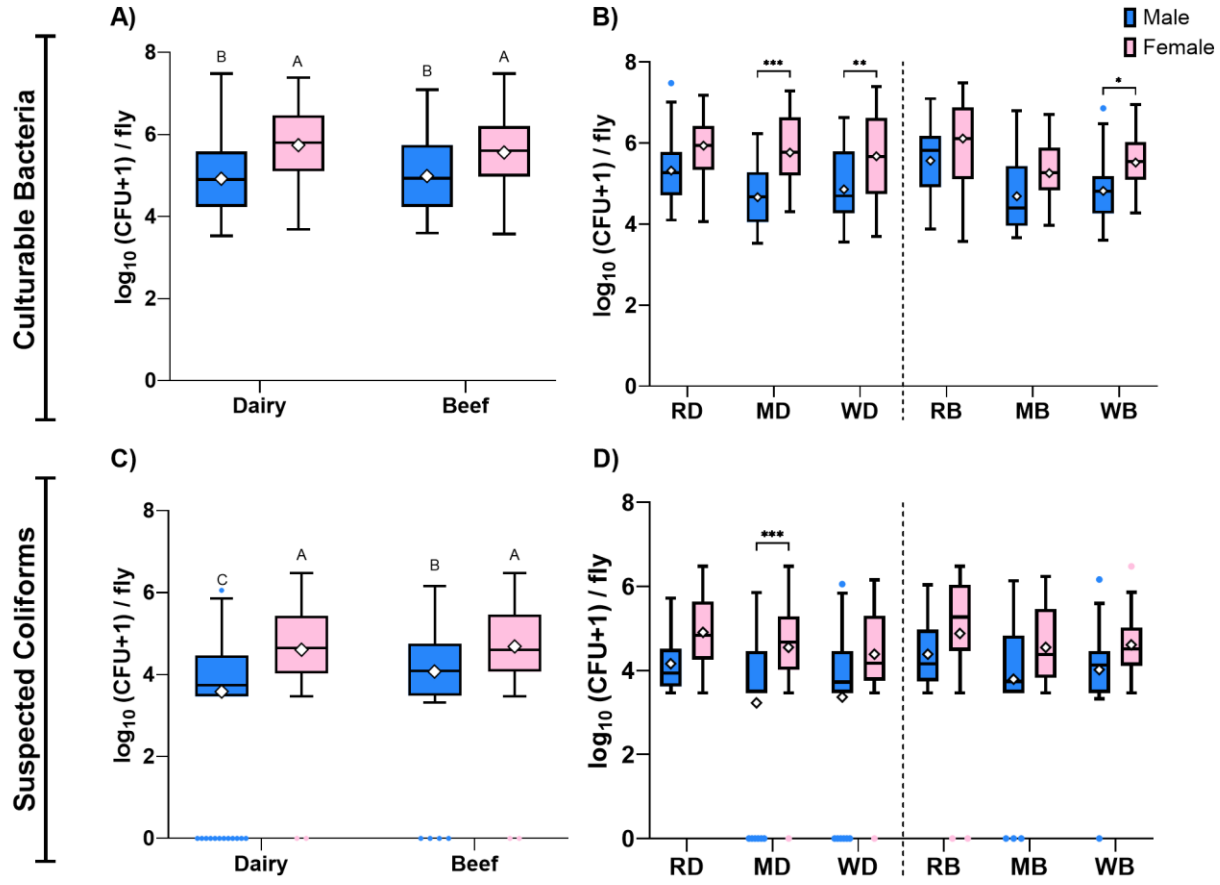

Figure 2.1. Abundance of bacteria from male and female flies across farm types

Mean abundance of culturable bacteria (grown on TSA, see text) and suspected coliforms (SC; grown on VRBA, see text) in male and female house flies within farm types (A, C) and locations (B, D). Differing letters in (A) and (C) indicate significant differences in pairwise comparisons of least square means (Tukey HSD, $P < 0.05$). For pairwise comparisons of least square means in (B) and (D): * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$. Box and whisker graphic overlay of the mean abundance values provides evaluation of the full range of CFUs, where the diamonds are the raw mean, solid middle bars are the median values, and the bars extending from the center represent the interquartile range.

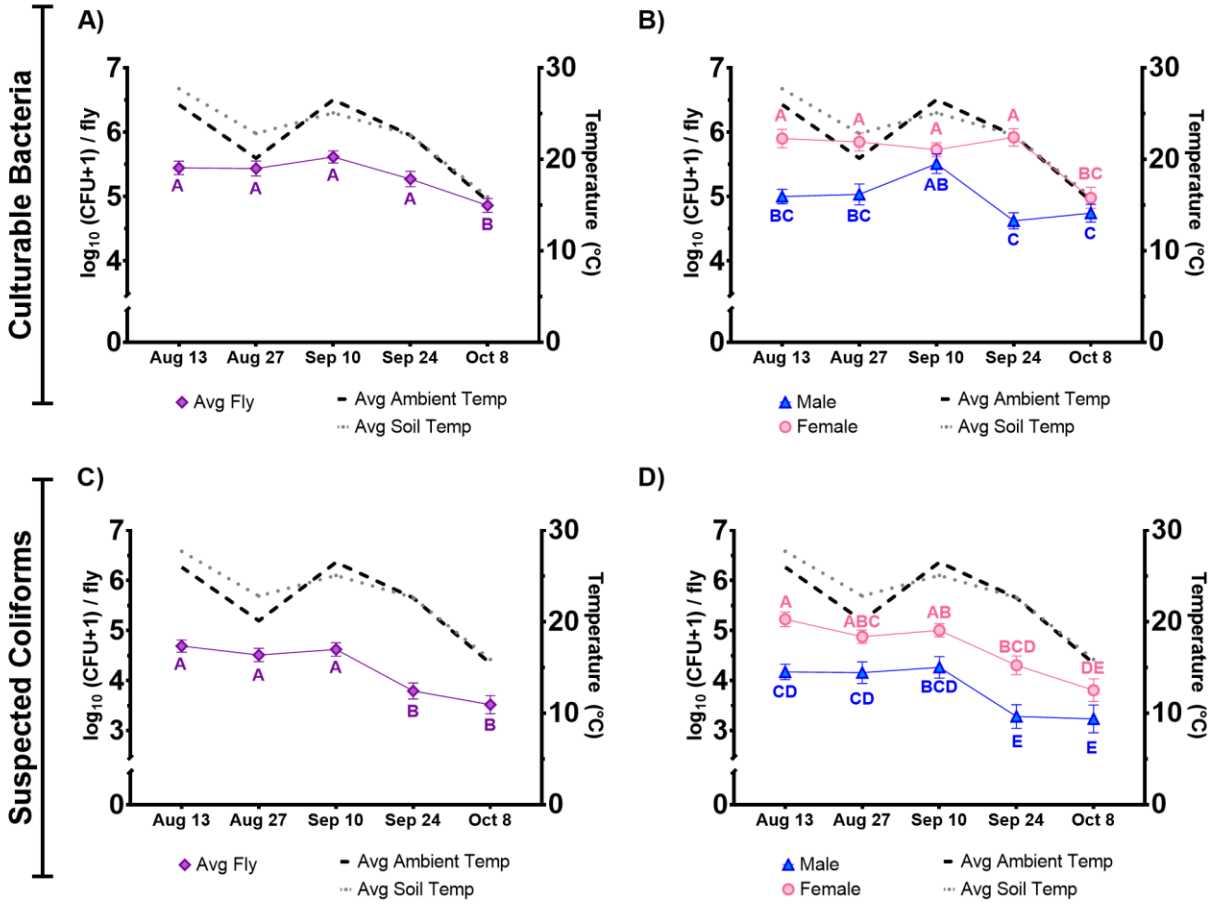

Figure 2.2. Relation between bacterial abundance in flies and environmental conditions

Relationship between abundance of culturable bacteria (grown on TSA, see text) and suspected coliforms (SC; grown on VRBA, see text) in flies irrespective of sex (A, C) and delineated by sex (male and female; B, D) with ambient and soil temperatures. Raw mean ± SEM is shown. Differing letters indicate significant differences in pairwise comparisons of least square means (Tukey HSD, $P < 0.05$).

Table 2.1. Climate variables used for prediction plots of factors influencing mean abundance of culturable bacteria and suspected coliforms carried by house flies

Parameter	Estimate	Standard Error	t Ratio	Prob > |t|
Culturable Bacteria				
Intercept	-0.6554	0.9883	-0.66	0.5076
Fly Sex [Female]	0.3498	0.0436	8.02	< 0.0001
Collection Soil Temp Avg	-0.2526	0.0572	-4.42	< 0.0001
Collection Avg Temp	0.2618	0.0485	5.40	< 0.0001
3D Avg Humidity	0.0732	0.0150	4.87	0.0038
3D Avg Precipitation	0.3498	0.0436	8.02	<0.0001
Suspected Coliforms				
Intercept	-1.4365	1.4127	-1.02	0.3099
Farm Type [Dairy]	-0.1414	0.0600	-2.35	0.0191
Fly Sex [Female]	0.4100	0.0600	6.83	< 0.0001
Collection Avg Temp	0.2852	0.0537	5.31	< 0.0001
3D Days of Precipitation	1.6464	0.4864	3.38	0.0008
3D Avg Humidity	0.0856	0.0271	3.16	0.0017
3D Avg Precipitation	0.0361	0.0076	4.71	<0.0001
1W Avg Soil Temp	-0.3757	0.0899	-4.18	< 0.0001

3D = Collection date and 2 days prior to the collection.

1W = Collection date and 6 days prior to the collection.

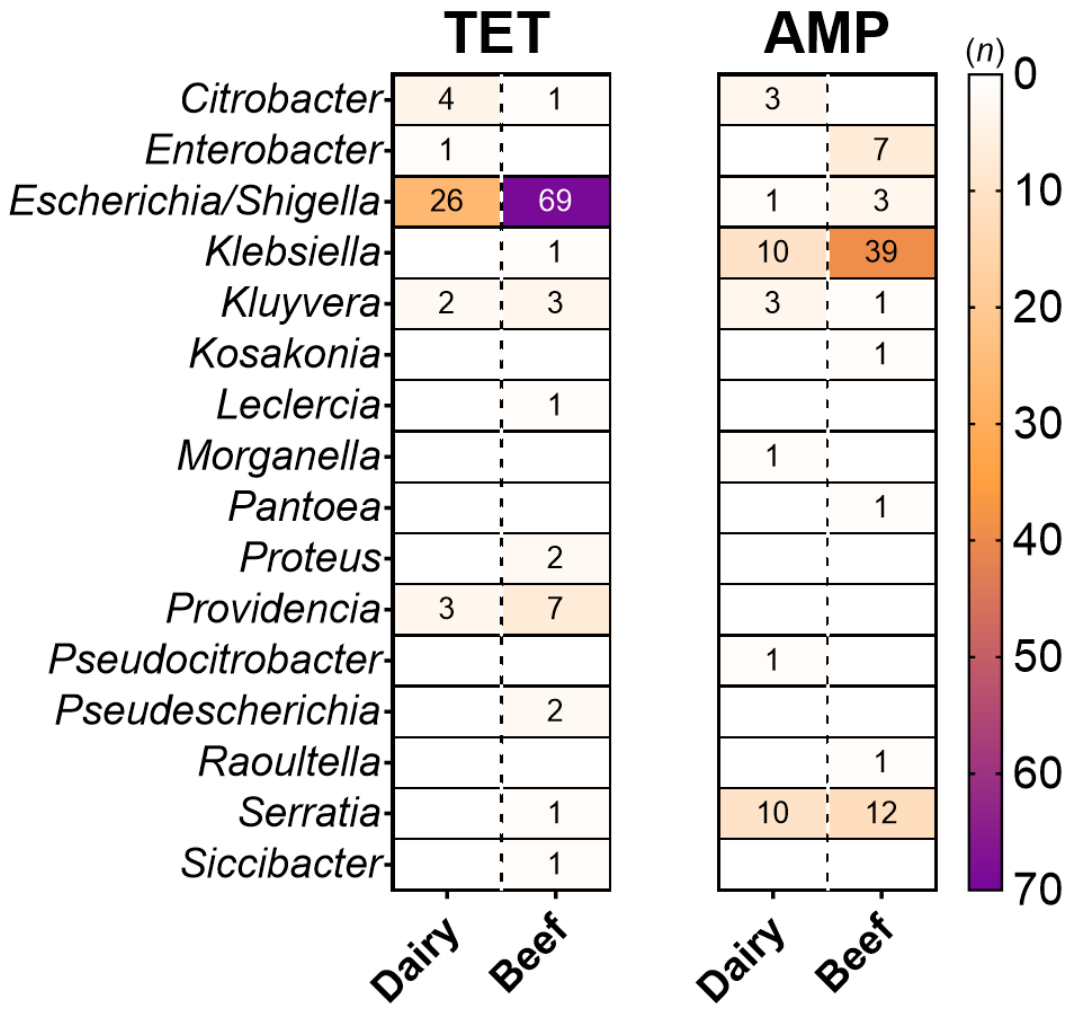

Figure 2.3. Antibiotic-resistant SC genera isolated from house flies by operation type

Number (n) of SC isolate genera cultured from house flies from beef and dairy operations with tetracycline (TET) or ampicillin (AMP) resistance phenotypes.

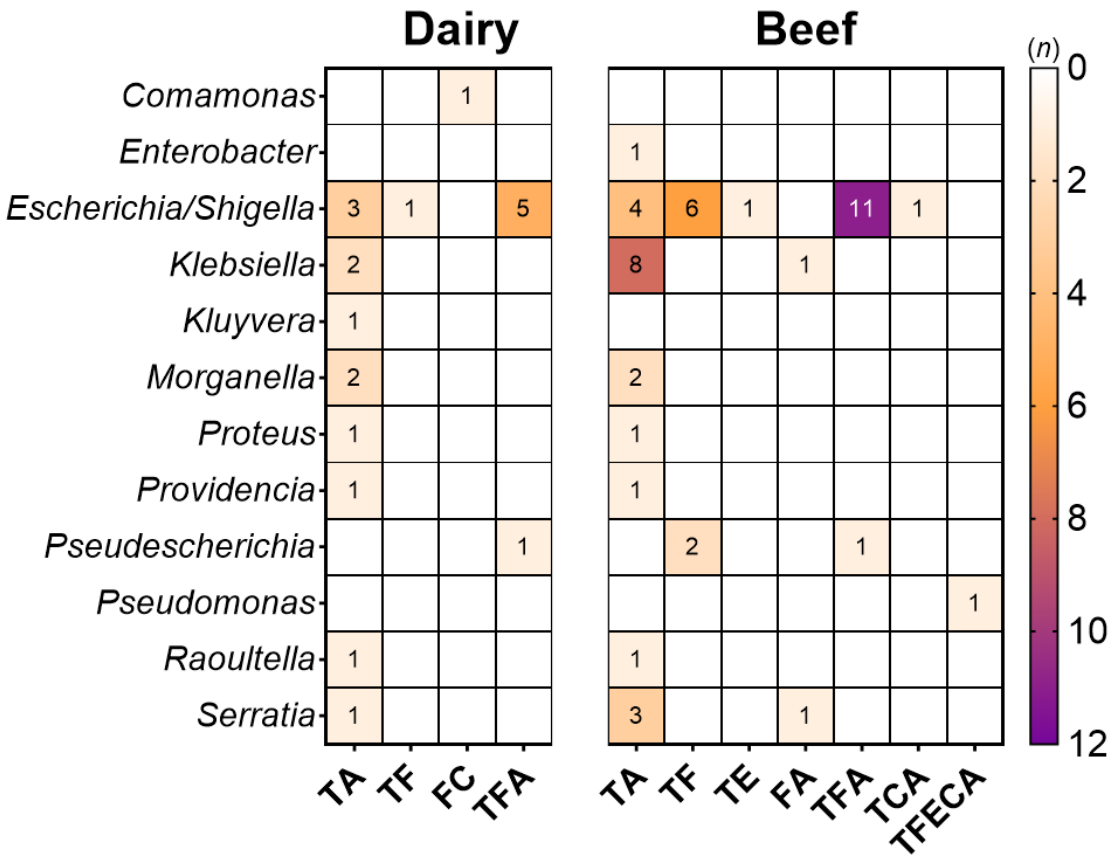

Figure 2.4. MDR SC genera from flies on beef and dairy farms

Number (n) of SC isolate genera with MDR profiles found in house flies from beef and dairy operations. Resistance phenotypes: T = tetracycline; F = florfenicol; A = ampicillin; C = ceftiofur; E = enrofloxacin.

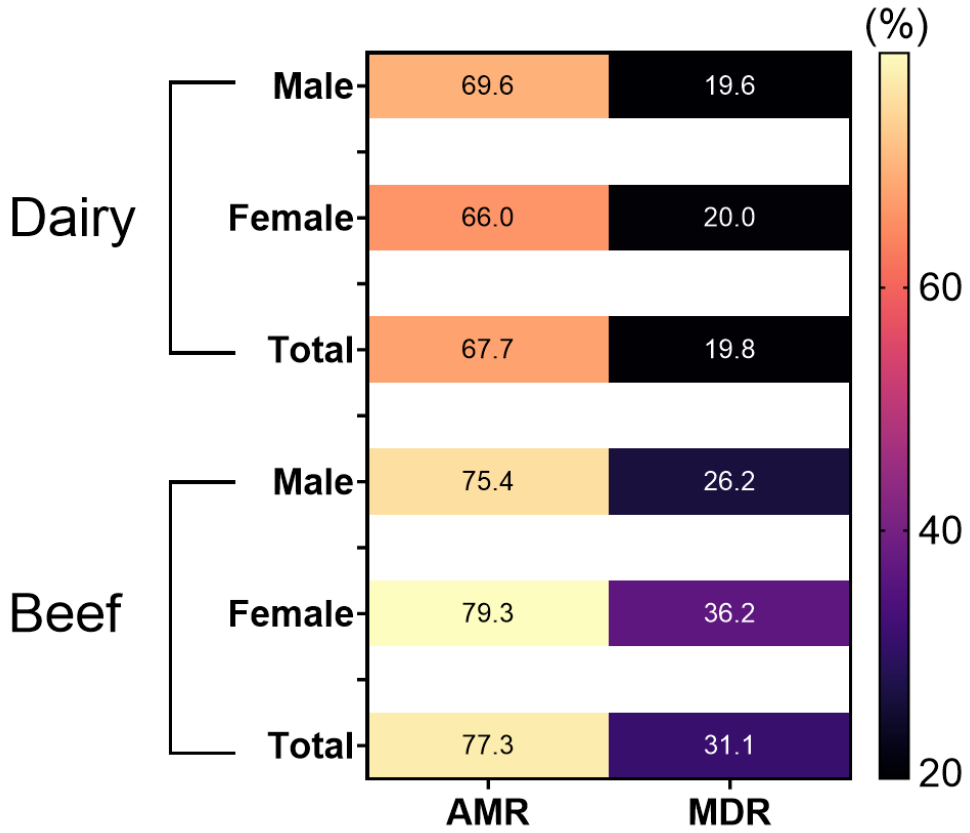

Figure 2.5. Prevalence of AMR and MDR SC isolates in house flies by sex and operation

Prevalence (%) of house flies carrying at least one SC isolate with resistance to ≥1 (AMR) or ≥ 2 (MDR) antibiotics. Beef: n = 61 male, n = 58 female; Dairy: n = 46 male; n = 50 female.

Chapter 3 - Adult house flies (Diptera: Muscidae) reflect site-specific antimicrobial resistant bacteria in confined beef cattle operation environments

In preparation as: Pickens V, Nayduch D, Purvis T, Hall B, Brendel L, Brooke G, Bird E, Olds C. Adult house flies (Diptera: Muscidae) reflect site-specific antimicrobial resistant bacteria in confined beef cattle operation environments.

Abstract

House flies (*Musca domestica* L.) frequent microbe-rich substrates such as animal manure to meet reproductive and nutritional needs. Recent studies propose the use of house flies in xenosurveillance, as they can accumulate pathogenic and antimicrobial resistant (AMR) bacteria during these interactions. However, more research is needed to understand how environmental factors influence their bacterial carriage, particularly AMR strains. This study investigated the effects of fly sex, location, and collection date on bacterial abundance in adult house flies across four beef cattle operations in Northeast Kansas. Antimicrobial resistant bacteria from flies, manure compost, manure patties, feed, and water were also compared between sites and sample types. After culturing samples onto MacConkey agar (MAC), colony forming units (CFUs) were enumerated and replica plated onto MAC infused with three different antibiotics. Distinct CFU morphotypes were isolated, identified, and susceptibility tested against tetracycline, florfenicol, enrofloxacin, ampicillin, and ceftiofur. Fly sex significantly influenced mean bacterial abundance in flies, with females carrying greater loads than males. Location, collection date, and their interaction also influenced bacterial abundance, with site-specific trends suggesting local environmental factors play a major role. Antimicrobial-resistant and

multidrug-resistant (MDR) bacteria were recovered from 97% and 74% of flies, respectively. Resistance phenotypes and taxa observed were more dependent on location than sample types. Within each site, fly isolates reflected resistance patterns found in environmental isolates. These results support the use of house flies as aggregate indicators for AMR surveillance in confined cattle and highlight their potential for identifying site-specific risks for fly-mediated bacterial transmission.

Introduction

Antimicrobial resistance (AMR) is an urgent global concern affecting public health, animal health, and food security. The prevalence of AMR bacterial pathogens continues to rise despite efforts to mitigate the spread of AMR over the past decade (Naghavi et al. 2024), emphasizing the need for better understanding of the development and transmission of AMR. As a One Health problem, action plans targeting AMR promote integrated research studying how resistance develops and spreads across human, animal, and environmental systems (CDC 2025). Surveillance of AMR in the food supply chain, particularly livestock, is key in revealing both sources and drivers of AMR to guide AMR mitigation strategies that can improve human and animal health protection.

House flies (*Musca domestica* L.) are persistent pests of livestock operations and globally recognized vectors of bacterial pathogens that threaten both human and animal health. House flies require a microbe-rich diet as larvae (Gerberich 1948, Greenberg 1954, Schmidtmann and Martin 1992, Zurek et al. 2000) developing in decomposing organic materials such as garbage waste (Paine 1912), manure (Khan et al. 2012), or plant matter (Cook et al. 2011). Adult house flies, particularly females, frequent these microbe-rich habitats to select oviposition sites, breed, and opportunistically feed off these resources (West 1951, Shah et al. 2016). During these

interactions, adult house flies acquire microorganisms via contact with their body surfaces or direct ingestion. These flies can subsequently expose animals to potential pathogens through contact with animals, ingestion by the animal, or transfer to the environment (Nayduch and Burrus 2017). House flies pose a significant risk for the movement of bacteria in confined cattle operations due to high animal densities and abundant microbe-rich substrates. As capable vectors of both pathogenic and antimicrobial resistant (AMR) bacteria, house fly associations within confined cattle operations can result in the infection of cattle through direct contact or environmental contamination (Ahmad et al. 2007), or even the infection of humans through food contamination (Macovei et al. 2008, Talley et al. 2009, Chakrabarti et al. 2010, Thomson et al. 2021).

The detection of AMR bacteria and antimicrobial resistance genes (ARGs) in house flies from confined cattle operations (Buma et al. 1999, Chakrabarti et al. 2010, Onwugamba et al. 2018, Neupane et al. 2020), as well as the demonstration of horizontal gene transfer of ARGs within the fly gut (Petridis et al. 2006, Akhtar et al. 2009, Fukuda et al. 2016, Gan et al. 2024), supports the role of house flies in AMR development and transmission. However, the extent to which the AMR bacteria harbored in house flies overlaps with other environmental reservoirs is still not well understood. Historically, investigations have been limited to either targeted surveillance of AMR *Escherichia coli* and *Salmonella spp.,* or genomic surveillance of the microbiota and ARGs found in house flies and cattle manure. These methods have excluded non-pathogen reservoirs of AMR present in the environment and do not encompass the extent by which house fly interactions in confined cattle operations may be contributing to AMR prevalence. Numerous studies demonstrate that flies acquire their gut microbiome locally from sources in their surrounding environment like animal excrement (Poudel et al. 2020, Neupane et

al. 2024), and that their environment also influences the ARGs detected in flies (Poudel et al. 2020, Yang et al. 2024). Shared serotypes of phenotypically resistant *E. coli* have been isolated from flies and cattle manure (Wadaskar et al. 2021, Hickman et al. 2022), and pathogenic or AMR *E. coli* introduced by infected animals have been later detected in flies following exposure (Marshall et al. 1990, Sanderson et al. 2006). Furthermore, house flies have been found to share up to 71.4% of the ARGs and mobile genetic element (MGE) genes identified in animal feces from the same area (Poudel et al. 2020). Considering that these findings indicate the acquisition of AMR pathogens or ARGs by flies following exposure to contaminated animal feces or an alternative environmental source, we therefore hypothesized that the microbial abundances and AMR bacteria carried by adult house flies are influenced by their environment.

We assessed the biotic and abiotic factors influencing the overall abundance of culturable Gram-negative bacteria carried by adult house flies in confined beef cattle operations in Kansas. Further, we attempted to identify potential linkages between flies and environmental sources of AMR Gram-negative bacteria in cattle operations by comparing the recorded species and resistance phenotypes of AMR bacteria cultured from flies and environmental substrates (manure compost, manure patties, feed, and water).

Materials and Methods

Sample Collection

Adult house flies (*Musca domestica* L.) and environmental substrates (manure compost, manure patties, feed, and water) were collected four times in the summer of 2020 from four Northeast Kansas beef cattle operations (one in Lyon, Marion, Riley and Washington counties). For each collection event, all operations were sampled within the same week, with Riley and Washington sampled on one day and Lyon and Marion sampled the second day. Collection dates

were as follows, Collection 1: August 4th & 6th; Collection 2: August 18th & 20th; Collection 3: September 15th & 17th and Collection 4: September 29th & October 1st. Animals at all four facilities were largely Angus and Angus cross cattle breeds. Three of the facilities were feedlots (Riley, Marion, and Washington) where animals were confined to pens and fed feed from a feed bunk. Lyon county site was a stocker operation where cattle were fed daily rations from feed bunk in pens but also had access to grazing pasture.

For all locations, two cattle pens located on opposite sides of the facility were selected for sampling of house flies, feed, and manure patties. The same pens were sampled during each visit unless no animals remained in the pen, at which point the closest pen with animals was selected (typically within 15 m of the original pen). At each pen, adult house flies (5 males and 5 females) were captured via sweep net from the feed bunk, anesthetized on ice, and sorted individually into sterile 1.5 ml microcentrifuge tubes with forceps. Three random fistfuls of feed were taken from the feed bunk with gloved hands and mixed in a sterile container. A sterile 50 ml conical tube was filled with water and sediment collected from the bottom of the pen's water trough using a bleached 30 ml plastic turkey baster. Finally, approximately 50 ml of fresh manure was collected from a single, undisturbed cow patty with a sterile tongue depressor and placed in a sterile 50 ml conical tube. Two samples of manure compost (approximately 50 ml) were also taken from compost piles outside the pens using a sterile tongue depressor. No manure compost samples were gathered for the Lyon site, as this facility does not compost manure.

For Week 3 and 4 of the study, cattle were not present in the Riley feedlot location but had other confined livestock still near the facility (swine, 265 m away; horses, 300 m away; dairy cattle, 485 m away; poultry, 690 m away). Therefore, house flies and environmental substrates were still found on site and collected as follows: 10 house flies (n = 5 males and 5 females) were

collected from the feed storage and 10 house flies (n = 5 males and 5 females) were collected from manure compost piles; water samples were collected from the same two water troughs as prior weeks; manure patty samples were collected from pen scrapings within the same pen as water samples; feed was collected from piles of rolled steam flake corn (1 sample) and hay/silage mix (1 sample) in storage area.

Estimation of Bacterial Abundance

House flies were transported on ice to the lab, where individual flies were hand homogenized in 500 µl of sterile phosphate buffer saline (PBS; 1.37 M NaCl, 27 mM KCl, 101.4 mM NaH_2PO_4, 17.6 KH_2PO_4, pH 7.4; Fisher Scientific, Pittsburg, PA) with a sterile pestle. Another 500 µl of sterile PBS was then added to the samples and briefly vortexed. Using 100 µl of the homogenate, ten-fold serial dilutions were prepared in sterile PBS and 100 µl of the sample homogenate and each dilution spread-plated in two replicates onto MacConkey agar (MAC; Becton Dickinson and Company, Sparks, MD, USA). Media plates were incubated at 37°C for 18 h. After incubation, colony forming units (CFUs) were counted if in the countable range (30-300 CFUs).

Bacterial abundances were estimated for environmental substrates solely to select master plates for replica plating onto antibiotic-infused media and subsequent selection of AMR isolates (see below). Feed samples were thoroughly mixed before adding 5 g of feed to 20 ml of PBS and mixing via inversion for 30 s. For both manure patty and manure compost samples, 2.5 g was added to 22.5 ml of sterile PBS and mixed via inversion for 30 s. To perform serial dilutions of water samples, 100 µl was taken directly from the 50 ml sample after vortexing for 10 s. All environmental samples were then serially diluted ten-fold in sterile PBS and 100 µl of the homogenate and each dilution spread-plated in duplicate onto MAC. After incubating the plates

at 37°C for 18 h, CFUs were enumerated if in the countable range (30-300 CFUs). This process was performed twice for each sample and the CFUs/ml averaged for each sample.

AMR Isolates from Flies and Environment

After CFU enumeration, sample cultures were replica plated onto antibiotic-infused MAC agar to select for isolates of suspected antibiotic resistance. Culture plates from a single dilution which produced 30-150 CFUs were used as the master plate for replica plating. If no growth was observed for higher dilutions of the sample, plates with 1-29 CFUs for the lowest sample dilution were used as the master plate for replica plating. During replica plating, colonies from each master plate were imprinted onto a sterile velvet cloth square (2.54 cm x 2.54 cm) affixed to a replica-plating tool (VWR International B.V., Amsterdam, The Netherlands). Imprints of each master plate were then pressed in succession onto MAC agar plates infused with tetracycline (T, 16 µg/ml), florfenicol (F, 8 µg/ml) and enrofloxacin (E, 0.25 µg/ml), respectively, and all antibiotic-infused plates incubated for 18 h at 37°C. For each sample, all colonies with distinct morphotypes and which grew on all three antibiotic-infused plates (hereafter, "screened isolates" for suspected resistance to tetracycline, florfenicol, and enrofloxacin) were streaked for isolation onto MAC agar and incubated overnight at 37°C. An isolated colony was then inoculated into 5 ml tryptic soy broth (TSB; Becton Dickinson and Company, Sparks, MD, USA), grown at 37°C and 100 rpm for 18 h, and cryo-preserved at -80°C in 25% glycerol stocks for downstream analysis.

Screened isolates were identified via the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technique according to manufacturer guidelines at the Kansas State Veterinary Diagnostic Lab (Manhattan, KS, USA) facilities. In summary, screened isolate glycerol stocks were partially thawed before pipetting 10 µl onto MAC,

streaking for isolation, and incubating overnight at 37°C. Performed in duplicate for each isolate, a single colony was picked with a sterile toothpick and smeared directly onto a MALDI steel target plate spot. After air drying, targets were then transported for MALDI-TOF MS analysis, where 1 µl of Bacterial Test Standard (Bruker Scientific LLC, Billerica, MA, USA) was smeared in replicate onto the target plate and dried at room temperature. All target spots were then overlayed with 1 µl HCCA matrix solution (Bruker Scientific LLC, Billerica, MA, USA) prepared with standard solvent (acetonitrile 50%, water 47.5% and trifluoroacetic acid 2.5%; Sigma-Aldrich, Saint Louis, MO, USA) and dried at room temperature before inserting into the Bruker MALDI-TOF Biotyper (Bruker Daltonics GmbH & Co. KG, Bremen, Germany) system. Using the MBT Compass Explorer interface (v.4.1.100), all measured mass spectra data were compared with reference patterns of the Bruker BDAL library. Log score values between 0 and 1.69 were considered as nonreliable identities and screened isolates considered as "unknown". Genus-level assignment was given to isolates if scores ranged from 1.70 to 1.99 (probable genus identification) or 2.00 to 2.29 (reliable genus identification and probable species identification). Finally, species-level assignment was recorded for isolates with a score between 2.30 and 3.00 (highly probable species identification).

Using the Kirby Bauer Disk Diffusion Method (Hudzicki 2009), antimicrobial susceptibility testing was performed for each isolate to confirm resistance to tetracycline (T), florfenicol (F), and enrofloxacin (E), and assess susceptibility to ampicillin (A) and ceftiofur (C). Isolated colonies from overnight cultures on TSA prepared for the MALDI-TOF analysis (see above) were mixed thoroughly into sterile water until the inoculum turbidity matched a 0.5 Remel^{TM} McFarland Equivalence Turbidity Standard (Remel, Lenexa, KS, United States). Inoculums were then spread onto Mueller Hinton (MH; Remel, Lenexa, KS) agar and a disk for

each antibiotic was added: 16 µg tetracycline (Becton Dickinson and Company, Sparks, MD), 30 µg florfenicol (Nufluor; VetLab, Palmetto Bay, FL, USA), 5 µg enrofloxacin (Baytril; VetLab, Palmetto Bay, FL, USA), 10 µg ampicillin (VetLab, Palmetto Bay, FL, USA), and 30 µg ceftiofur (Naxcel; VetLab, Palmetto Bay, FL, USA). After incubating plates at 37°C for 18 h, the zones of inhibition for each antibiotic were measured using a BIOMIC V3 (Giles Scientific, Santa Barbara, CA, United States). Zone diameters were interpreted for susceptibility according to the 2020 Clinical and Laboratory Standards Institute (CLSI) VET01S breakpoints correlating with the isolate species or order classification assigned by MALDI-TOF MS analysis (i.e. *Klebsiella pneumoniae*, *Escherichia coli*). Isolates that did not belong to a bacterial species or order with standardized CLSI breakpoints for a given antibiotic were interpreted using the same breakpoints for Enterobacterales. Interpretation of zone diameters for enrofloxacin susceptibility was the only exception, as the CLSI zone diameter breakpoints for enrofloxacin susceptibility of *Pseudomonas aeruginosa* were used for all isolates belonging to the order Pseudomonadales.

In downstream analysis, we investigated whether isolates had resistance to a given antibiotic, regardless of co-resistance to other antibiotics (i.e, “any-A” refers to an isolate that had resistance to ampicillin). To evaluate the combined AMR phenotypes observed for an isolate, we created “AMR profiles,” which use concise letter codes to distinguish whether an isolate was resistant to a single (i.e., “A” refers to an isolate had resistance only to ampicillin) or multiple antibiotics (i.e., “AEF” describes an isolate had resistance to ampicillin, enrofloxacin, and florfenicol).

Statistical Analysis

All statistical methods were performed using JMP Pro Statistical Software (JMP®, Version 17.0, SAS Institute Inc., Cary, NC, USA). Average CFUs/fly for 315 house flies were

estimated from CFU enumeration of plated sample dilutions as described above and log10 transformed before analysis. If neither plated replicate for a sample yielded CFUs within the countable range, adjustments were made for the following conditions. Samples with dilutions which jumped from 'too numerous to count' (TNTC; >300 CFUs) on highest plated dilution down to 'too few to count' (TFTC; < 30 CFU) on the lowest usable dilution were assigned 301 CFUs for the highest dilution with TNTC of CFUs. If samples had a dilution with TFTC went down to a dilution with no growth, a value of 29 CFUs was assigned to the highest dilution with TFTC. Zero CFUs were assigned only if no growth was observed on all plated dilutions. Once log transformed, bacterial abundances in flies were analyzed using the linear regression and ANOVA approaches within the Standard Least Squares Personality of Fit Model to investigate the effects of fly sex, collection events, location, and their interactions. Pairwise *post hoc* comparisons of least square means estimates using the Tukey honestly significant difference (HSD) test were performed to assess differences between fly sex across locations and differences between fly sex across collection events.

Results

Factors Associated with Bacterial Abundances in House Flies

Three hundred and fifteen house flies (n = 155 females and n = 160 males) were collected, with bacterial abundances ranging from 0 to 3.00×10^6 CFUs per fly. No culturable bacteria were recovered from 41 house flies, of which 65.9% (n = 27) were males observed evenly across collection sites and dates. Overall, female house flies carried significantly greater mean bacterial abundances ($7.32 \pm 2.87 \times 10^5$ CFUs/fly; F = 44.4069, df = 1, $p < 0.0001$) than male flies ($3.92 \pm 1.91 \times 10^4$ CFUs/fly). Similarly, female flies were observed to carry significantly greater mean bacterial abundances than male flies for most collection events

(Collection 1: t = 1.318, df = 0.340, p = 0.0032; Collection 2: t = 1.092, df = 0.335, p = 0.0272; Collection 4: t = 1.397, df = 0.342, p = 0.0015), but the interaction of fly sex and collection event was not statistically significant (F = 0.5709, df = 3, p = 0.6346). Additionally, mean bacterial abundances from female flies were greater than male flies within all locations, but only significantly greater for Lyon (t = 1.236, df = 0.342, p = 0.0086) and Riley (t = 1.417, df = 0.337, p = 0.0009) flies (Table 3.1). Collection event (F = 3.7964, df = 3, p = 0.0108) and location (F = 4.3505, df = 3, p = 0.0051) had a significant overall effect on mean bacterial abundances within flies, as well as the interaction of collection event and location (F = 3.5867, df = 9, p = 0.0003; Figure 3.1). Trends in mean bacterial abundances from flies within a location between collection events were variable between sites, with Marion flies typically carrying greater mean abundances than other locations. The interaction of fly sex, collection event, and location did not have a significant overall effect (F = 0.5621, df = 9, p = 0.8277). Notably, trends in mean bacterial abundances observed for each environmental sample type at each location did not appear to reflect the overall location effect observed for mean abundance in house flies (Appendix Figure B.1).

Cross-Location Comparisons of AMR Bacteria in House Flies

Cultures from a total of 237 house flies (Lyon: n = 62 flies; Marion: n = 53 flies; Riley: n = 65 flies; Washington: n = 57 flies) underwent replica plating onto antibiotic-infused MAC agar to select "screened isolates," or bacterial isolates from each fly sample with distinct morphotypes and suspected MDR. After replica plating, 388 screened isolates of distinct morphotypes from each sample were subcultured from approximately 70% of flies (n = 165/237) from all collection sites and weeks, with a range of 1 to 10 screened isolates selected per fly. Screened isolates were obtained from only 2 flies for the Marion site during Collection 3 due to sample losses in a

laboratory accident. Additionally, sampling bias during the selection of bacterial colonies, as well as the inaccuracy of using antibiotic-infused media for predicting AMR, resulted in the inability to measure the prevalence of flies carrying MDR bacteria throughout the study. Of the 165 flies from which screened isolates were selected, disk susceptibility testing indicated that 97.0% (n = 160) of house flies carried at least one AMR isolate and 74.5% (n = 123) flies carried at least one MDR isolate. The location with the highest proportion of flies carrying MDR isolates was Lyon (94.7%; n = 36/38), with as many as 8 MDR isolates cultured from a single fly, followed by Riley (78.8%; n = 41/52), Washington (62.2%; n = 28/45), and Marion (60.0%; n = 18/30).

Despite observable growth for all 388 screened isolates on tetracycline- (T), florfenicol- (F), and enrofloxacin- (E) infused MAC, only 5.2% (n = 20) of the screened isolates from flies were interpreted as triple-resistant to all three antibiotics after disk susceptibility testing. Nonetheless, 93.3% (n = 362/388) of isolates from house flies were AMR and 58.2% (n = 226/388) were MDR to the five antibiotics evaluated. The cumulative number of antibiotics to which bacterial isolates from flies were resistant varied between locations (Figure 3.2). Most isolates from Riley (62.1%; n = 64/103) and Lyon (84.4%; n = 92/109) flies were identified as MDR, whereas fewer than half of the isolates from flies at Washington (38.5%; n = 45/117) and Marion (42.4%; n = 25/59) were MDR bacteria. Larger proportions of isolates from Marion flies were resistant to four antibiotics (8.5%; n = 5/59) than isolates from Riley (0%; n = 0/103) or Washington (0.9%; n = 1/117) flies, but Marion isolates were also the largest proportion of isolates with no resistance to antibiotics (16.9%; n =10/59). In contrast, the largest proportion of isolates with resistance to four (19.3%; n = 21/109) and five (4.6%; n = 5/109) antibiotics were carried by Lyon flies, as well as the lowest proportion of isolates with no resistance (0.9%; n =

1/109). Notably, resistance to all five antibiotics was only observed in isolates from four Lyon flies.

Any resistance to ampicillin (any-A), tetracycline (any-T), florfenicol (any-F) and ceftiofur (any-C), which solely evaluates an isolate's phenotype for the indicated antibiotic (see Methods for details), was observed in isolates from flies collected at all locations. However, fly isolates with any resistance to enrofloxacin (any-E) were recorded for all sites except Riley. When evaluating resistance to each antibiotic among isolates, 71.9% of AMR isolates had any-A resistance (*n* = 279/388), 63.9% any-T resistance (*n* = 248/388), 26.5% any-F resistance (*n* =103/388), 12.6% any-C resistance (*n* = 49/388), and 6.7% any-E resistance (*n* = 26/388). Twenty total AMR profiles were observed, of which seven profiles were shared in fly isolates from all locations (A, T, AC, AT, AF, TF, ATF; Figure 3.3). The widest variety of AMR profiles was observed in bacteria isolated from Lyon flies (*n* = 17), followed by Marion (*n* = 10) and finally Riley (*n* = 9) and Washington (*n* = 9) flies. Unique AMR profiles were observed for fly isolates from Lyon (F, TE, TEF, ATEFC), Riley (AFC), and Washington (ATE) locations, but not Marion. Overall, AT was the most frequently observed AMR profile in isolates from house flies (27.6%; *n* = 107/388), followed by A (21.1%; *n* = 82/388), T (15.7%; *n* = 61/388), and ATF (8.8%; *n* = 34/388). A, T, and AT represented the greatest proportion of AMR profiles seen in isolates from flies collected at Marion (55.8%; *n* = 33/59), Riley (64.1%; *n* = 66/103), and Washington (81.2%; *n* = 95/117), whereas A, AT, and ATF were the greatest proportion of AMR profiles observed in isolates from Lyon flies (47.7%; *n* = 52/109).

Common Taxa of AMR Bacteria in House Flies

Forty different taxa were assigned to the 362 AMR isolates from flies, of which over 75% belonged to only 8 taxa: *Escherichia coli* (25.4%; *n* = 92/362), *Klebsiella pneumoniae* (15.7%; *n*

= 57/362), *Enterobacter cloacae* (8.3%; *n* = 30/362), *Providencia stuartii* (7.2%; *n* = 26/362), *Providencia* (*Proteus*) *rettgeri* (6.9%; *n* = 25/362), *Proteus mirabilis* (6.4%; *n* = 23/362), *Ochrobactrum intermedium* (4.7%; *n* = 17/362), and *Pseudomonas sp.* (3.3%; *n* = 12/362). These taxa comprised most of the AMR isolates from flies within each location as well, but variation in the number of isolates belonging to each taxon was observed due to either location or antibiotic resistance (Figure 3.4). For example, the most common taxonomic assignment of isolates from flies with any-A resistance from flies collected at Lyon (*n* = 23/89) and Marion (*n* = 10/34) was *E. coli* but, for Washington and Riley, was *K. pneumoniae* (*n* = 24/76) and *P. rettgeri* (*n* = 13/80), respectively. *Escherichia coli* was again the most common taxon of fly isolates with any-C resistance from Lyon (*n* = 11/26) and Marion (*n* = 3/6) flies, whereas more fly isolates with any-C resistance from Riley (*n* = 5/12) and Washington (*n* = 2/5) were classified as *O. intermedium*. Across all locations, the widest variety of bacterial taxa from flies were any-A resistant (Lyon: *n* = 22; Marion: *n* = 11; Riley: *n* = 21; Washington: *n* = 16), and the lowest variety of bacterial taxa were any-E resistanct (Lyon: *n* = 6; Marion: *n* = 1; Riley: *n* = 0; Washington: *n* = 4). Of the 99 recorded combinations of taxa with any-A, any-T, any-E, any-F, or any-C resistance (i.e., *Achromobacter mucicolens* with any-A resistance), 66.7% (*n* = 66) were observed in isolates from flies collected at two or more different locations. Seventy-eight different combinations of taxa paired with AMR profiles were found in isolates from house flies, with the greatest number of unique combinations in isolates from flies at Lyon (*n* = 38), followed by Riley (*n* = 17), Washington (*n* = 7), and Marion (*n* = 4).

Thirty-two different taxa were identified from the 226 MDR isolates from flies, with roughly 75% belonging to six major taxa: *E. coli* (25.7%; *n* = 58/226), *K. pneumoniae* (14.2%; *n* = 32/226), *P. rettgeri* (12.8%; *n* = 29/226), *P. stuartii* (11.5%; *n* = 26/226), *Pseudomonas sp.*

(5.3%; *n* = 12/26), and *O. intermedium* (5.3%; *n* = 12/226). Pairings of taxonomic classifications with AMR profiles measured for MDR fly isolates varied between locations (Table 3.2). Fifty-eight combinations of taxa and AMR profiles were found for MDR house fly isolates, of which 70.0% (*n* = 40) were unique to flies within a single location, largely represented by Lyon flies (57.35%; *n* = 23/40). AT-resistant *P. rettgeri* (*Proteus*) represented the greatest proportion of MDR isolates from Marion (16.0%; *n* = 4/25) and Riley (20.3%; *n* = 13/64) flies, in contrast to AT-resistant *K. pneumoniae* from Lyon flies (14.1%; *n* = 13/92) and AT-resistant *P. stuartii* from Washington flies (24.4%; *n* = 11/45). Only four taxonomic assignment/AMR profile combinations were shared by MDR isolates from flies at all locations: *E. coli* with TF or ATF resistance profiles, ATF-resistant *K. pneumoniae*, and AT-resistant *P. rettgeri.* Of the MDR isolates from flies, the largest variety of AMR profiles was found in *E. coli* isolates from Lyon (*n* = 7), Marion (*n* = 5), and Washington flies (*n* = 3), and in *Pseudomonas sp.* isolates from Riley flies (*n* = 3). No cases were observed where all AMR profiles observed for a single taxon were shared amongst MDR isolates from flies at all locations. For example, MDR *E. coli* isolates from flies at all locations had eight different resistance profiles overall (AT, TF, TE, ATF, TEF, ATFC, ATEF, ATEFC), but only AT and ATF resistance profiles were shared amongst MDR fly isolates from all locations (Appendix Figure B.2). Additionally, *E. coli* was the only taxon of fly isolates with the ATEFC resistance profile, of which all isolates were from flies collected at Lyon.

Comparison of AMR Bacteria from House Flies to Environmental Samples

A total of 341 screened isolates (manure compost: *n* = 39; manure patty: *n* = 86; feed: *n* = 149; water: *n* = 67) were selected from 63.6% (*n* = 14/22) of manure compost samples, 64.5% (*n* = 20/31) of manure patty samples, 92.3% (*n* = 24/26) of feed samples, and 78.6% (*n* = 22/28) of

water samples that were replica plated onto antibiotic-infused media. One or more AMR isolates were cultured from 100% of manure compost, manure patty, feed, and water samples, whereas one or more MDR isolates were cultured from more than 70% of manure compost samples (71.4%; *n* = 10/14), manure patty samples (75.0%; *n* = 15/20), feed samples (87.5% *n* = 21/24), and water samples (77.3%; *n* = 17/22). Overall, 302 (88.6%) environmental isolates were AMR, and 170 (49.9%) were MDR. The cumulative number of antibiotics to which isolates from an environmental substrate were phenotypically resistant varied between locations and sample types (Figure 3.5). Similar to what was observed for isolates cultured from house flies, Lyon had the greatest proportion of MDR isolates from environmental substrates, excluding manure compost (manure patty = 100%; feed = 71.1%; water = 82.4%). Additionally, isolates from Lyon water samples were the only isolates from environmental substrates resistant to all five antibiotics.

Any-A and any-T resistance was observed in all environmental sample types within locations, but some sample types did not have any-E, any-F, or any-C resistance (Figure 3.6). Unlike house fly isolates, AMR isolates from Marion manure compost did not have any-E- or any-F resistance, while AMR isolates from Washington manure compost, manure patty, or water samples also lacked any-E (manure compost and water), any-C (manure compost and manure patty), or any-F (water) resistance. As observed in Riley house flies, isolates from all environmental sample types lacked any-E resistance. Additionally, Riley water samples lacked isolates with any-F resistance. Similar to observations for isolates from house flies, the greatest proportion of AMR isolates from manure compost (*n* = 30/61), feed (*n* = 100/232), and water (*n* = 50/120) had any-A resistance, whereas any-T resistance was most common for AMR isolates from manure patties (*n* = 62/145). The total taxonomic classifications of AMR isolates from feed (*n* = 27), water (*n* = 21), manure compost (*n* = 14), and manure patties (*n* = 9) varied between

different locations and sample types (Figure 3.6). For example, AMR isolates from Lyon manure patties were solely identified as *E. coli*, whereas AMR isolates from Marion, Washington, and Riley manure patties belonged to two (*E. coli* and *Pseudomonas putida*), three (*E. coli*, *Escherichia sp., Klebsiella oxytoca*), or seven different taxa (*E. cloacae, Enterobacter sp., E. coli, Escherichia sp., K. pneumoniae, O. intermedium, Pseudomonas fulva*), respectively. The largest proportion of AMR isolates overall from manure patties (78.3%; n = 47/60) and water (26.8%; n = 15/56) were *E. coli*, in contrast to *K. pneumoniae* (19.8%; n = 25/126) being predominant from feed and *O. intermedium* (27.8%; n = 10/36) from manure compost. The eight major taxa of AMR isolates observed in AMR house fly isolates were also recovered in one or more environmental samples, although proportions were variable between locations and sample types (Figure 3.6). Manure compost was the only environmental substrate from which AMR isolates belonging to all the major taxa observed in house flies were recovered. In contrast, six of the eight major taxa from house fly isolates were observed in feed and water isolates (*E. coli*, *K. pneumoniae*, *E. cloacae*, *P. stuartii*, *P. mirabilis*, *O. intermedium*), and four of the major taxa were observed in manure patty isolates (*E. coli*, *K. pneumoniae*, *E. cloacae*, *O. intermedium*).

Overall, 19 different AMR profiles of bacterial isolates were observed for manure compost, manure patty, feed and water samples. Within a location, house fly isolates were observed to share most AMR profiles observed in isolates from environmental samples (Lyon: n = 12/13; Marion: n = 10/14; Riley: n = 9/12; Washington: n = 6/8). For samples collected from Lyon and Washington, isolates from house flies carried additional AMR profiles not observed in environmental substrates from the same location (Lyon: AF, TE, AEF, TEF, AEFC; Washington: AC, ATF, AEFC). Marion was the only location where no AMR profiles were shared amongst isolates from all sample types. In contrast, all Lyon samples had isolates with TF, ATF, ATFC,

and ATEF resistance profiles, all Riley samples had isolates with A, T, and AT resistance profiles, and all Washington samples had isolates with T and AT resistance profiles. TEF and AEF were the only AMR profiles unique to house fly isolates and were cultured from only Lyon house flies.

Isolates from house flies shared 52.4% (n = 43) of the 82 total unique combinations of taxa and AMR profiles observed for isolates from environmental samples, although these isolates were not consistently recovered across locations and collection dates (Appendix Table B.1-B.4). Within Lyon, Marion, Riley, and Washington, fly isolates shared 51.6% (n = 16/31) , 37.1% (n = 13/35), 50.0% (n = 16/32), and 62.5% (n = 15/24) of each taxa/AMR profile combination observed for isolates from collected environmental substrates, respectively (Figure 3.7). *E. cloacae* with A resistance, TF-resistant *E. coli*, and *K. pneumoniae* with A and AT resistance were the only taxa/AMR profile combinations shared between flies and environmental samples at all locations. Overall, *E. coli* isolates had the widest variety of AMR profiles for isolates observed in both flies and environmental samples (T, TF, AT, ATF, ATEF, ATFC), followed by *K. pneumoniae* (A, AF, AT, ATF). ATEFC resistance was only observed in Lyon bacterial isolates: four *E. coli* isolates from flies and one *Proteus hauseri* isolate from water.

Discussion

This study investigated the effects of fly sex, location, and date of collection event on estimated abundances of culturable enteric bacteria carried by house flies at confined beef cattle operations. Further evaluation determined which AMR enterica bacteria were shared between house flies and environmental samples (manure patties, manure compost, water, and feed) to infer associations with flies . As observed in previous studies (Neupane et al. 2020, Pickens et al. 2025), female house flies carried significantly greater overall bacterial abundances than male

flies in confined cattle operations. Within each location bacterial abundances also were greater in females compared to males in this study, although not statistically significant for flies collected from Marion or Washington counties. No significant interactions were found between fly sex and collection event, fly sex and location, or all three factors combined, suggesting that sex-based differences in bacterial carriage were generally consistent across time and among cattle operations. We observed the same trend for the differences in bacterial abundance across fly sex among most collection events and locations, however this finding contrasts with prior studies that reported significant interactions between fly sex and variables such as location, county, and/or collection date (Neupane et al. 2020, Pickens et al. 2025). This discrepancy may be due in part to the differences in culture media used in this study compared to the prior studies (e.g., violet red bile agar vs. MAC), as Pickens et al. (2025) noted that the factors influencing overall bacteria CFUs cultured from flies differed from factors affecting suspected coliform CFUs, and differences between culture media influenced the bacterial species recovered from samples. Neupane et al. (2020) provided evidence that larger-sized house flies (more commonly observed for female flies) does not correlate with higher mean abundances of culturable bacteria. Instead, female house flies are believed to carry greater bacterial loads because of their frequent exposure to microbe-rich substrates while seeking out potential oviposition sites (Shah et al. 2016, Thomson et al. 2017), as well as feeding preferences that help them acquire the essential nutrients required for proper egg development (Glaser 1923, Neupane et al. 2023).

Collection event, location, and their interaction significantly influenced the mean abundance of culturable enteric bacteria carried by house flies. Interestingly, temporal trends of bacterial loads in house flies varied among cattle operations, suggesting site-specific factors played a strong role. Given how house flies acquire bacteria from their environment and

considering that all operations were located within similar climate zones, we suspected that facilities with similar management practices, particularly the management of environmental substrates, would produce flies with similar bacterial loads. Marion and Washington were the most alike in terms of feed mixture, cattle density, manure management, and infrastructure. However, while the mean abundance of bacteria cultured from flies at Marion stayed consistent across collection events, Washington flies had substantial fluctuations in mean bacteria abundances measured between collection events. Additionally, there was no clear overall trend in mean bacterial abundance carried by house flies from our collection sites to suggest climate as a significant contributor as previously predicted (Pickens et al. 2025). We therefore believe other environmental or management factors, like fly population densities and the influx or efflux of cattle, could contribute to the observed site-specific variance. Notably, collection events 3 and 4 at the Riley cattle operation occurred after the feedyard was emptied (cattle were removed), yet flies did not have lower bacterial abundances than flies collected at earlier events. The abundance of bacteria flies would have carried if the cattle were not removed remains unknown. The abundance of culturable enteric bacteria in house flies may have been affected by differences in bacterial composition or availability of one or more of these substrates (i.e., sources of microbes), but our sampling of environmental substrates (manure compost, manure patties, feed, and water) was insufficient to assess trends in bacterial abundances across locations and collection events within these resources.

Our results provide evidence that house flies can be used to reveal site-specific patterns of AMR phenotypes observed for environmental enteric bacteria in confined beef cattle operations. Lyon flies carried a substantially larger proportion of MDR bacteria than flies from any other site, particularly those with resistance to three or four antibiotics. This was also the

only site with isolates resistant to all five antibiotics (see Figure 3.2). We speculate that this phenomenon is related to management and operation practices at this location, which tends to house high-risk calves that often have diseases that may require extensive antibiotic usage, although those records were not available for this study. Larger proportions of MDR bacteria were also observed in environmental sample types collected from Lyon, but the proportion of MDR bacteria observed in house flies did not always match with each environmental sample type collected from other operations (see Figure 3.5). Additionally, resistance phenotypes and AMR profiles of isolates from different sample types did not seem to depend on the sample type, but rather the location from which they originated. For example, only 7 of the 20 total AMR profiles observed in isolates from house flies were found in isolates from flies at all locations. A challenging aspect of AMR surveillance is that resources are limited, and it is difficult to determine which sources should be sampled for AMR surveillance in food production systems and the environment. We argue that house flies, although not entirely perfect representatives of the environment, may serve as aggregate sampling tools that provide a more efficient and comprehensive survey of AMR within confined cattle operations than manure patties, manure compost, feed, or water alone. Frequent interactions of adult house flies with diverse environmental sources harboring AMR bacteria have led to the use of these organisms as sentinels for the surveillance of AMR bacteria in urban and agricultural settings, including confined cattle operations. Future research that incorporates pooled fly samples for subsequent culture would aid in the assessment of environmental risks for AMR, monitor the threat house flies pose for the development and transmission of AMR, and identify higher risk locations where further AMR mitigation strategies should be implemented.

To the best of our knowledge, this is the first study to attempt comparisons of AMR bacteria in house flies versus a variety of environmental substrates at confined beef cattle operations. We selectively cultured AMR bacteria from 97% (n = 160/165) of house flies and 100% of manure compost, manure patty, feed, and water samples. Additionally, MDR bacteria were recovered from at least 70% of all sample types. While resistance phenotypes of isolates from environmental samples varied by sample types within a location, isolates from house flies represented most of the resistance phenotypes to each antibiotic observed for AMR bacteria in environmental isolates from the same site. Isolates from flies shared 52% of the 82 different combinations of AMR profiles and bacterial taxa in environmental samples, and more notably shared at least 70% of the AMR profiles observed overall for isolates from environmental samples at each location. Greater proportions of AMR profiles and taxa shared between isolates from flies and environmental substrates observed within locations, suggesting site specificity of AMR profiles of bacterial taxa cultured from flies and environmental substrates. Furthermore, a lower number of AMR profiles/taxa combinations are shared amongst isolates from the environmental samples alone versus the environment and flies, bolstering the utility of house flies as aggregate sampling tools for AMR surveillance in confined cattle operations (see Figure 3.7).

There are several caveats and limitations associated with our study design and approach. Due to our low sampling of environmental substrates and difficulty with the replica plating method for resistance prediction, it is possible that, 1) we missed recovering isolates from environmental samples that would have shared phenotypes or taxa observed in fly isolates, and/or 2) we did not culture other AMR phenotypes or taxa which may not be present in house flies. Additionally, the use of MAC agar as our selective media for culture eliminated the

evaluation of bacteria, particularly Gram-positive strains, that could have been evaluated across the samples, and likely also are reservoirs for AMR. Nonetheless, our study successfully demonstrated that culturable AMR bacteria from house flies overlap in both the taxa and resistance phenotypes present in environmental reservoirs of confined cattle operations.

This study demonstrates the potential of house flies as a bridge for AMR bacteria between environmental sources at beef cattle operations, as well as their potential to serve as sentinels for AMR surveillance in confined cattle operations. Fly sex, location, and collection event significantly affected the bacterial load carried by house flies, suggesting these variables may alter bacterial transmission dynamics. Additionally, AMR phenotypes and bacterial taxa observed in house flies and environmental substrates varied by location, identifying site-specific trends that may guide targeted AMR mitigation efforts. Whole genome sequencing of the AMR *E. coli* isolates identified in this study is underway and will more definitively help in assessing their genetic relatedness among samples while also determining genomic elements contributing to their observed phenotypic resistance.

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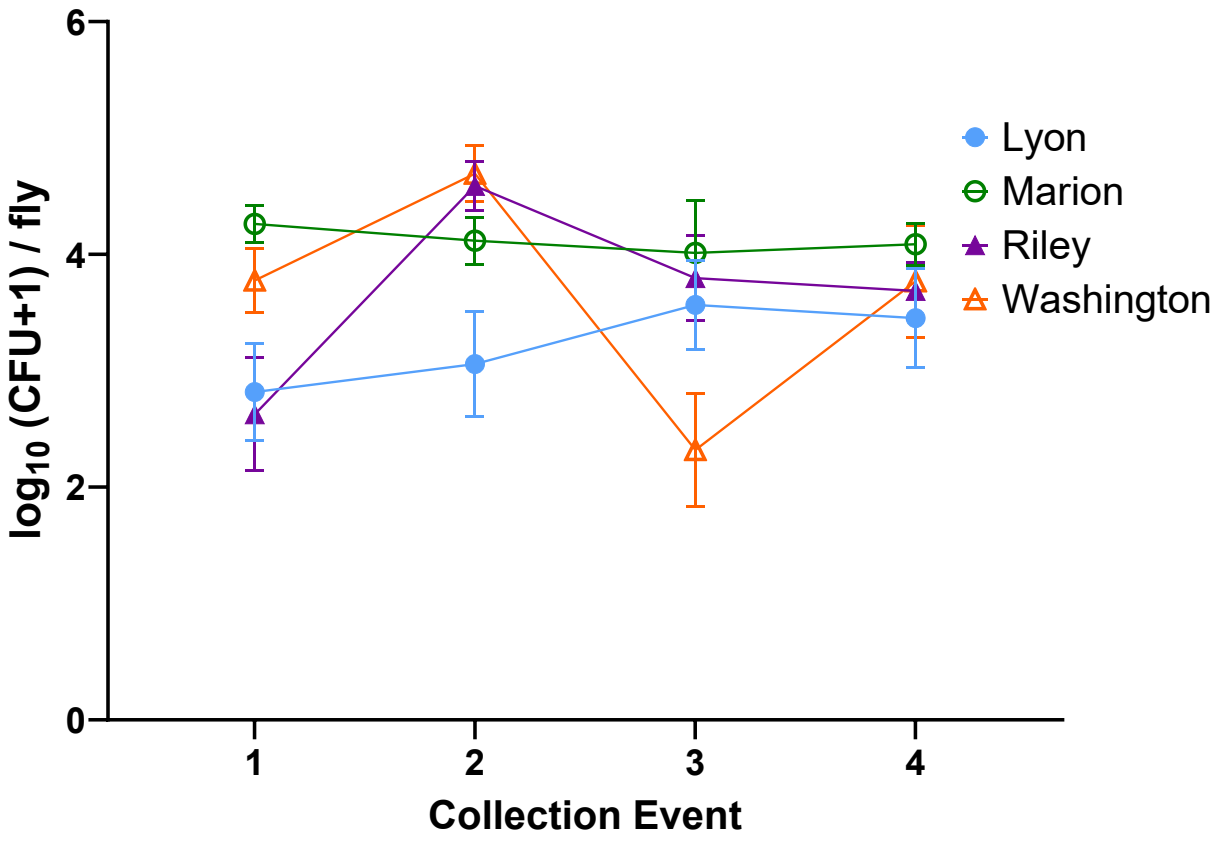


Figure 3.1. Bacterial abundances in house flies across four collection events at four locations

Bacteria were cultured from house fly homogenate onto MacConkey agar, and abundances (mean + SEM) measured in house flies from each location over collection events are shown. Collection 1: August 4th & 6th; Collection 2: August 18th & 20th ; Collection 3: September 15th & 17th; Collection 4: September 29th & October 1st. Washington and Riley samples were collected Aug. 4th, Aug. 18th, Sep. 15th, and Sep. 29th. Lyon and Marion samples were collected Aug. 6th, Aug. 20th, Sep. 17th, and Oct. 1st.

Table 3.1. Mean culturable bacteria abundance by fly sex and location

Mean abundances of bacteria cultured on MacConkey agar from male and female flies collected at each location.

Location	Sex	N	Mean $\log_{10}$(CFU+1) ± SE/fly
Lyon	F	28	3.87 ± 0.25^{A}
	M	30	2.64 ± 0.24^{D}
Marion	F	29	4.54 ± 0.24^{A}
	M	29	3.70 ± 0.24^{ABC}
Riley	F	30	4.40 ± 0.13^{A}
	M	30	2.98 ± 0.24^{CD}
Washington	F	29	4.16 ± 0.24^{AB}
	M	29	3.14 ± 0.24^{BCD}

CFU = colony forming units.

*Differing letters indicate significant differences in pairwise comparisons of least square means (Tukey HSD, $p < 0.05$).

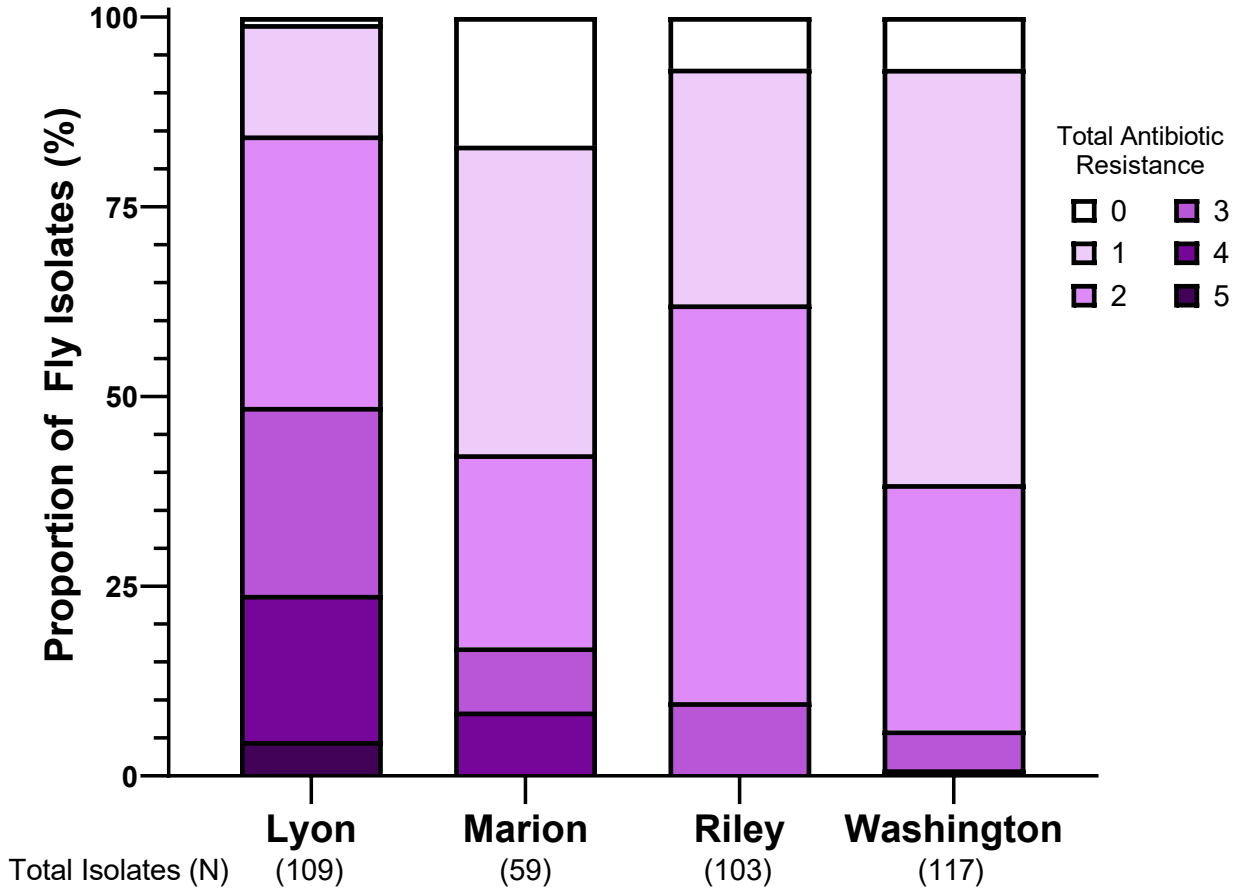


Figure 3.2. Antibiotic resistance profiles of bacterial isolates from house flies by location of beef cattle operation

Proportions (%) of bacterial isolates from flies at each location with phenotypic resistance to different totals of antibiotics (0-5 of a total of 5 antibiotics tested). The number of screened isolates from flies at each location tested for susceptibility (N) are in parentheses below the county names.

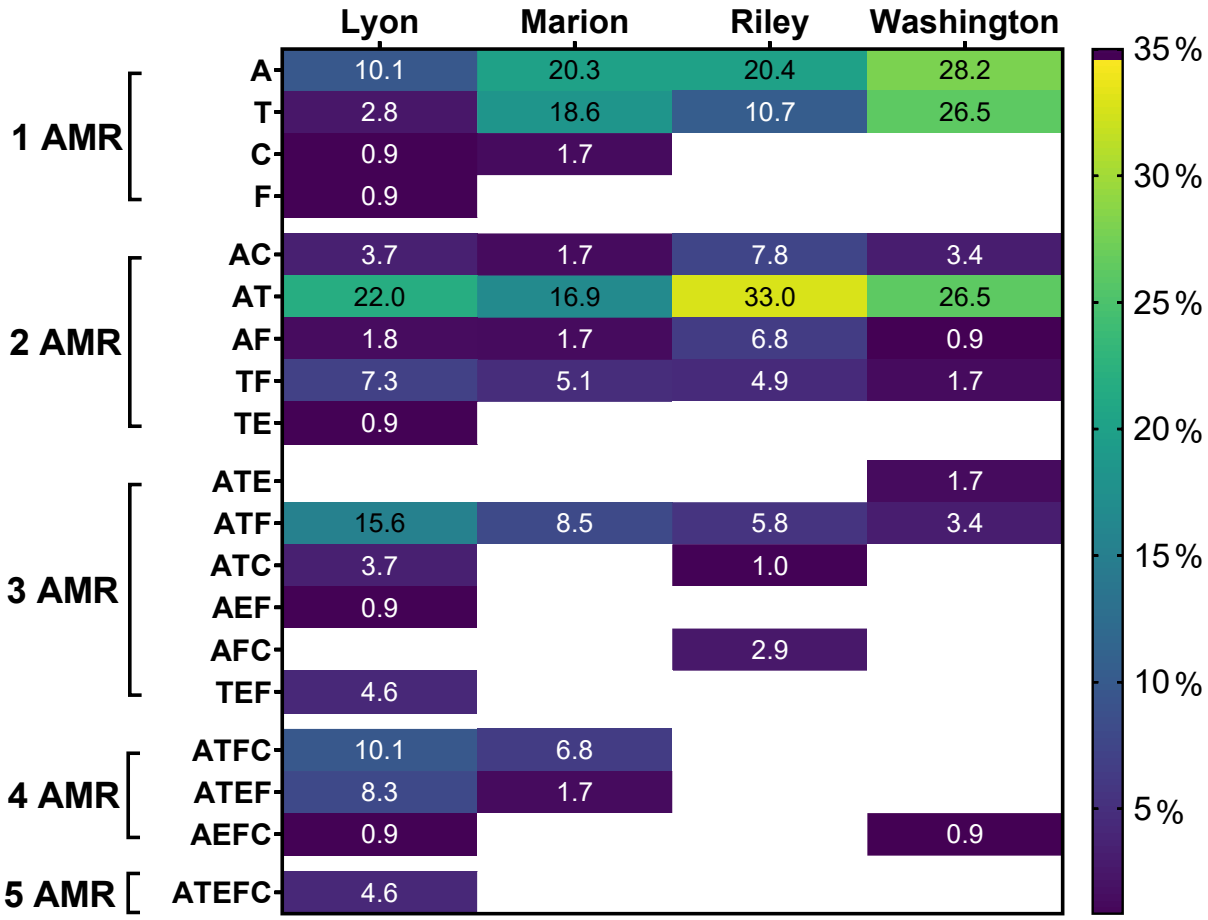


Figure 3.3. AMR profiles of fly isolates by location

Proportions (%) of AMR isolates from flies at Lyon (N = 109), Marion (N = 59), Riley (N = 103), and Washington (N = 117) beef cattle operations with observed AMR and MDR profiles. A= ampicillin, T= tetracycline, E= enrofloxacin, F= florfenicol, C= ceftiofur.

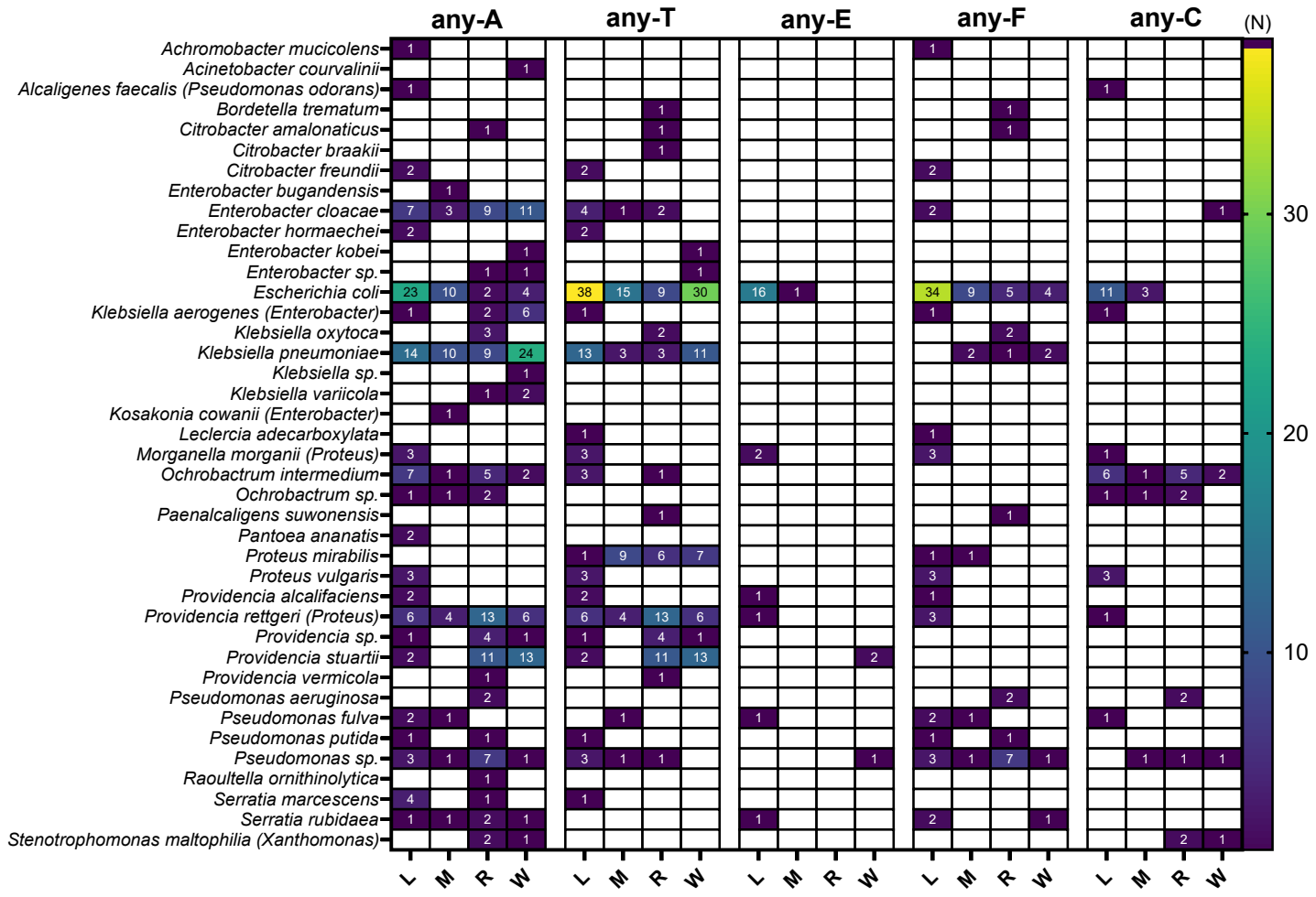


Figure 3.4. Taxonomic assignments of AMR bacteria cultured from house flies by location and antibiotic

Total isolates (N) from flies collected at Lyon (L), Marion (M), Riley (R), and Washington (W) locations assigned to bacterial taxa and found phenotypically resistant to ampicillin (A), tetracycline (T), enrofloxacin (E), florfenicol (F), or ceftiofur (C) are shown. "Any-X" refers to isolates that had resistance to the indicated antibiotic, regardless of other observed phenotypes for the isolate (see Methods for details).

Table 3.2. Taxonomic assignments of MDR bacteria cultured from house flies by location and resistance profile

Numbers of multidrug-resistant (MDR) isolates from flies collected at each location (Lyon, Marion, Riley, Washington) assigned to bacterial taxa and AMR profiles.

Taxa	AMR Profile	Lyon	Marion	Riley	Washington
Achromobacter mucicolens	AF	1			
Alcaligenes faecalis (Pseudomonas odorans)	AC	1			
Bordetella trematum	TF			1	
Citrobacter amalonaticus	ATF			1	
Citrobacter freundii	ATF	2			
Enterobacter cloacae	AC				1
	AT	2	1	2	
	ATF	2			
Enterobacter hormaechei	AT	2			
Enterobacter kobei	AT				1
Enterobacter sp.	AT				1
Escherichia coli	AT		3		2
	TF	6	2	3	2
	TE	1			
	ATF	7	3	2	2
	TEF	5			
	ATEF	5	1		
	ATFC	6	3		
	ATEFC	5			
Klebsiella aerogenes (Enterobacter)	ATFC	1			
Klebsiella oxytoca	ATF			2	
Klebsiella pneumoniae	AF		1	1	
	AT	13	2	3	9
	ATF		1		2
Leclercia adecarboxylata	TF	1			
Morganella morganii (Proteus)	ATFC	1			
	ATEF	2			
Ochrobactrum intermedium	AC	2		4	2
	ATC	3		1	
Ochrobactrum sp.	AC	1	1	2	
Paenalcaligens suwonensis	TF			1	
Proteus mirabilis	TF	1	1		
Proteus vulgaris	ATFC	3			
Providencia alcalifaciens	AT	1			

	ATEF	1			
Providencia rettgeri (Proteus)	**AT**	2	4	13	6
	ATF	2			
	ATC	1			
	ATEF	1			
Providencia sp.	**AT**	1		4	1
Providencia stuartii	**AT**	2		11	11
	ATE				2
Providencia vermicola	**AT**			1	
Pseudomonas aeruginosa	**AFC**			2	
Pseudomonas fulva	**AF**	1			
	ATF		1		
	AEFC	1			
Pseudomonas putida	**AF**			1	
	ATF	1			
Pseudomonas sp.	**AF**			5	
	AFC			1	
	ATF	3		1	
	AEFC				1
	ATFC		1		
Serratia marcescens	**AT**	1			
Serratia rubidaea	**AF**				1
	AEF	1			
Stenotrophomonas maltophilia (Xanthomonas)	**AC**			2	1

A= ampicillin, T= tetracycline, E= enrofloxacin, F= florfenicol, C= ceftiofur

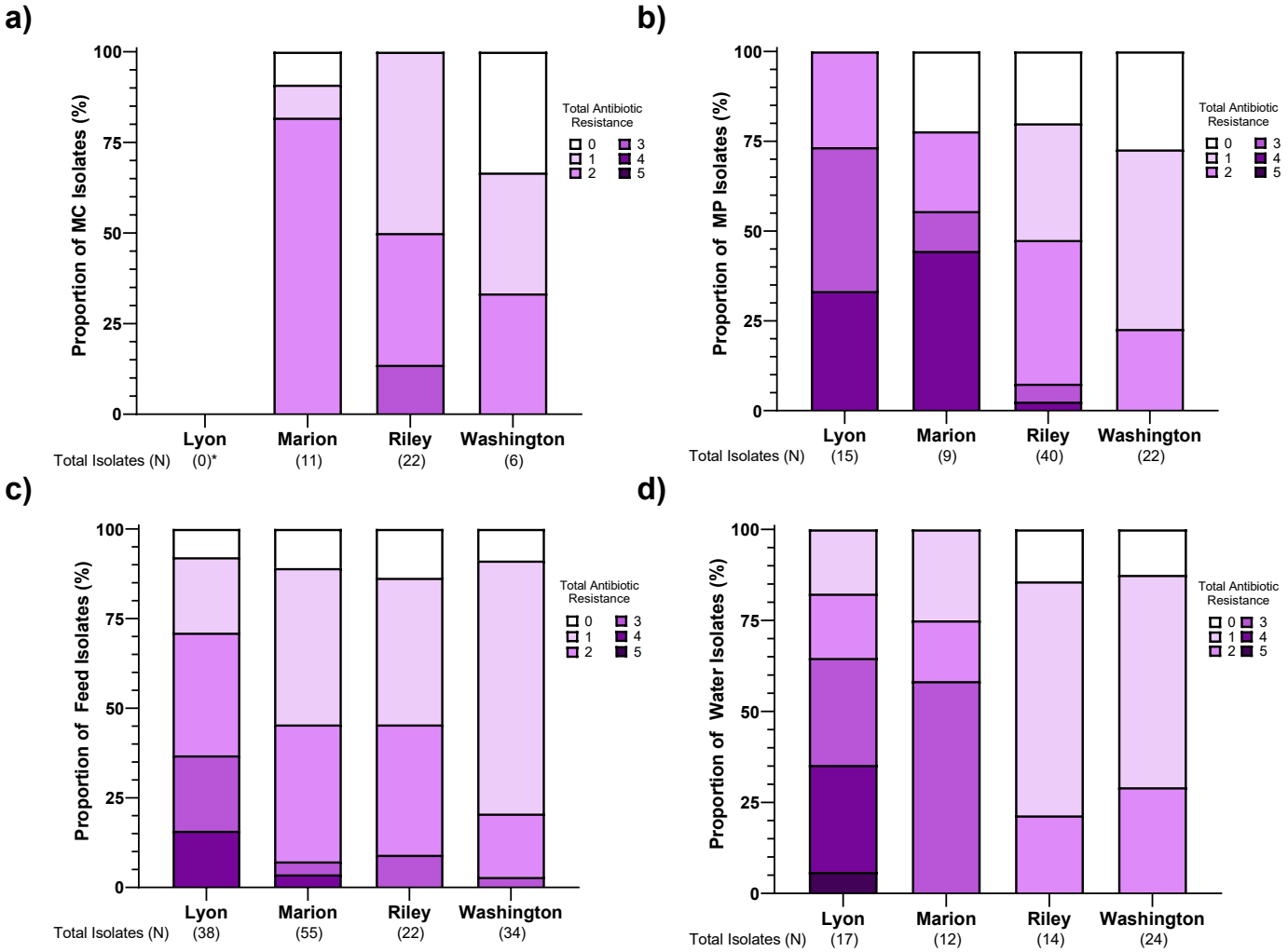
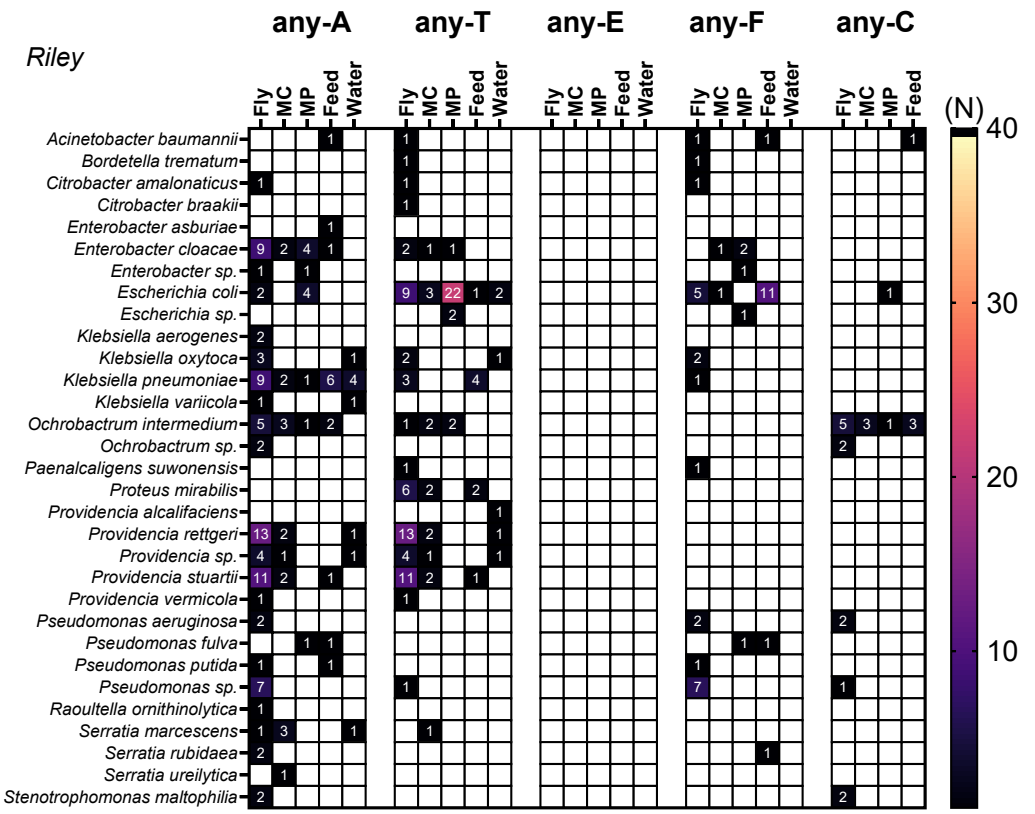
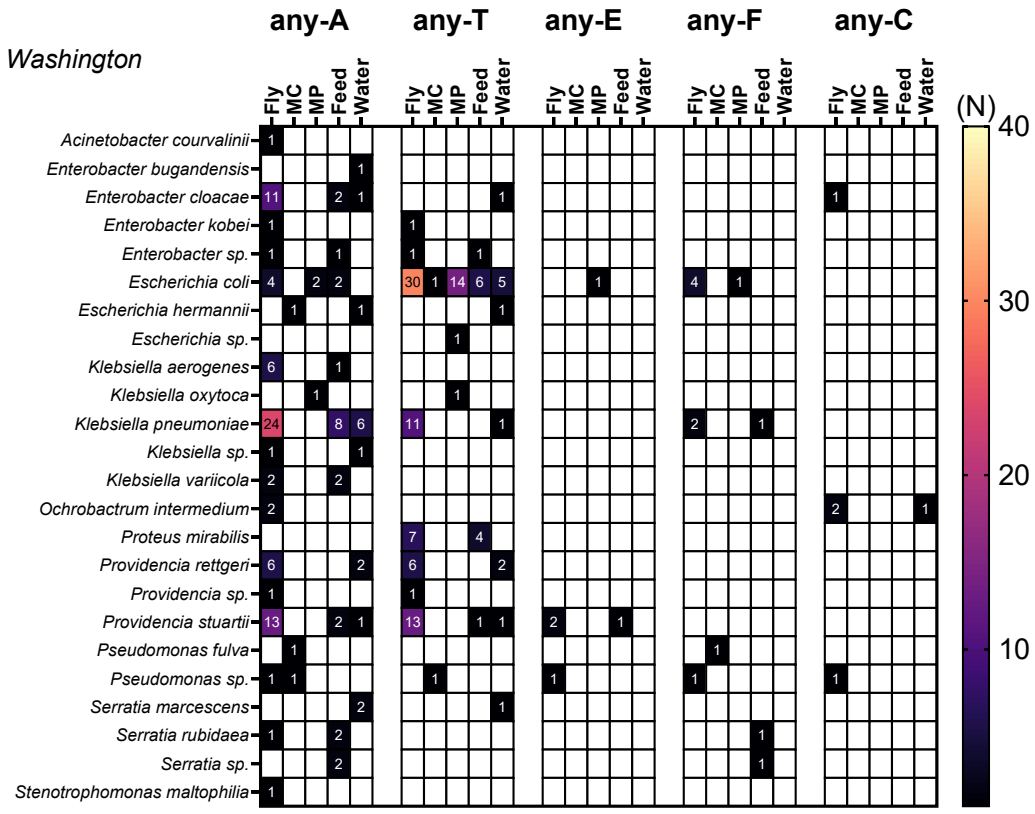
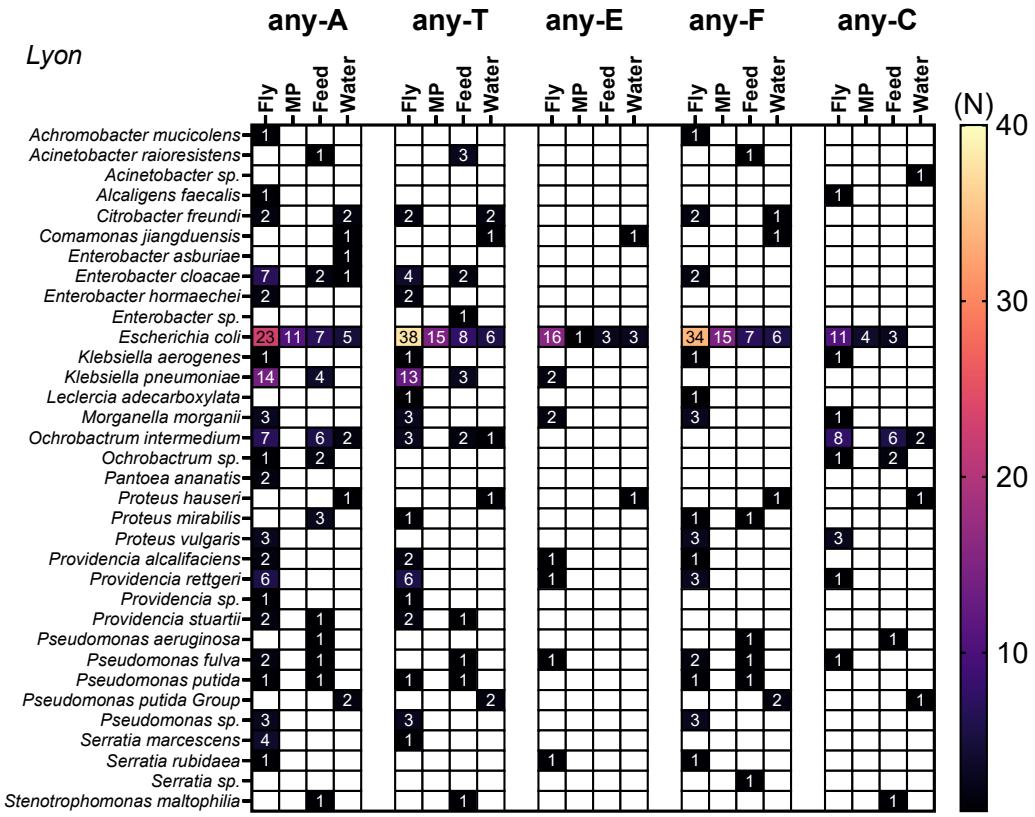


Figure 3.5. AMR profiles of environmental isolates by sample type and location

Proportion (%) of isolates from manure compost (MC; a), manure patty (MP; b), feed (c), and water samples (d) at each location with phenotypic resistance to different totals of antibiotics (0-5). *Lyon did not have MC to be sampled.


Riley
any-A
any-T
any-E
any-F
any-C
Fly
MC
MP
Feed
Water
(N)
40
30
20
10
Acinetobacter baumannii
Bordetella trematum
Citrobacter amalonaticus
Citrobacter braakii
Enterobacter asburiae
Enterobacter cloacae
Enterobacter sp.
Escherichia coli
Escherichia sp.
Klebsiella aerogenes
Klebsiella oxytoca
Klebsiella pneumoniae
Klebsiella variicola
Ochrobactrum intermedium
Ochrobactrum sp.
Paenalcaligens suwonensis
Proteus mirabilis
Providencia alcalifaciens
Providencia rettgeri
Providencia sp.
Providencia stuartii
Providencia vermicola
Pseudomonas aeruginosa
Pseudomonas fulva
Pseudomonas putida
Pseudomonas sp.
Raoultella ornithinolytica
Serratia marcescens
Serratia rubidaea
Serratia ureilytica
Stenotrophomonas maltophilia


Washington
any-A
any-T
any-E
any-F
any-C
Fly
MC
MP
Feed
Water
(N)
40
30
20
10
Acinetobacter courvalinii
Enterobacter bugandensis
Enterobacter cloacae
Enterobacter kobei
Enterobacter sp.
Escherichia coli
Escherichia hermannii
Escherichia sp.
Klebsiella aerogenes
Klebsiella oxytoca
Klebsiella pneumoniae
Klebsiella sp.
Klebsiella variicola
Ochrobactrum intermedium
Proteus mirabilis
Providencia rettgeri
Providencia sp.
Providencia stuartii
Pseudomonas fulva
Pseudomonas sp.
Serratia marcescens
Serratia rubidaea
Serratia sp.
Stenotrophomonas maltophilia


Lyon
any-A
any-T
any-E
any-F
any-C
Fly
MP
Feed
Water
(N)
40
30
20
10
Achromobacter mucicolens
Acinetobacter raioresistens
Acinetobacter sp.
Alcaligens faecalis
Citrobacter freundii
Comamonas jiangduensis
Enterobacter asburiae
Enterobacter cloacae
Enterobacter hormaechei
Enterobacter sp.
Escherichia coli
Klebsiella aerogenes
Klebsiella pneumoniae
Leclercia adecarboxylata
Morganella morganii
Ochrobactrum intermedium
Ochrobactrum sp.
Pantoea ananatis
Proteus hauseri
Proteus mirabilis
Proteus vulgaris
Providencia alcalifaciens
Providencia rettgeri
Providencia sp.
Providencia stuartii
Pseudomonas aeruginosa
Pseudomonas fulva
Pseudomonas putida
Pseudomonas putida Group
Pseudomonas sp.
Serratia marcescens
Serratia rubidaea
Serratia sp.
Stenotrophomonas maltophilia

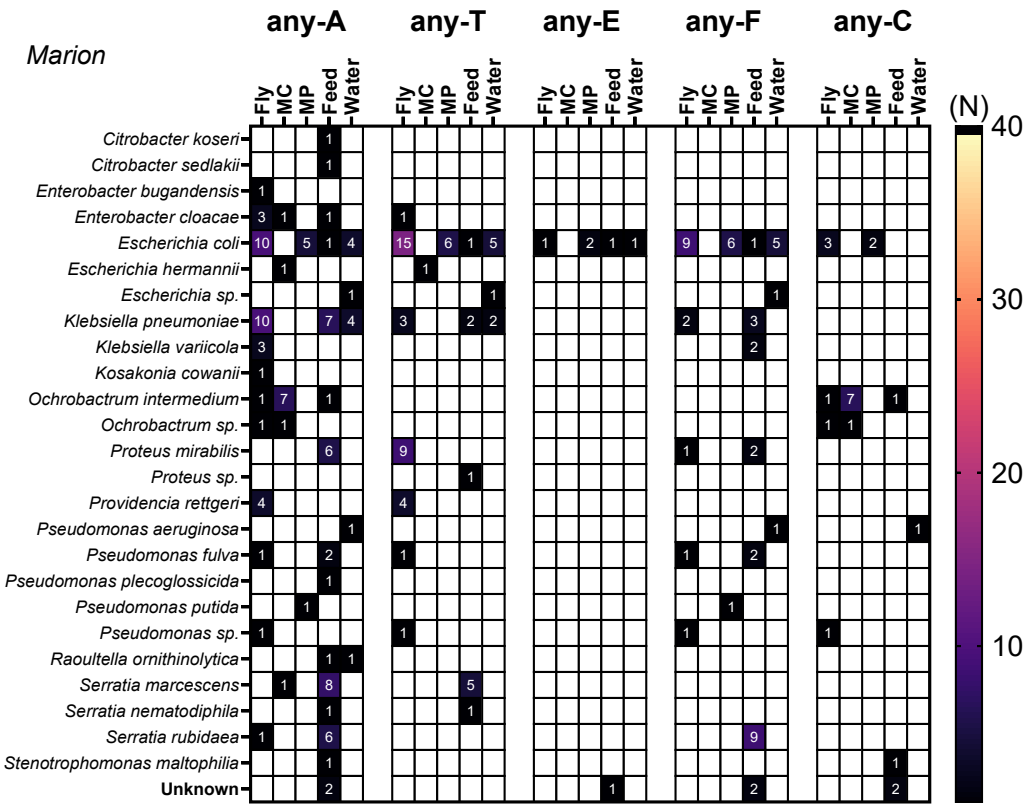
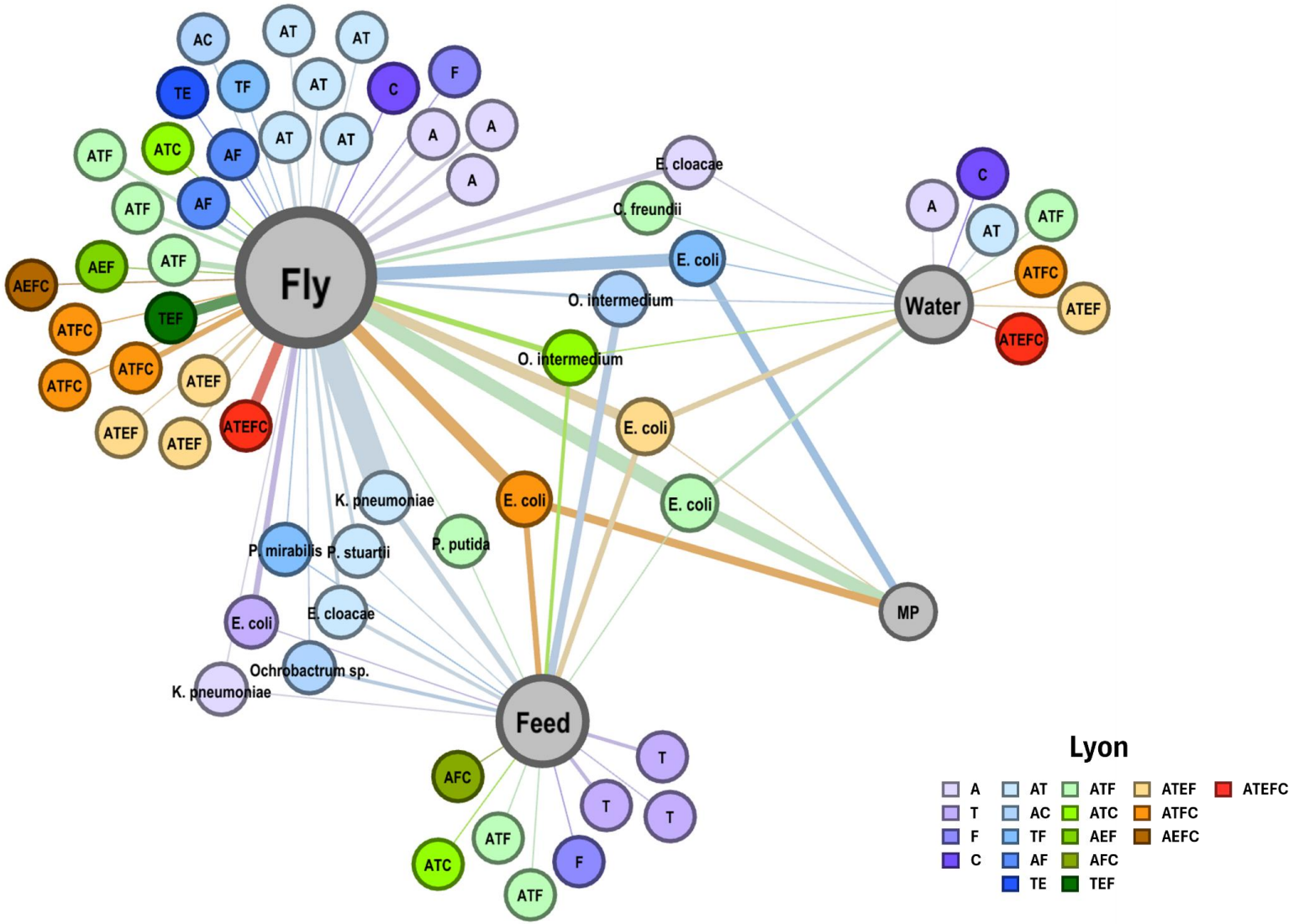

Marion
any-A
any-T
any-E
any-F
any-C
Fly
MC
MP
Feed
Water
(N)
40
30
20
10
Citrobacter koseri
Citrobacter sedlakii
Enterobacter bugandensis
Enterobacter cloacae
Escherichia coli
Escherichia hermannii
Escherichia sp.
Klebsiella pneumoniae
Klebsiella variicola
Kosakonia cowanii
Ochrobactrum intermedium
Ochrobactrum sp.
Proteus mirabilis
Proteus sp.
Providencia rettgeri
Pseudomonas aeruginosa
Pseudomonas fulva
Pseudomonas plecoglossicida
Pseudomonas putida
Pseudomonas sp.
Raoultella ornithinolytica
Serratia marcescens
Serratia nematodiphila
Serratia rubidaea
Stenotrophomonas maltophilia
Unknown

Figure 3.6. Taxonomic assignments of AMR bacteria cultured from house fly and environmental samples by each location and antibiotic

Total isolates (N) from flies and environmental samples collected at each location assigned to bacterial taxa and found phenotypically resistant to ampicillin (A), tetracycline (T), enrofloxacin (E), florfenicol (F), or ceftiofur (C) are shown. MC = manure compost; MP = manure patty. "Any-X" refers to isolates that had resistance to the indicated antibiotic, regardless of other observed phenotypes for the isolate (see Methods for details).


Fly
Water
Feed
MP
E. cloacae
C. freundii
E. coli
O. intermedium
O. intermedium
E. coli
E. coli
E. coli
K. pneumoniae
P. mirabilis
P. stuartii
P. putida
E. coli
E. cloacae
Ochrobactrum sp.
K. pneumoniae
AC
AT
AT
TE
TF
AT
C
F
AT
AT
A
A
ATF
ATC
AF
AF
A
ATF
AEF
ATF
AEFC
TEF
ATFC
ATFC
ATFC
ATEF
ATEF
ATEF
ATEFC
C
A
AT
ATF
ATFC
ATEF
ATEFC
AFC
ATF
ATC
F
ATF
T
T
T
Lyon
A
T
F
C
AT
AC
TF
AF
TE
ATF
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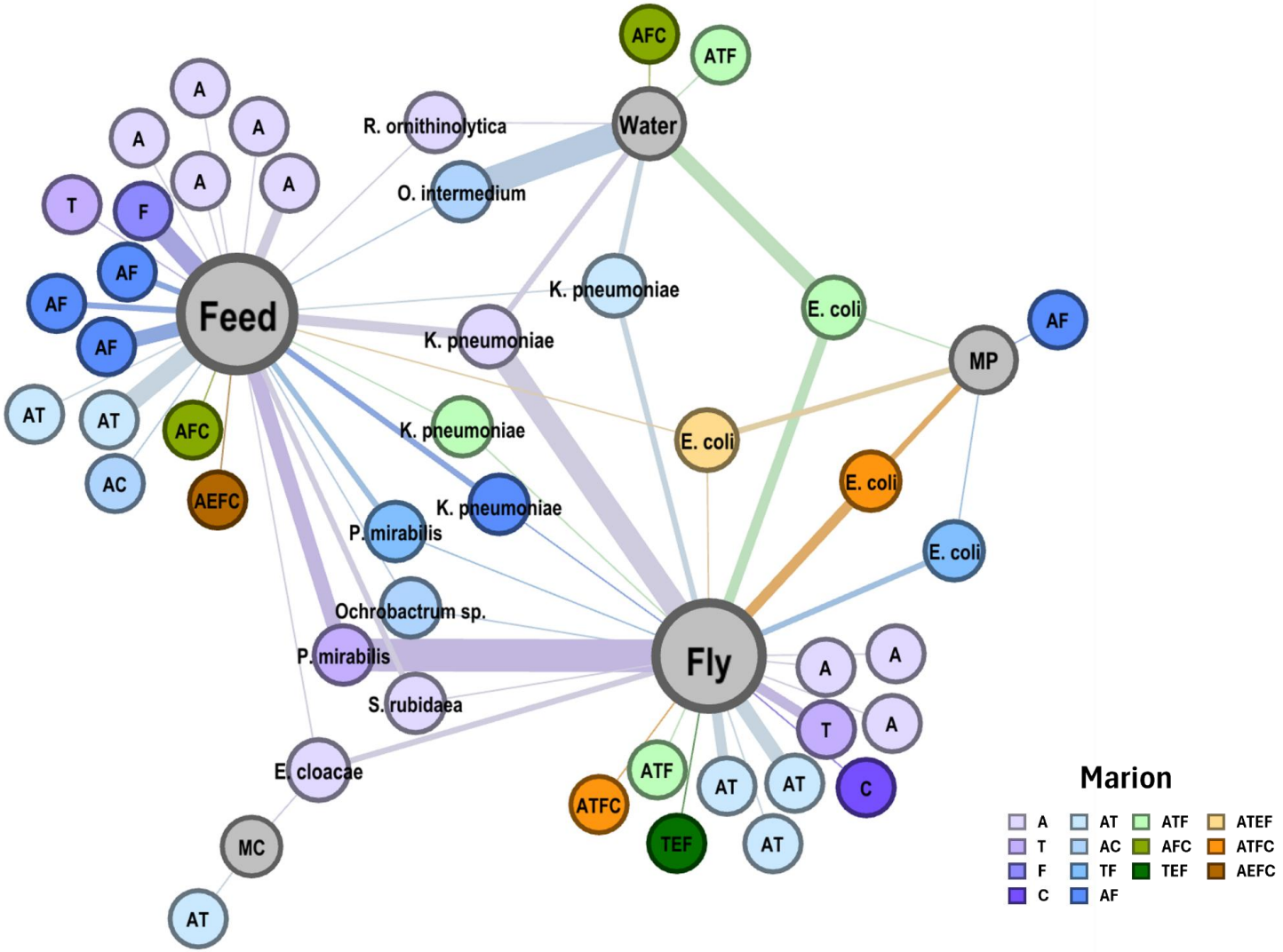
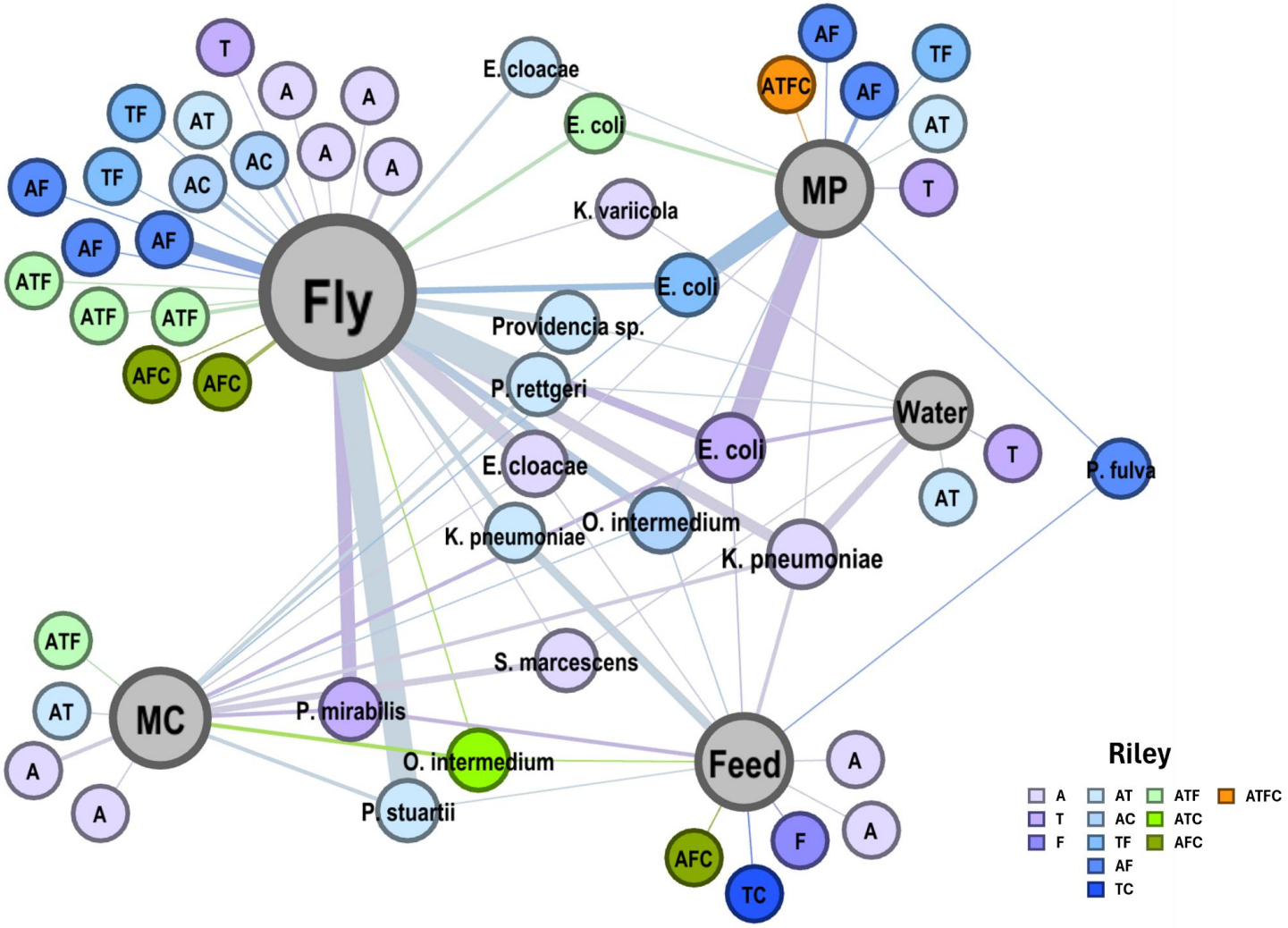
AFC
TEF
ATEF
ATFC
AEFC
ATEFC


Feed
Water
Fly
MP
A
T
F
C
AF
AT
AC
AFC
AEFC
ATF
ATFC
TEF
ATEF
AFC
ATF
R. ornithinolytica
O. intermedium
K. pneumoniae
K. pneumoniae
K. pneumoniae
K. pneumoniae
P. mirabilis
Ochrobactrum sp.
P. mirabilis
S. rubidaea
E. cloacae
MC
E. coli
E. coli
E. coli
E. coli
AF
Marion
A
AT
ATF
ATEF
T
AC
AFC
ATFC
F
TF
TEF
AEFC
C
AF


Fly
MP
Water
Feed
MC
E. cloacae
E. coli
K. variicola
E. coli
Providencia sp.
P. rettgeri
E. coli
E. cloacae
K. pneumoniae
O. intermedium
K. pneumoniae
P. fulva
S. marcescens
P. mirabilis
O. intermedium
P. stuartii
Riley
A
T
F
AT
AC
TF
AF
TC
ATF
ATC
AFC
ATFC

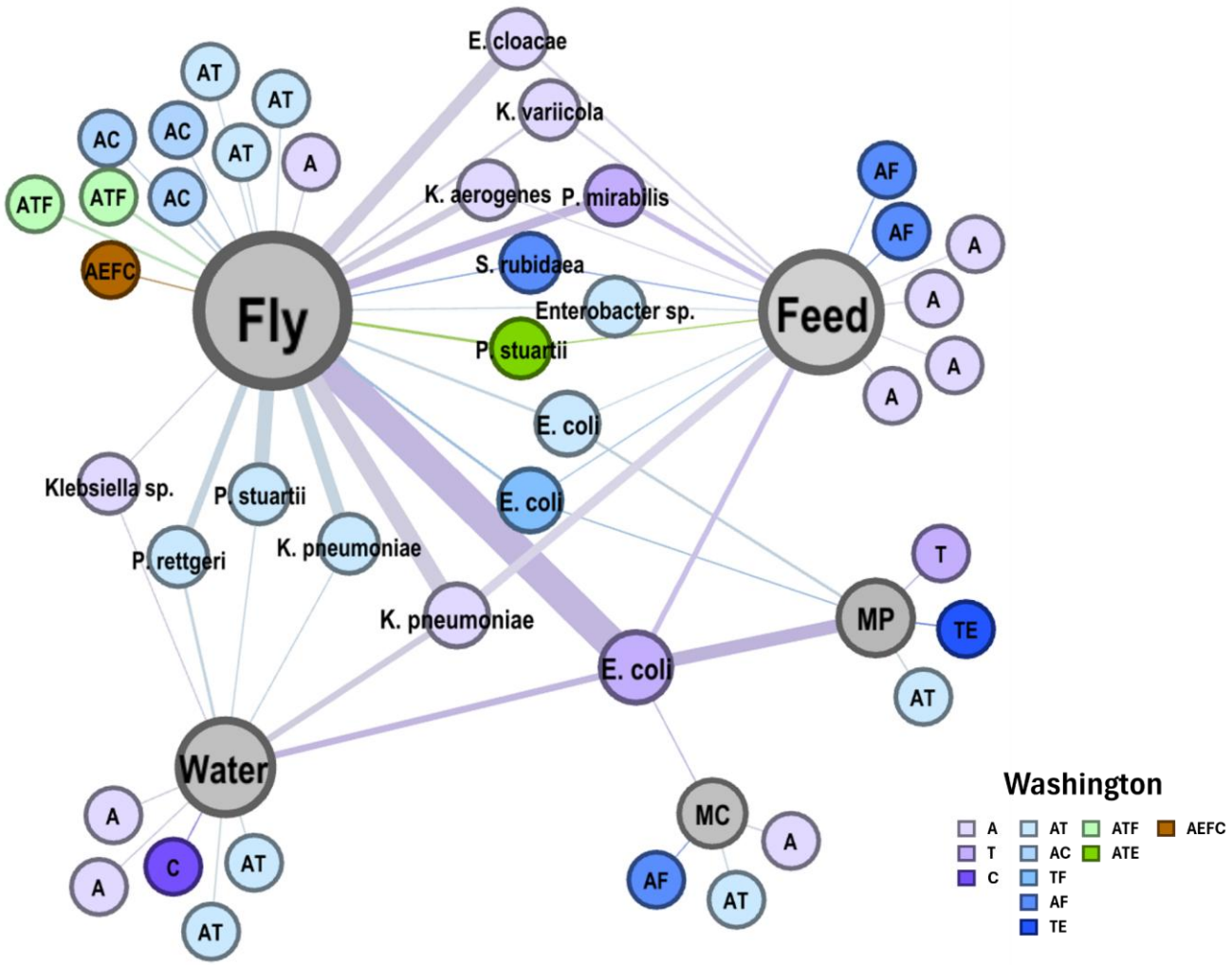


Figure 3.7. Relationships between AMR profiles of bacteria taxa cultured from flies and environmental samples by each location

Isolate taxa and AMR profile combinations shared amongst flies and environmental samples collected at each location. Differing colors indicate specific AMR/MDR profiles indicated in the legend, and weights of edges are proportional to the number of isolates. A= ampicillin, T= tetracycline, E= enrofloxacin, F= florfenicol, C= ceftiofur, MC = manure compost, MP = manure patty.

Chapter 4 - Bridging the gap: Comparative genomics of antimicrobial resistant *Escherichia coli* carried by house flies (Diptera: Muscidae), manure, feed, and water sources at Kansas beef cattle operations

In preparation as: Pickens V, Bird E, Olds C, Nayduch D. Bridging the gap: Comparative genomics of antimicrobial resistant *Escherichia coli* carried by house flies (Diptera: Muscidae), manure, feed, and water sources at Kansas beef cattle operations.

Abstract

Adult house flies (*Musca domestica* L.) are common pests of confined cattle operations, as well as reservoirs and vectors of pathogenic and antimicrobial-resistant (AMR) bacteria. Previously, genomic comparisons between AMR bacteria carried by house flies and environmental sources have prioritized animal manure, excluding alternative environmental sources of AMR bacteria frequented by flies such as animal feed. This study assessed the genetic relatedness between AMR *Escherichia coli* carried by house flies, cattle manure patties, feed, and water collected from a beef cattle stocker operation in Kansas. Whole genome sequencing was performed on 66 *E. coli* isolates from house flies and environmental samples that were phenotypically resistant to one or more antibiotics: ampicillin, florfenicol, enrofloxacin, ceftiofur, and tetracycline. Based upon 2,997 core genes, phylogenetic tree reconstruction of isolate genomes revealed clustering between *E. coli* isolated from flies and all environmental sample types, suggesting the sharing of AMR *E. coli* between flies and co-located environmental samples. Genomes of *E. coli* isolates from house flies shared 90% of the antimicrobial resistance genes (ARGs) detected in environmental isolates. Notably, ARGs such as *bla*$_{\text{CMY-2}}$ and *tet*(A),

previously demonstrated to be transferred horizontally within the house fly gut, were identified on conjugative or mobilizable plasmids within *E. coli* from flies, feed, and manure patties. These findings provide evidence for the role of house flies in the carriage and dispersal of AMR *E. coli* and associated ARGs, as well as indicates that house flies may contribute to the development of AMR in beef cattle environments. Furthermore, house flies stand to serve as a tool for AMR/ARG surveillance in beef cattle farms.

Introduction

Antimicrobial resistance (AMR) is a threat to global health and food security. Since the World Health Organization (WHO) committed to the AMR global action plan in 2015, national action plans have implemented regulations targeting antibiotic usage in clinal and agricultural settings for the mitigation of emerging AMR pathogens. Nevertheless, historical trends in global AMR burden from 1990 to 2021 show human deaths associated with and attributable to bacterial AMR increasing worldwide and are forecasted to continue increasing through 2050 (GBD 2021 Antimicrobial Resistance Collaborators 2024). Research investigating the environmental reservoirs and mechanisms by which AMR bacteria or antimicrobial resistance genes (ARGs) are exchanged among bacteria is essential in mitigating the emergence and dispersal of AMR bacteria. However, the persistence and spread of AMR within the environment are amongst the least understood components of One Health which attempts to link together sources of emerging AMR with clinical cases in human and animal health (Robinson et al. 2016).

The influx of antibiotic residues and resistance genes to the environment from industrial, clinical, and agricultural waste exerts selective pressure for the development and persistence of AMR bacteria (Reinthaler et al. 2003, Diwan et al. 2010, Ji et al. 2012). Environmental and commensal bacteria are reservoirs of ARGs (Robinson et al. 2016), which can transfer to human

and animal pathogens via horizontal gene transfer of mobile genetic elements such as transposons, plasmids, and bacteriophages (D'Costa et al. 2006, Wellington et al. 2013). The mechanisms by which bacteria which have recently acquired AMR from food animal production systems make their way into human and animal clinical cases is complex, but can involve nosocomial, foodborne or environmental acquisition routes of infection (Graham et al. 2024).

Filth flies, particularly house flies, are an additional, albeit understudied, route by which AMR bacteria within the environment may both develop resistance and eventually disseminate to humans and animals. House flies, *Musca domestica* L., frequent microbe-rich substrates in urban and rural environments around the globe for reproductive and nutritional needs (Gerberich 1948, West 1951, Greenberg 1954, Shmidtmann and Martin 1992, Zurek et al. 2000, Alam and Zurek 2004, Shah et al. 2016, Thomson et al. 2017). During these interactions, adult house flies acquire bacteria from environmental substrates through direct ingestion or contact with their external surfaces. In confined beef cattle operations, house flies have an abundance of environmental resources rich in microorganisms for in breeding and nutritional needs, from which they can acquire bacteria, including AMR strains, and thereby pose a significant threat to animal health as bacterial reservoirs and vectors (Nayduch et al. 2023). Resistant and pathogenic bacteria have been isolated or molecularly identified in house flies from confined cattle operations (Buma et al. 1999, Chakrabarti et al. 2010, Onwugamba et al. 2018, Pickens et al. 2025) and can be disseminated by flies to cattle and feed (Ahmad et al. 2007, Ghosh and Zurek 2015). Adult house flies also facilitate the horizontal transfer of ARGs between both Gram-positive and Gram-negative bacteria within their alimentary canal. Petridis et al. (2006) demonstrated that genes encoding chloramphenicol resistance or toxins were transferred horizontally between *Escherichia coli* in the house fly crop and gut via plasmid exchange or phage transduction.

Detection of *Enterococcus faecalis* transconjugants from both the fly labellum and digestive tract within 24 hours after exposure to donor and recipient strains further demonstrated the plasmid-mediated transfer of *tet*(M) between *E. faecalis* (Akhtar et al. 2009). Additionally, donor, recipient, and transconjugant *E. faecalis* were detected in the drinking water of flies, indicating transfer by flies to environmental surfaces after exposure to the contaminated food source. Plasmid-mediated horizontal transfer of third-generation cephalosporin resistance genes $bla_{\text{CTX-M}}$ and $bla_{\text{CMY-2}}$ was demonstrated not only between donor and recipient strains of *E. coli*, but also to other enteric bacteria present in the fly gut, such as *Achromobacter* sp. and *Pseudomonas fluorescens*, without the pressure of antibiotic usage (Fukuda et al. 2016). These findings highlight the role house flies play a role in the development, prevalence, persistence and dissemination of AMR bacteria.

In addition to facilitating the horizontal transfer of ARGs within their gut, filth flies have been able to acquire strains of AMR *E. coli* from infected swine and cattle, however the mode of acquisition, i.e., animal manure, the animals themselves or other environmental sources, was not clear (Marshall et al. 1990). Shared resistance phenotypes have been observed for *E. coli* and *Salmonella* isolated from house flies and cattle manure (Wadaskar et al. 2021), and metagenomic surveillance of AMR in house flies and various animal feces revealed that 71.4% of the ARGs and mobile genetic elements found in dog, poultry, dairy cattle, and beef cattle feces were also present in house flies (Poudel et al. 2020). These studies provide evidence for shared microbiota with AMR (and the genetic basis of their AMR, i.e. their ARGs or "resistomes") between flies and sources like animals or their waste but did not inspect the genetic relatedness of strains to more conclusively confirm transfer or acquisition of identical strains. A study incorporating whole genome sequencing of AMR *E. coli* cultured from multiple sources at swine facilities

implicated house flies as disseminators of AMR *E. coli* from pig feces due to close genomic relationships between isolates from flies and pig feces (Behrens et al. 2022). Because this method has not been applied to other animal production systems or other environmental sources of AMR bacteria house flies interact with, our present study compared the genomes of AMR *E. coli* cultured from house flies and several co-located environmental sources (manure patties, feed, and water) within confined beef cattle operations. The aim was to better understand the relatedness of these strains and to elucidate the role of house flies in the acquisition and potential dissemination of AMR *E. coli* among these sources.

Materials and Methods

Isolate Selection

For this study, a subset of 67 AMR *Escherichia coli* isolates cultured from house flies, manure patties, feed, and water collected from a single beef cattle operation (Lyon) were selected from our previous study (Chapter 3) for whole genome sequencing (WGS). In summary, 10 adult female and 10 adult male house flies were collected from feed bunks four different times at the Lyon beef cattle operation from August through October of 2020. In addition, two samples of cattle manure patties, feed, and water were collected. Samples were individually homogenized and serially diluted in sterile phosphate buffer saline (PBS; PBS; 1.37 M NaCl, 27 mM KCl, 101.4 mM NaH_2PO_4, 17.6 KH_2PO_4, pH 7.4; Fisher Scientific, Pittsburg, PA, USA) before spread plating onto MacConkey (MAC) agar and incubating at 37°C for 18 h. Plated sample dilutions with 30-150 colony forming units (CFUs) were replica-plated in succession onto plates of tetracycline- (16 µg/ml), florfenicol- (8 µg/ml) and enrofloxacin-infused (0.25 µg/ml) MAC agar and the antibiotic-infused plates incubated for 18 h at 37°C. For each sample, bacterial colonies

of distinct morphotypes which grew on all three antibiotic-infused plates were sub-isolated and cryo-preserved at -80°C as 25% glycerol stocks for downstream identification using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and susceptibility testing against five antibiotics via Kirby-Bauer disk diffusion: ampicillin, tetracycline, enrofloxacin, florfenicol, and ceftiofur. Finally, the selected subset of 67 AMR *E. coli* isolates were subjected for whole genome sequencing (WGS), as described below, to compare the genomic relatedness and resistomes (i.e., collection of ARGs in the genome) of isolates from house flies (n=38), and co-located cattle manure patties (n=14), feed (n=8), and water (n=7) sources from the same confined cattle operation.

DNA Isolation and Whole Genome Sequencing

Selected isolates were cultured from 20% glycerol stocks that had been cryo-preserved at -80°C. Frozen isolate stocks were thawed and vortexed for 3-5 s, followed by pipetting 10 µl of stock onto tryptic soy agar (TSA; Becton Dickinson and Company, Sparks, MD, USA) and streaking for isolation with a sterile loop. After incubating the plate at 37°C for 18 h, an isolated colony was selected and again streaked onto TSA and incubated at 37°C for 18 h. For total gDNA extraction an isolated colony was inoculated into 4 ml of tryptic soy broth (TSB; Becton Dickinson and Company, Sparks, MD, USA) and incubated at 37°C with 100 rpm for 20 h. Total genomic DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol with the Gram-negative bacteria pretreatment protocol and adding 4 µl of RNase A (100 mg/ml; Qiagen, Hilden, Germany) to each sample. A quality control (QC) check was performed on DNA extracts for purity (A260/280 of 1.8-2.0 desired) using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany), as well as DNA concentration (minimum of 10 ng/µl) using the dsDNA High

Sensitivity Kit and Qubit 4 fluorometer (Invitrogen, Carlsbad, CA, USA). DNA samples were submitted to Novogene (Sacramento, CA, USA) for library construction and paired-end 150 bp sequencing with the Illumina NovaSeq 6000 System (Illumina Inc., San Diego, CA, USA). Metadata and final QC reports for DNA extracts and sequencing statistics of each bacterial isolate are provided (Appendix Tables C.1 and C.2).

Genome Annotation and Comparative Genomics

Unless otherwise specified, all software tools were executed within Apptainer version 1.3.1 (Kurtzer et al. 2017; Apptainer Container Project 2021). Short-read sequence data were first processed using BALROG-ISO version 1.0.0 (Bird 2025). BALROG-ISO performed quality control using FastQC version 0.12.1 (Andrews 2010) and fastp version 0.20.1 (Chen et al. 2018; OpenGene 2020), followed by removal of human contamination with the NCBI Human Read Removal Tool version 2.2.1 (NCBI 2023), sequence data assembly with SPAdes version 3.15.5 (Prjibelski et al. 2020; Center for Algorithmic Biotechnology 2022) in "--isolate" assembly mode, genome completeness assessment with QUAST version 5.2.0 (Gurevich et al. 2022; Mikheenko et al. 2023) and BUSCO version 5.8.2 (Manni et al. 2021; EZlab 2024), and taxonomy prediction of each genome using version 2.4.0 of the Genome Taxonomy Database Toolkit (GTDB-Tk; Chaumeil et al. 2022, Australian Centre for Ecogenomics 2024) with GTDB database version r220 (Parks et al. 2022). Additionally, all genomes were annotated with Prokka version 1.14.6 (Seemann 2014, Seeman 2019) and plasmid sequences identified using Plasmer version 0.1-20220816 (Nekokoe 2022, Zhu et al. 2023). Antimicrobial resistance genes (ARGs) were predicted for each genome using three different methods: AMRFinderPlus version 4.0.19 (Feldgarden et al. 2021, NCBI 2025) with database version 2024-12-18.1 (Feldgarden et al. 2019), Resistance Gene Identifier (RGI) version 6.0.3 (Arpcard 2023) with version 4.0.0 of the

Comprehensive Antibiotic Resistance Database (CARD; Alcock et al. 2023), and ResFinder version 4.6.0 (Bortolaia et al. 2020, Center for Genomic Epidemiology n.d.b.) with database version 2.5.1 (Zankari et al. 2012; Center for Genomic Epidemiology 2023). AMR results were consolidated using hAMRonization version 1.1.8 (Maguire et al. 2024, Mendes et al. 2024), and quality control metrics were summarized with MultiQC version 1.28 (Ewels et al. 2016, MultiQC 2025).

Following BALROG-ISO processing, ubiquitous genes were identified using Panaroo version 1.5.2 (Tonkin-Hill et al. 2020; Tonkin-Hill 2025) set to a threshold of 99% presence across all bacterial isolate genomes. Ubiquitous genes were aligned using MAFFT version 7.526 (Katoh and Standley 2013; SnapGene 2018). Using a threshold calculated with Tukey's outlier test (entropy = 0.046), the alignment was filtered to remove genes exceeding the Block Mapping and Gathering with Entropy (BMGE). With the filtered ubiquitous gene alignment, a maximum-likelihood phylogenetic tree was constructed by IQ-TREE version 2.4.0 (Minh et al. 2020, IQ-TREE 2025) using a GTR+G substitution model and 1,000 ultrafast bootstrap replicates. Visualization of genome relatedness was performed using the Interactive Tree of Life (iTOL) webtool (Letunic and Bork 2019).

Prior to estimation of resistance gene prevalence in isolate genomes, all gene hits predicted by AMRFinder, Resfinder, and RGI were screened for pseudogenes. Using a custom tool (https://github.com/edwardbirdlab/edwardbirdlab-tools), the inferred open reading frame of each predicted gene hit was extracted to determine if nonsense mutations were present (i.e., an early stop codon). Genes were removed from further analysis if they contained an early stop codon resulting in a truncated protein less than 90% of the expected length. ARG hits were filtered for at least 90% coverage and identity, and ARG gene symbols were standardized across

all databases. Unique ARG hits for each isolate were consolidated from all 3 databases, selecting result outputs first for AMRFinderPlus. If an ARG hit was not found by AMRFinderPlus, the ARG hit from CARD was selected or, if an ARG hit was not found by AMRFinderPlus or CARD, from ResFinder (compiled final ARG list provided in supplementary file "final_arg_list_ch4.csv"). Associations between recorded resistance phenotypes and ARGs identified for each class of the antibiotics tested were investigated, with particular emphasis on ARGs categorized by AMRFinderPlus as "core" genes (highly curated and well-characterized ARGs known to be directly involved with AMR). All isolates were surveyed for eight specific genes (hereafter "targeted ARGs") which have previously been demonstrated to be horizontally transferred between bacteria within the fly gut (Petridis et al. 2006, Akhtar et al. 2009, Fukuda et al. 2016, Gan et al. 2024): *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM Group}}$, *mcr-8,* *tet*(A), and *tet*(M). Additionally, plasmids and mobile genetic elements (MGEs) were identified from the assembled genomes generated by BALROG-ISO using plasmidVerify (Antipov et al. 2019, Center for Disease Epidemiology n.d.a.) and MobileElementFinder version 1.0.3 (Johansson et al. 2021, Johansson 2023), respectively. Reciprocal best gene hits were identified for shared plasmids between isolates from house flies and environmental sources that carried targeted ARGs using DIAMOND v2.1.11 (Buchfink et al. 2015). Syntenic blocks were identified with MCScanX v0.1 (Wang et al. 2012) and visualized with SynViso (https://github.com/kiranbandi/synvisio). Detailed commands for each analysis are available in the accompanying GitHub repository (https://github.com/edwardbirdlab/Bact_Iso_2019_WGS).

Results

General Characterization of *E. coli* Genomes

Of the 67 bacterial genome sequences prepared in this study, one fly isolate (EC506) was removed due to later classification by GTDB-Tk as a non-*E. coli* species. FastQC, QUAST and BUSCO quality assessments of the whole genome sequencing, adapter removal, trimming, and genome assemblies are summarized in Appendix Tables C.3 and C.4. Prior to trimming, the number of raw reads per *E. coli* isolate ranged from 6.54 to 44.34 million reads, with a mean number of 15.59 million reads per isolate (1,000x coverage), and the percentage or reads with average Q-scores above 30 ranged between 91.03-98.07% (mean Q30 = 97.41%). Overall, a low proportion of reads were trimmed per isolate (mean = 0.45%), with a maximum trimmed read proportion of only 2.12%, resulting in a final mean read total of 15.52 million reads per isolate. Assembled *E. coli* genomes averaged a total length of 5.16 Mb, mean N50 of 187.84 Kb, and mean L90 of 40.27, with a range of 4.71-5.53 Mb in length and range in N50 and L90 of 67.45-672.53 Kb and 9-97 contigs per genome, respectively. A median of 399 total contigs (range = 182-1378 total contigs) were assembled for isolate genomes, with the largest genome contigs per isolate ranging in length from 211.94 Kb to 1598.61 Kb (mean = 478.87 Kbp). The assembled 66 *E. coli* genomes were found highly complete from the 874 BUSCO groups within the enterobacteriaceae_odb12 lineage dataset (BUSCO score range = 99.5-99.8%), with 64 isolates missing one BUSCO and two isolates missing two BUSCOs.

E. coli Subtyping and Cross-Origin Relatedness

Forty-one serotypes were identified from the 66 isolates composed of 27 different O- and 23 different H-antigens (Appendix Table C.5). The O-antigen was not identified for five isolates and not distinguishable for another six isolates, while the H-antigen could not be identified for

one isolate. Antigens O8 and H23 were the most common O- and H-types of isolates (O8 = 14/66; H23 = 11/66), and these isolates originated from flies, manure patties, and feed. Notably, one isolate from a fly and five isolates from a manure patty belonged to serogroup O103, one of the "big six" non-O157 STEC serogroups (O26, O45, O103, O111, O121, and O145) considered to be a significant risk for foodborne disease outbreaks (Alharbi et al. 2022). Flies shared isolate serotypes O103: H2 and O128: H2 with manure patties, serotypes O8: H23 and O8: H7 with manure patties and feed, and serotype O91: H23 with water. Fourteen isolates from flies and manure patties were identified as Enterohemorrhagic (EHEC), Enteropathogenic (EPEC), Enterotoxigenic (ETEC), and/or Shiga toxin-producing (STEC) *E. coli* pathotypes. Of the 14 isolates, four were hybrid pathotypes STEC/ETEC (n = 1 fly isolate), STEC/EHEC (n = 1 fly isolate), and EPEC/ETEC (n = 2 patty isolates). All 66 *E. coli* isolates possessed the *hlyE* virulence gene, and isolates from flies and manure patties additionally shared the *eae*, *ehxA*, and *sta1* (Figure 4.1). However, *stx1* was found only in fly isolates (n = 2), while *hlyA* was found only in a manure patty isolate.

Panaroo identified 3,218 ubiquitous genes across the 66 *E. coli* genomes. Using Tukey's outlier test, an entropy threshold of 0.046 was determined for BMGE filtering, resulting in the removal of 221 genes from the ubiquitous genome alignment. This yielded a final alignment of 2,997 ubiquitous genes. Two isolates from the same fly, EC454 and EC461, were found to be identical across all 2,997 ubiquitous genes. The resulting phylogenetic tree was rooted at the midpoint (Figure 4.2), with most isolates (n = 65) clustering on one side of the root and classified by GTDB-Tk as *E. coli*, corresponding to the GTDB-defined representative clade that includes the type strain. In contrast, isolate EC498 fell on the other side of the root and was identified as *Escherichia coli F*, indicating it belongs to a divergent genomic lineage within the *E. coli* species

complex of GTDB. Isolates from same fly, manure patty, feed, or water sample collected on the same collection dates did not always cluster together, clustering instead with other isolates of similar AMR phenotypes. Isolates cultured from flies shared clades intermixed with isolates from water, feed, and manure patties, most often those which shared similar collection dates and AMR phenotypes. However, some clades were also composed of isolates which did not originate from the same sample type or share the same AMR phenotypes (i.e. EC658, EC468, EC485).

Resistance and Virulence Gene Characterization for *E. coli* from Flies and Environment

MultiAMR analysis and filtering for 90% coverage and sequence identity resulted in a total of 5,926 resistance and virulence gene hits from the CARD, NCBI, and ResFinder databases. Pseudogene filtering removed 630 hits, leaving 5,296 total hits across all isolates. Compared with CARD and AMRFinder, ResFinder had a higher proportion of pseudogenes for both the overall total hits per database (CARD = 0%; AMRFinder = 1.81%; ResFinder = 53.40%) and number of unique genes identified per database (CARD = 0%; AMRFinder = 0.76%; ResFinder = 28.26%). Overall, more total hits were observed with the CARD database (Appendix Figure C.1), but more unique resistance/virulence genes were observed with the AMRFinder database. However, for individual isolates, more unique gene hits were occasionally observed when using results from CARD versus AMRFinder. A total of 194 unique virulence, heavy metal resistance, and AMR genes were identified across all isolates, with 13.40% of genes shared from all three databases and 8.25% of genes shared amongst only two databases (largely CARD and AMRFinder). Notably, only five genes were uniquely identified by ResFinder, while 89 and 57 genes were identified only by AMRFinder or CARD, respectively. After consolidating the outputs from MultiAMR analysis, resistance and virulence genes were found in all 66

isolates, with a range of 50-89 hits and an average of approximately 64 hits per isolate. Additionally, all 66 isolates had one or more plasmids carrying resistance/virulence genes, with an average of 13.15% (range = 1.96-31.08%) of all the resistance and virulence genes identified per isolate predicted to originate from plasmids versus the chromosome.

Out of the 193 virulence, heavy metal resistance, and AMR genes identified, 116 (60.10%) were ARGs (compiled ARGs in supplementary file "final_arg_list_ch4.csv"). Manure patty, feed, and water *E. coli* isolates had 106 ARGs identified, of which 90.57% were also found in *E. coli* isolates from house flies. Isolates from house flies shared the highest proportion of ARGs with water isolates (n = 72/73 ARGs; 98.63%), followed by feed isolates (n = 85/88 ARGs; 96.59%) and manure patty isolates (n = 90/98 ARGs; 91.84%). No isolates from flies carried $bla_{\text{TEM-1B}}$ despite its carriage in isolates from all environmental sample types. Other ARGs found in environmental isolates that were not present in the genomes of fly isolates were *aadA17*, bla_{TEM}, $bla_{\text{TEM-206}}$, $bla_{\text{TEM-214}}$, $bla_{\text{TEM-248}}$, *mef*(C), and *mph*(G) from manure patty isolates, and $bla_{\text{CTX-M-15}}$ and $bla_{\text{EC-13}}$ from feed isolates.

Sixty-five of the isolates carried ARGs predicted to originate from plasmids, but most ARG hits were predicted to originate from the chromosome (87.74%; n = 3,135/3,573 hits; Figure 4.3). Multi-class ARGs, or ARGs which confer resistance to multiple antibiotic classes, represented the largest proportion of total ARG hits observed (56.18%; n = 1,951/3,473) and were predicted to only originate from the chromosome in all but two isolates. Most ARG classes observed in environmental *E. coli* isolates were also found in house fly isolates, but ansamycin ARGs were only observed in one house fly isolate. ARGs for aminocoumarins, nitroimidazoles, peptides, and phosphonic acids were found in 100% of isolates and all predicted to originate only from the chromosome. In contrast, ARGs for lincosamides and trimethoprims were predicted to

originate exclusively from plasmids and were only found in a low proportion of isolates across all sample types. Beta-lactam, quinolone, and tetracycline ARGs of both chromosomal and plasmid origin were observed in isolates from all sample types, although plasmid-originated ARGs were typically in lower proportions of isolates than chromosomal ARGs for those classes. Interestingly, plasmid-origin ARGs conferring sulfonamide resistance were found in most isolates from flies (n = 26/37), manure patty (n = 14/15), feed (n = 7/8), and water (n = 4/5) samples, but chromosomal sulfonamide ARGs were observed exclusively in house fly isolates.

ARG Genotype and Phenotype Correlations

Due to database limitations for our tested antibiotics, correlations between observed ARG hits and phenotypic resistance to each of our five antibiotics were conducted at the drug class level (tetracycline = tetracyclines; ampicillin and ceftiofur = beta-lactams; florfenicol = phenicols; enrofloxacin = quinolones). Interestingly, all 66 isolates had ARG hits for all four antibiotic classes, irrespective of whether they were found to have displayed phenotypic resistance to the corresponding specific antibiotic. For example, five isolates lacked phenotypic resistance to florfenicol, possessing only single or dual resistance to tetracycline and enrofloxacin. Nevertheless, 13 different ARGs for phenicol resistance (including multidrug ARGs) were found overall, with nine of them observed in all 66 isolates. Additionally, no single ARG appeared to be associated with functional phenicol resistance in the selected isolates. Of the five isolates that weren't phenotypically resistant to florfenicol, all five lacked *mdt*M and *cmlA1*, suggesting that these genes would be associated with functional florfenicol resistance. However, these ARGs were present in only 1 or 5 of the total isolates which were phenotypically florfenicol-resistant, respectively.

Most ARG hits for the tested antibiotics also conferred resistance to multiple antibiotic classes. Overall, 75.42 % of tetracycline ARG hits (n = 850/1,127), 76.93% of quinolone ARG hits (n = 1,137/1,478), 85.88% of beta-lactam ARG hits (n = 1,143/1,331), and 91.10% of phenicol ARG hits (n = 655/719) also can confer resistance to other antibiotic classes. Considering that most isolates carried multiclass ARGs regardless of the observed phenotypes, the prevalence of the top 5 non-multiclass ARGs in isolates was investigated to assess potential correlations between observed resistance phenotypes and commonly observed ARGs in isolates (Fig. 4.4). The proportion of *E. coli* isolates carrying the top ARGs observed for each antibiotic class for each AMR resistance phenotype profile was dependent on the antibiotic class, with isolates belonging to the same AMR phenotype profiles not always sharing the same ARGs. Notably, fly *E. coli* isolates carried all the top 5 ARGs observed in environmental isolates for each antibiotic class.

The subunits of antibiotic efflux pumps for tetracyclines (*emrK* and *emrY*) and quinolones (*emrA* and *emrR*) were the only non-multiclass ARGs shared among all isolates, despite just 22 isolates being phenotypically enrofloxacin-resistant. At least 8 different non-multiclass ARGs were identified for the tetracycline, beta-lactam, and quinolone antibiotic classes, whereas *floR* and *cmlA1* were the only two non-multiclass ARGs identified for phenicols. Interestingly, though, 89.39% of isolates carrying *floR* and 7.58% carrying *cmlA1*. Two isolates from house flies with ATF resistance phenotype profiles had neither *floR* nor *cmlA1* but carried multiclass ARGs. Compared to the other antibiotic classes, the prevalence of the top 5 ARGs for beta-lactam resistance was highly variable between sample types and AMR phenotype profiles.

Hits in AMRFinder for "core" genes, or ARGs expected to influence phenotypic resistance, did not correlate with measured resistance phenotypes. Two isolates that displayed only tetracycline resistance had *floR*, a core gene expected to impart chloramphenicol/florfenicol resistance. However, only 57 of the 61 isolates with phenotypic resistance to florfenicol had *floR*, whereas all 61 isolates shared 8 other ARGs of chromosomal origin which can confer quinolone resistance. Further investigation of isolates with ARG hits for quinolone resistance also revealed that isolates from manure patty (n =2), house fly (n = 2), and feed (n = 1) all carried the core gene *qnrB19* which was predicted to be carried by the conjugative plasmid Col440I and translated protein of this gene in our samples was nearly identical to the reference protein sequence from *qnrB19.* However, only the manure patty isolates displayed phenotypic resistance to enrofloxacin (Appendix Figure C.2). Of the 24 ARGs conferring resistance to quinolones found in both manure patty isolates, only one ARG, *parC*, was not also shared in the fly and feed isolates. AMRFinder considers *parC* another core gene for quinolones and, for both manure patty isolates, was predicted to originate in the chromosome.

ARGs Associated with Fly-Mediated Horizontal Gene Transfer

Eight ARGs were specifically surveyed for identification on the isolate genomes due to facilitation of horizontal gene transfer within the house fly gut in prior studies. No isolates carried *mcr-8*, *bla*$_{\text{CTX-M-2}}$, or *bla*$_{\text{CTX-M-14}}$. However, sixty-three isolates (95.5%) were carriers of one or more of five remaining ARGs: *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM Group}}$, *tet*(A), and *tet*(M). Overall, 60 isolates (90.9%) carried *tet*(A), 20 isolates (30.3%) carried *tet*(M), 19 isolates (28.8%) carried *bla*$_{\text{TEM Group}}$, 13 isolates (19.7%) carried *bla*$_{\text{CMY-2}}$, and 1 isolate (1.5%) carried *bla*$_{\text{CTX-M-15}}$. All sample types had one or more isolates that carried *bla*$_{\text{TEM Group}}$, *tet*(A), and *tet*(M), whereas *bla*$_{\text{CMY-2}}$ was only found in isolates from flies, manure patties, and feed. *bla*$_{\text{CTX-}}$

$_{M\text{-}15}$ was only found in an isolate cultured from feed, and this same *E. coli* isolate carried the highest number of targeted ARGs (*bla*$_{CTX\text{-}M\text{-}15}$, *bla*$_{TEM\ Group}$, *tet*(A), *tet*(M)). Overall, approximately half of the isolates carried two targeted ARGs (53.0%; n = 35/66), followed by 31.8% (n = 21/66) carrying 1 targeted ARG and 7.6% (n = 5/66) carrying 3 targeted ARGs.

Of the 5 targeted ARGs found in the *E. coli* isolates, *bla*$_{CTX\text{-}M\text{-}15}$ was the only ARG not predicted to be on a plasmid. Overall, 113 total ARG hits were recorded, with 98.2% (n = 111) of the ARGs located on a plasmid and 2 hits (*bla*$_{CTX\text{-}M\text{-}15}$ and *tet*(M)) located on the chromosome. PlasmidFinder was unable to assign an identity for plasmids carrying *bla*$_{TEM\ Group}$ or *tet*(M), but 100% (n = 19/19) of the plasmids carrying *tet*(M) were predicted to originate from *E. coli*, while plasmids carrying *bla*$_{TEM\ Group}$ ARGs were predicted to originate from five taxonomic groups: *E. coli* (n = 13/19), *Escherichia fergusonii* (n = 2/19), *Klebsiella pneumoniae* (n = 2/19), Enterobacteriaceae (n = 1/19), and *Salmonella* (n = 1/19; Figure 4.5). Similarly, unidentified plasmids carrying *bla*$_{CMY\text{-}2}$ were predicted to originate from either *E. coli* (n = 5/13) or *Salmonella enterica* (n = 2/13) and unidentified plasmids carrying *tet*(A) were predicted to originate from eight taxonomic groups: Enterobacterales (n = 5/48), Enterobacteriaceae (n = 11/48), *E. coli* (n = 18/48), *E. fergusonii* (n = 6/48), Gammaproteobacteria (n = 2/48), *K. pneumoniae* (n = 2/48), Proteobacteria (n = 3/48), and *S. enterica* (n = 1/48). Classifiable plasmids identified as carriers of one or more of the targeted ARGs included three conjugative plasmids (IncA/C$_2$, IncFII, Incl1) and one mobilizable plasmid (IncQ1). Plasmids IncA/C$_2$ and IncFII were both identified as carriers of *bla*$_{CMY\text{-}2}$ and *tet*(A), while Incl1 was only a carrier of *bla*$_{CMY\text{-}2}$ and IncQ1 was only a carrier of *tet*(A). One IncA/C$_2$ plasmid from a feed isolate was predicted to have originated from *K. pneumoniae*, but all other classified plasmids with the targeted ARGs were predicted to have originated from *E. coli*.

Two known plasmids carrying targeted ARGs were successfully identified in *E. coli* isolates from flies and an environmental source. IncA/C_2 plasmids carrying *tet*(A) and *bla*$_{CMY-2}$ were found in isolates from both a fly (EC581) and feed sample (EC1248), while Incl1 plasmids carrying *bla*$_{CMY-2}$ were found in isolates from both a fly (EC1679) and manure patty (EC658). The Incl1 plasmids in EC1679 and EC658 that were predicted to originate from *E. coli* did not carry additional ARG hits, and the *bla*$_{CMY-2}$ sequences were nearly identical (Appendix Figure C.3). In contrast, the IncA/C_2 plasmids from EC581 and EC1248 were predicted to originate from *E. coli* and *K. pneumoniae*, respectively, and had additional ARG hits for *aph*(3")-*lb*, *aph*(6)-*ld*, and *sul2* at identical positions on the plasmid. The IncA/C_2 plasmid from EC581 additionally carried *floR*, which was absent in IncA/C_2 from EC1248. The *bla*$_{CMY-2}$ nucleotide sequences from EC581 and EC1248 were identical, while *tet*(A) differed by ~22 nucleotides at the 3' end, resulting in different predicted proteins (Appendix Figures C.4 and C.5). A full alignment of *bla*$_{CMY-2}$ sequences from all four isolates confirmed near identity despite originating from *E. coli* isolates collected from different sample types (Appendix Figure C.6).

High syntenic conservation was observed between two large regions of the IncA/C_2 plasmids from EC581 (fly origin) and EC1248 (feed origin), notably composing 92.42% of the plasmid sequence for EC581 (Figure 4.6). The first region spanned 66.8 kb and included 84 genes, among them *bla*$_{CMY-2}$. The second region spanned 14.8 kb composed of 21 genes, such as *tet*(A), *aph*(3")-*lb*, *aph*(6)-*ld*, and *sul2*. The gene content, order, and orientation were conserved in both regions, with no structural rearrangements. Mobile Element Finder identified evidence of transposable element-mediated mobility, indicating that the composite transposon cn_46712_IS26 and insertion sequences IS26 and ISEc9 in both plasmids were located at the same relative positions near the shared ARGs for EC581 and EC1248 on IncA/C_2. However, the

plasmids differ in the later region, with the IncA/C_2 plasmid from isolate EC1248 lacking *floR* and various mobile elements such as the composite transposon cn_42415-IS26 and insertion sequence ISVsa3. Instead, the later region of the IncA/C_2 plasmid in EC1248 has the unit transposon Tn3411 and extends approximately 63 kb longer than the IncA/C_2 plasmid in EC581.

Discussion

As capable vectors of bacteria, and due to their global distribution, the potential of house for use in the surveillance of pathogenic and/or AMR bacteria in the environment has been emphasized (Zurek and Ghosh 2014, Nayduch and Burrus 2017, Onwugamba et al. 2018, Gwenzi et al. 2021, Yin et al. 2022, Usui et al. 2022, Nayduch et al. 2023, Neupane et al. 2024). Confined cattle operations offer the opportunity to assess both the development and transfer of AMR in the environment using house flies. Yet, to the best of our knowledge, no studies have compared AMR bacteria found in house flies to co-located environmental substrates at cattle operations other than in animal feces. The aim of this study was to assess the genetic relatedness of AMR *E. coli* carried by house flies versus the variety of environmental sources they typically frequent at beef cattle operations (manure, feed, and water).

Overall, we observed strong evidence that house flies could be used for surveillance of AMR *E. coli* found in manure patties, feed, and water sources at beef cattle operations. House flies carried AMR *E. coli* closely related to strains from these environmental substrates, even when collected on different dates. Additionally, isolates from flies shared approximately 90% of the 106 different ARGs also observed in environmental isolates and reflected ARGs observed for all antibiotic classes to which environmental isolates had ARGs. This included not only the detection of ARGs from antibiotic classes that were not found in all sample types (i.e., ansamycin ARGs were only found in isolates from flies, manure patties, and feed) but also trends

in predicted genomic context for these ARGs (chromosome or plasmid encoded). Notably, *bla*$_{\text{TEM-1B}}$ was the only ARG found in isolates from all environmental sources that was not found in isolates from flies, despite its distribution in the genomes of *E. coli* isolated from the environment, humans, and animals (Farooq et al. 2025). Thus, the use of house flies to survey AMR in confined cattle settings could potentially overlook some ARGs exclusive to only environmental substrates. Further research would help, however, to identify the gaps of using house flies in place or in concurrence with environmental substrates, like whether the findings of this study translate to other bacterial species or systems house flies interact with. Depending on the aims of future AMR surveillance studies, this information could be used to indicate whether house flies offer a more efficient and representative sample of environments.

House flies explore a wide range of resources in cattle operations and likely acquire and disseminate bacteria among these substrates. As reported in Chapter 3, it was observed that manure compost, manure patties, feed, and water each harbored AMR bacteria with distinct taxa and resistance profiles, many of which were also recovered from house flies. These findings suggest a potential exchange of AMR *E. coli* and ARGs among house flies and environmental substrates. Prior research has typically focused on the role of house flies in transporting AMR bacteria off cattle operations to fresh produce for human consumption (Macovei et al. 2008, Chakrabarti et al. 2010, Pace et al. 2017, Thomson et al. 2021). However, the specific routes, frequency, and conditions driving the acquisition and dissemination of bacteria via house flies within cattle operations remain poorly understood. Ahmad et al. (2007) demonstrated that house flies were capable vectors of AMR *E. coli* O157:H7 to cattle in enclosed pens but did not assess the complex behaviors and substrate interactions that occur between flies and their environment in field settings. In natural environments, these interactions may substantially alter both

acquisition and transmission dynamics and therefore animal health risks. The results of this study indicate that house flies share AMR *E. coli* found in cattle manure, feed, and water. Furthermore, a few studies have assessed the potential for house flies to vector bacteria acquired from animal manure in laboratory settings (Talley et al. 2009, Wasala et al. 2013, Pace et al. 2017, Thomson et al. 2017). A field study by Ghosh and Zurek (2015) identified clonal matches of *E. coli* and *Klebsiella* spp. shared between house flies and beef cattle feed but did not investigate other environmental substrates or the source from which flies acquired the strains. In the current study, we typically observed the highest densities of house flies on feed within confined cattle operations, and contaminated feed presents a direct route for livestock to ingest AMR pathogens. Animal feeds may provide a route for directly exposing cattle to newly developed AMR pathogens shed by house flies via regurgitation or defecation spots. Future research should attempt to determine how flies contribute to the movement and amplification of AMR bacteria to feed and other environmental substrates that may put cattle, and ultimately the food supply, at risk.

To gain insight into the potential of house flies to facilitate resistance gene transfer in beef cattle operations, we surveyed isolates from field samples for eight "target" ARGs, which were previously demonstrated to be horizontally transferred within the fly gut in laboratory assays (Petridis et al. 2006, Akhtar et al. 2009, Fukuda et al. 2016, Gan et al. 2024). Of the targeted ARGs, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM Group}}$, *tet*(A), and *tet*(M) were observed in one or more isolates across sample types. Except for *bla*$_{\text{CTX-M-15}}$, most of the targeted ARGs found were predicted to be of plasmid origin largely carried by unidentifiable plasmids. More importantly, candidate plasmids (IncQ1, IncA/C$_2$, IncFII, and Incl1) carrying the targeted and additional ARGs were identified in both fly and environmental isolates that were not previously assessed in

HGT studies within the fly gut. The carriage of *tet*(A) by IncQ1, a mobilizable plasmid, in a fly isolate suggests that this ARG either was acquired and/or is transferrable to other bacteria with the assistance of initiators. More importantly, the carriage of *bla*$_{CMY-2}$ and *tet*(A) on the conjugative plasmids IncA/C$_2$, IncFII, and Incl1 in *E. coli* from flies, manure patties, and/or feed indicates that these ARGs can readily transfer between bacteria, even though it may originate from different sources.

The syntenic conservation of genetic features observed between the IncA/C$_2$ plasmids carrying *bla*$_{CMY-2}$ and *tet*(A), as well as other ARGs, in *E. coli* isolates from a fly (EC581) and feed (EC1248) indicate strong structural similarity associated with composite transposons. This conservation suggests the possibility of a prior horizontal transfer event of these ARG operons between *E. coli* now isolated from flies and cattle feed during our survey. However, the absence of the ARG *floR* and composite transposon cn_42415-IS26 in EC1248, along with the placement of the unit transposon Tn3411 and the subsequent sequence, suggests divergence between the regions that were non-syntenic has occurred. Considering that multiple ARGs, such as *aph*(3")-*lb*, *aph*(6)-*ld*, and *sul2*, were shared on IncA/C$_2$ from EC581 and EC1248, there may be additional candidates for further investigation of HGT of ARGs between bacteria in the alimentary canal of house flies. Investigation of these candidate plasmids and ARGs could aid in our understanding of the role house flies play in the development and transfer of resistance in confined cattle or other animal production settings.

A few limitations of this study are worth consideration for their implications on these findings and for future studies. First, the isolates collected were biased toward more highly antibiotic-resistant strains of *E. coli* from a single collection site. Isolates were obtained by selective culturing on antibiotic-infused MAC agar, and all isolates originated from a farm that

had a greater prevalence of MDR isolates than three other farms (Chapter 3). Therefore, it is inconclusive whether the same patterns observed between *E. coli* isolated from flies and environmental substrates in our study would be seen for other AMR bacterial species or *E. coli* with different resistance profiles, particularly if collected from different farms. Second, the isolates analyzed in this study came from relatively small samples of the total abundance of flies, manure, feed, and water present at the collection site. Samples were not collected evenly from the environment, collecting only two samples per environmental type on each collection date compared to ten fly samples per date. Additionally, we examined multiple *E. coli* isolates from the same fly, manure, feed, or water samples. Collecting a larger number of samples from each substrate type and potentially pooling samples would benefit future studies aiming to generate more representative data from confined cattle operations. Third, to predict whether genes were of plasmid origin, plasmids were not isolated directly from bacterial isolates but instead relied on predictive models for plasmid identification. The predicted plasmid identities are more biased toward well-characterized plasmids, and because we sampled environmental *E. coli*, this likely contributed to a higher proportion of unidentifiable plasmids. Nevertheless, plasmids carrying targeted ARGs in field-collected *E. coli* that have not previously been investigated for horizontal gene transfer within the fly gut were identified. Future investigations could use improved methods to more accurately determine which plasmids in field settings carry these and other ARGs shared in flies and environmental sources, aiding prediction and experimental demonstration of horizontal gene transfer. Finally, observed phenotypes were compared between antibiotic susceptibility testing to the antibiotic classes predicted from AMRFinderPlus core genes, which are expected to have a phenotypic effect. It was observed that AMRFinderPlus core predictions often poorly represented actual phenotypes, indicating that the presence of these

ARGs alone is not always indicative of phenotypic resistance, even when located on the same plasmid and well-aligned with sequences from isolates known to express the phenotype.

In summary, this study showed that adult house flies carry AMR *E. coli* that are very closely related to AMR *E. coli* observed in cattle manure, feed, and water samples from confined beef cattle. Furthermore, similarities in the trends of ARGs observed in isolates from flies versus manure, feed, and water, including the presence of ARGs in both fly and environmental isolates which have been previously observed to horizontally transfer within the fly gut were observed. These findings indicate that house flies play a role in the ecology of antibiotic-resistant bacteria within confined cattle operations and support their potential for use in AMR surveillance.

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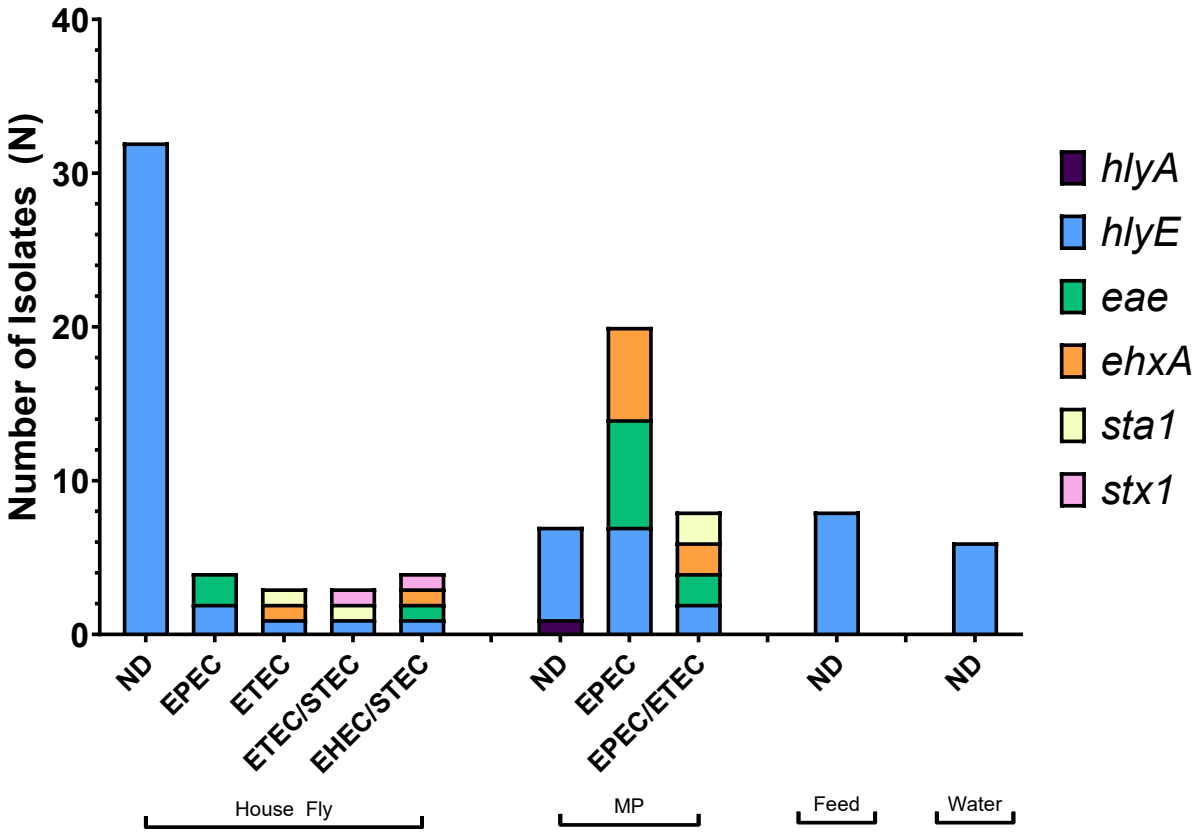


Figure 4.1. Distribution of *E. coli* pathotypes across house flies and environmental samples

Number of isolates from house flies, manure patties (MP), feed, and water samples assigned to each category of *E. coli* pathotype (EPEC, ETEC, EHEC, STEC) and carrying virulence genes. ND = pathotype not determined.

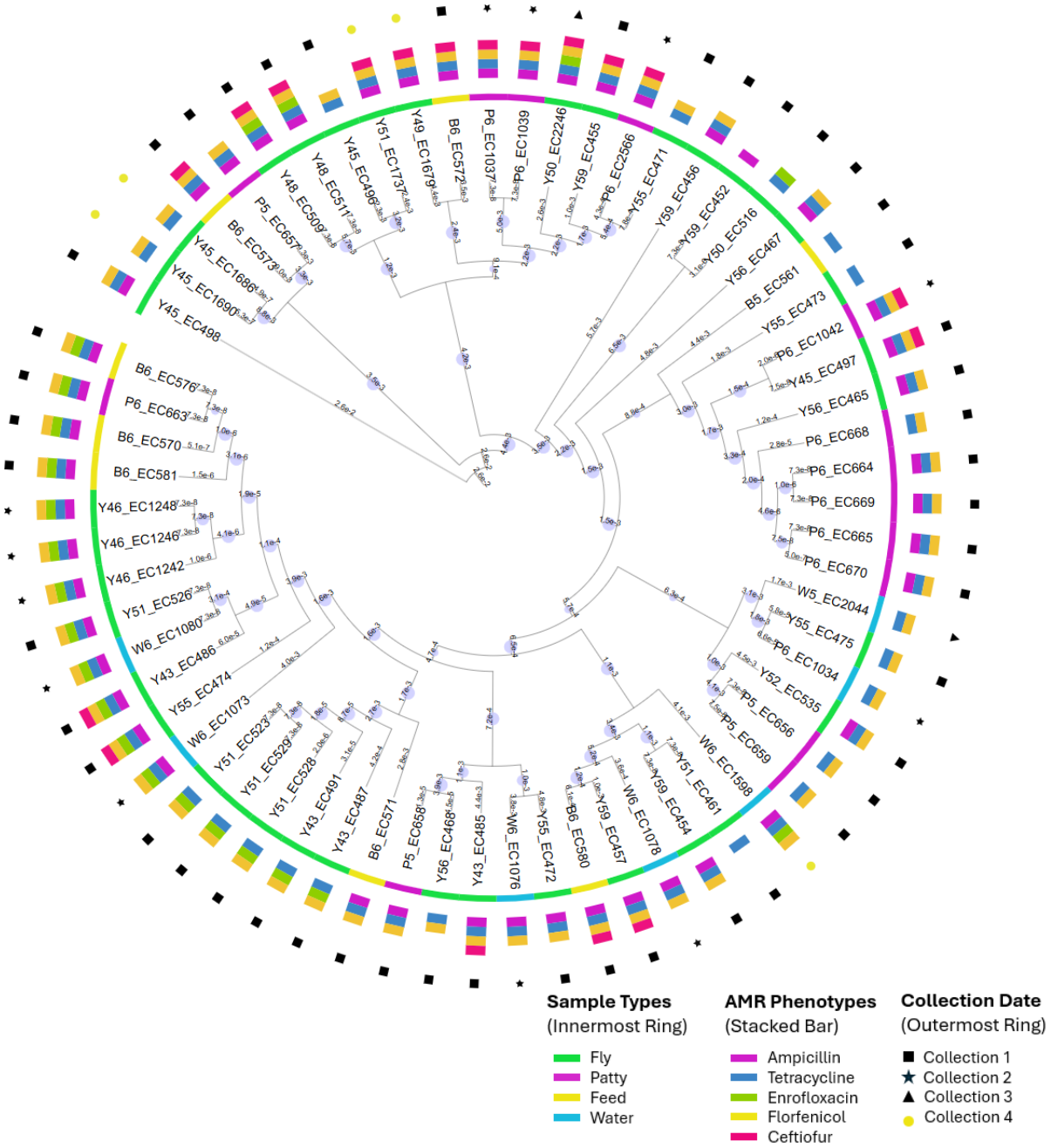


Figure 4.2. Maximum likelihood tree of AMR *E. coli* isolates from different sample types and collection dates

Maximum likelihood phylogeny of *E. coli* isolates from different sample types and collection dates. Innermost ring indicates sample type from which isolates originated color coded as indicated in the legend and named accordingly (Y=fly, P=patty, B=feed, W=water); stacked bar in middle ring indicates the measured resistance phenotypes of isolates for each antibiotic, color coded as indicated in legend; shapes around outer ring represent different collection dates of isolates as indicated in legend.

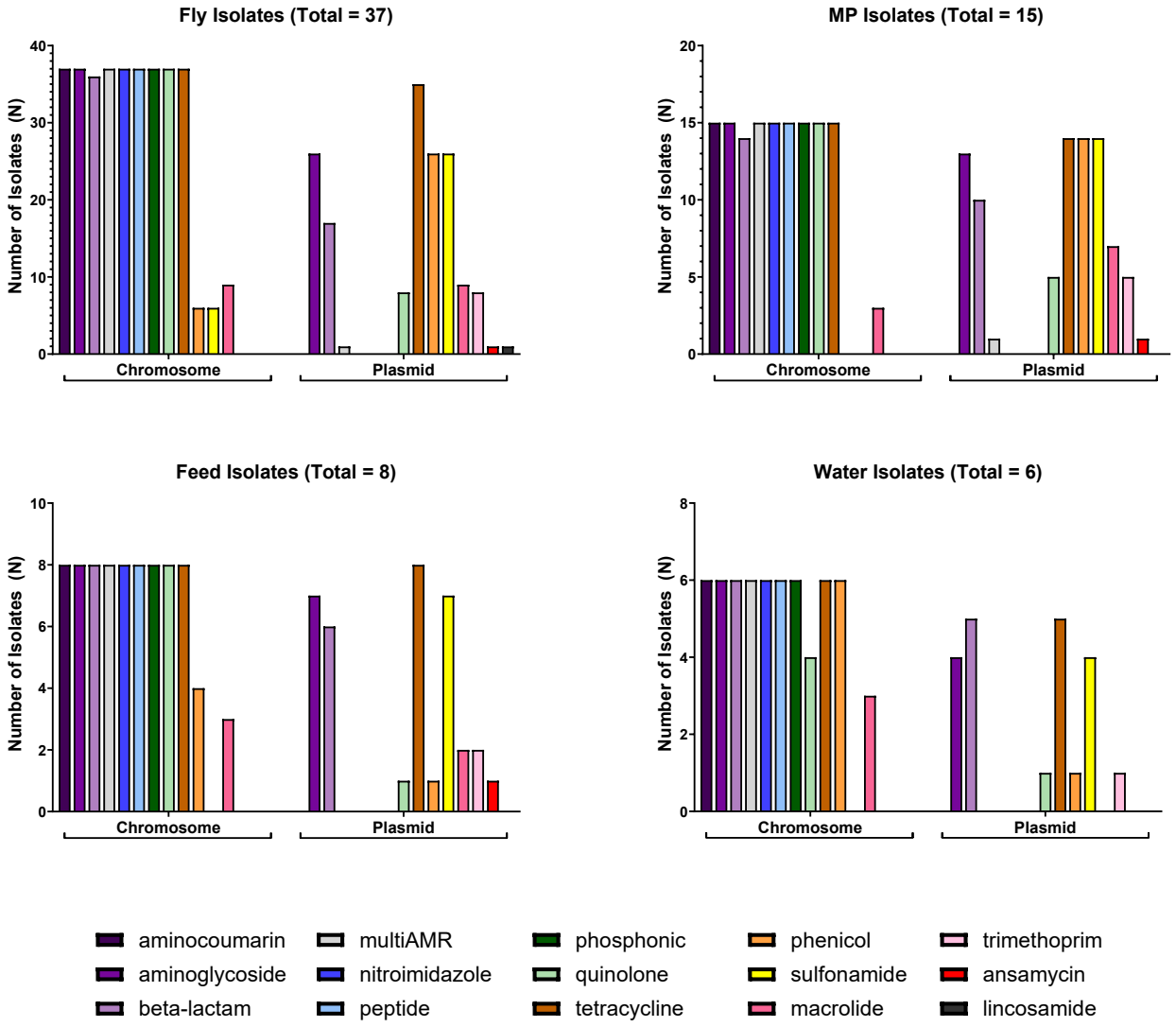
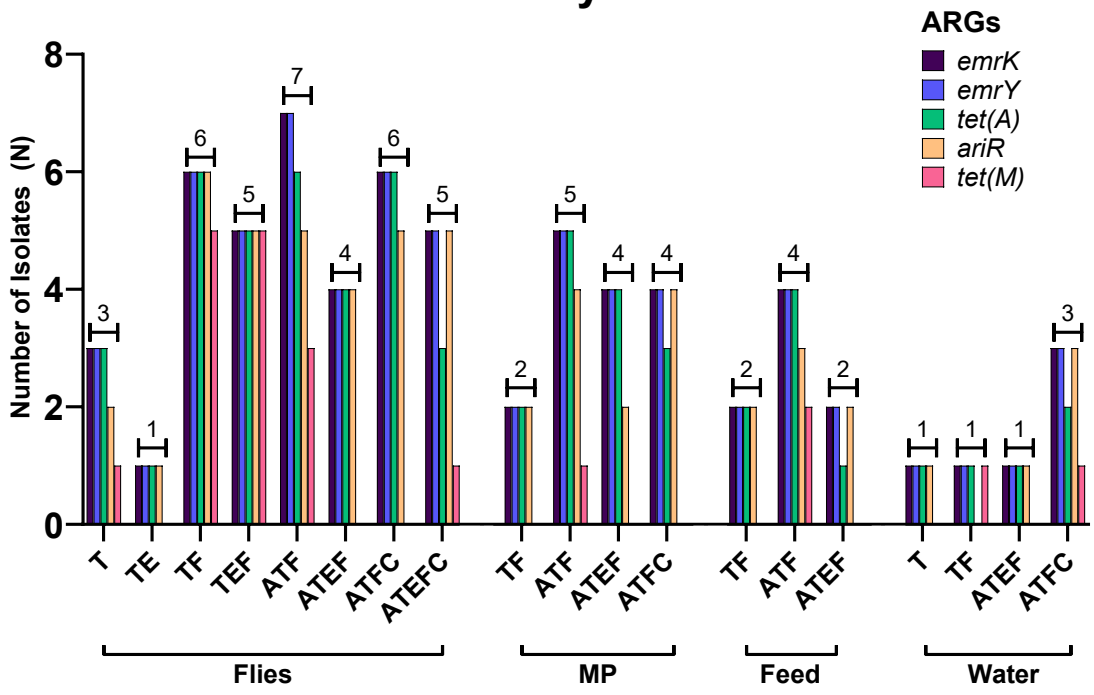


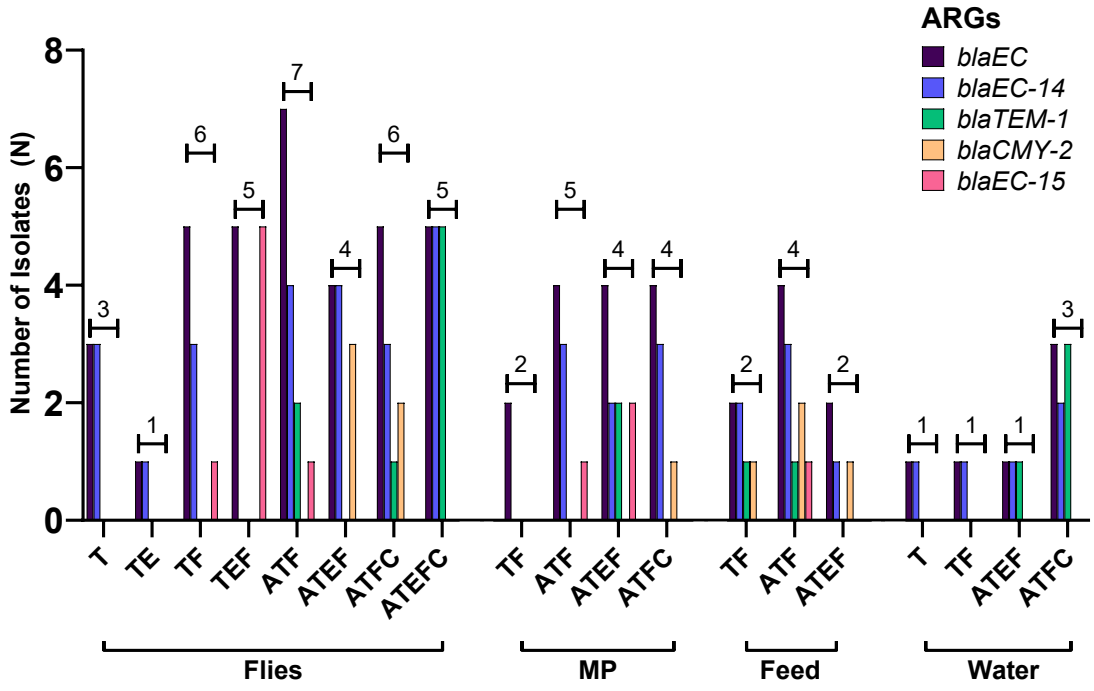
Figure 4.3. ARG-carrying *E. coli* isolates by sample type and genetic origin

Number of *E. coli* isolates from each sample type harboring ARGs allocated to different antibiotic classes and predicted to be of either plasmid or chromosomal origin.

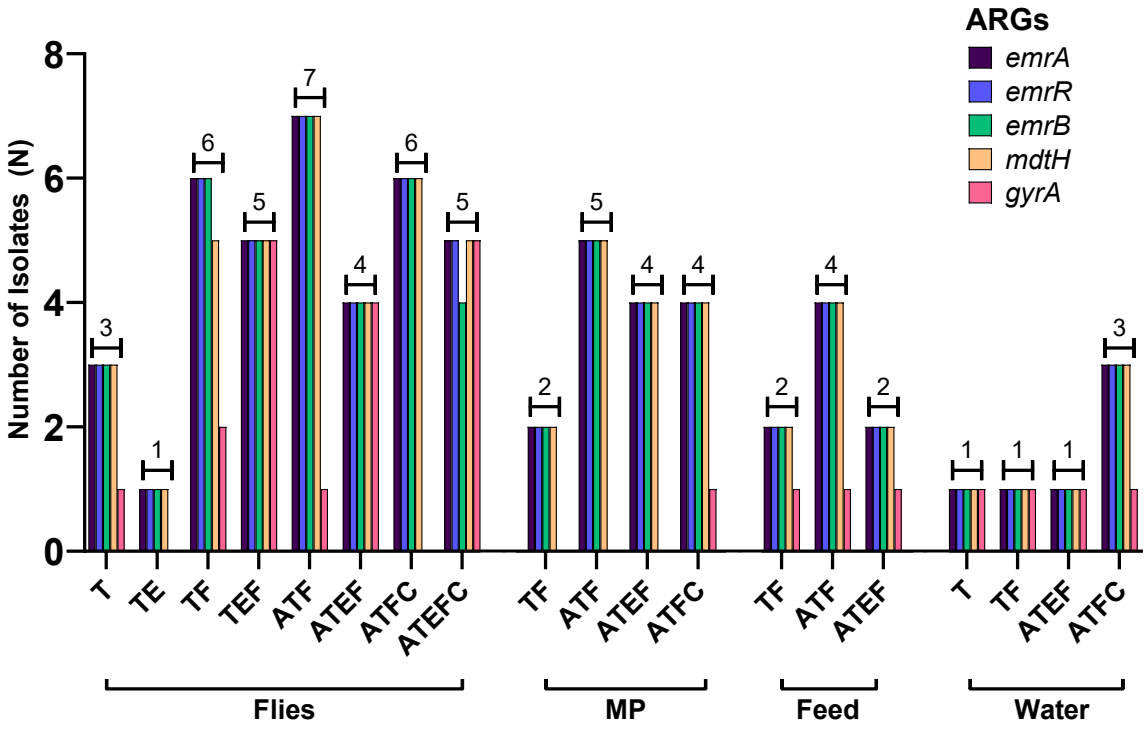
a)


Tetracyclines
ARGs
emrK
emrY
tet(A)
ariR
tet(M)
Number of Isolates (N)
0
2
4
6
8
3
1
6
5
7
4
6
5
2
5
4
4
2
4
2
1
1
1
3
T
TE
TF
TEF
ATF
ATEF
ATFC
ATEFC
TF
ATF
ATEF
ATFC
TF
ATF
ATEF
T
TF
ATEF
ATFC
Flies
MP
Feed
Water

b)


Beta-Lactams
ARGs
blaEC
blaEC-14
blaTEM-1
blaCMY-2
blaEC-15
Number of Isolates (N)
0
2
4
6
8
3
1
6
5
7
4
6
5
2
5
4
4
2
4
2
1
1
1
3
T
TE
TF
TEF
ATF
ATEF
ATFC
ATEFC
TF
ATF
ATEF
ATFC
TF
ATF
ATEF
T
TF
ATEF
ATFC
Flies
MP
Feed
Water

c)


Quinolones
ARGs
emrA
emrR
emrB
mdtH
gyrA
Number of Isolates (N)
0
2
4
6
8
3
1
6
5
7
4
6
5
2
5
4
4
2
4
2
1
1
1
3
T
TE
TF
TEF
ATF
ATEF
ATFC
ATEFC
TF
ATF
ATEF
ATFC
TF
ATF
ATEF
T
TF
ATEF
ATFC
Flies
MP
Feed
Water

d)

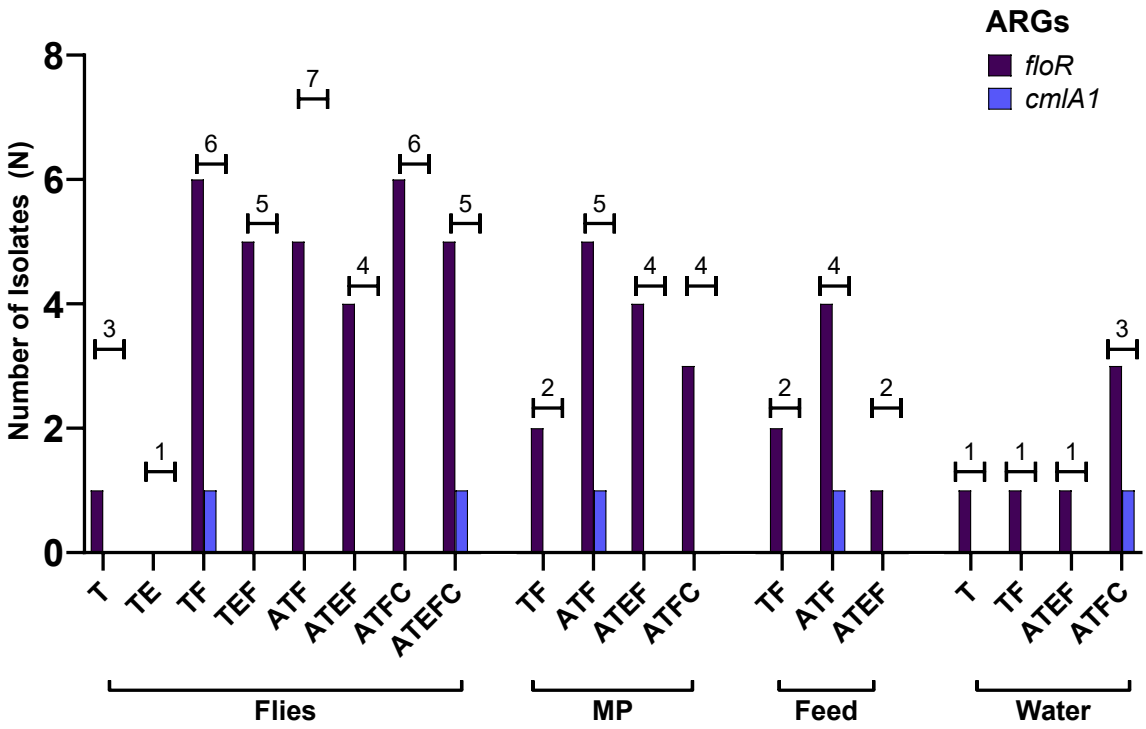

Phenicols
ARGs
floR
cmlA1
Number of Isolates (N)
0
2
4
6
8
3
1
6
5
7
4
6
5
2
5
4
4
2
4
2
1
1
1
3
T
TE
TF
TEF
ATF
ATEF
ATFC
ATEFC
TF
ATF
ATEF
ATFC
TF
ATF
ATEF
T
TF
ATEF
ATFC
Flies
MP
Feed
Water

Figure 4.4. Distribution of top ARGs among *E. coli* with distinct AMR phenotypes and sample type origins

Number of *E. coli* isolates from each sample type (flies, manure patty (MP), feed and water) harboring the top 5 non-multiclass ARGs observed for phenotypically tested antibiotic classes: tetracycline (a), beta-lactams (b), quinolones (c), and phenicols (d). Numbers above plotted bars indicate the total number of isolates observed for the specified AMR phenotype from that sample type. AMR phenotype profiles of isolates are shown as letter code combinations along x-axis. A= ampicillin, T= tetracycline, E= enrofloxacin, F= florfenicol, C= ceftiofur.

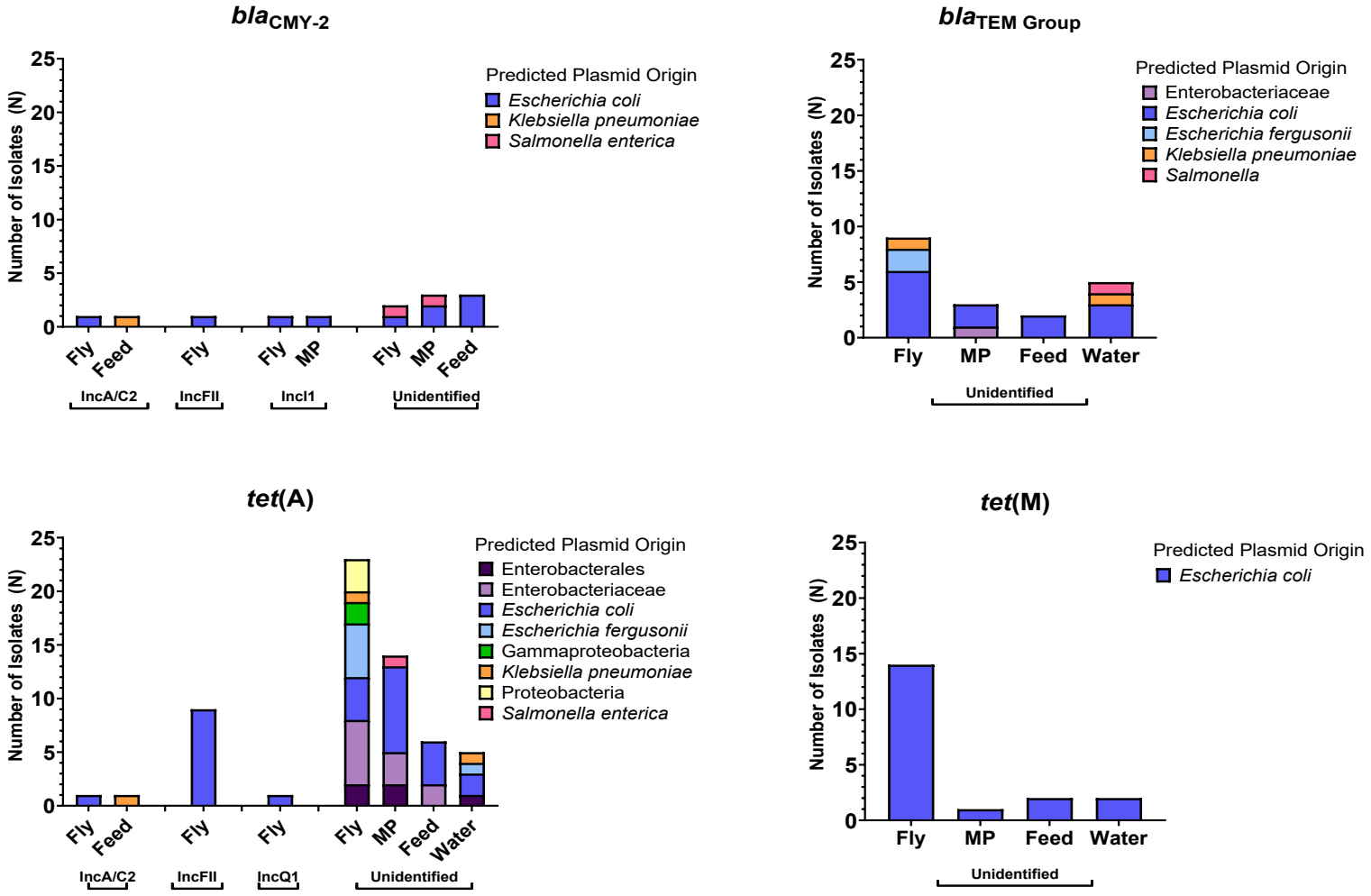


Figure 4.5. ARGs associated with horizontal gene transfer on plasmids in isolates by sample type and plasmid origin

Number of isolates from different sample types carrying ARGs previously associated with horizontal gene transfer among bacteria within the fly gut (*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM Group}}$, *tet*(A), and *tet*(M)) on identified (IncA/C2, IncFII, IncI1, IncQ1) or unidentified plasmids of predicted taxonomic origins.

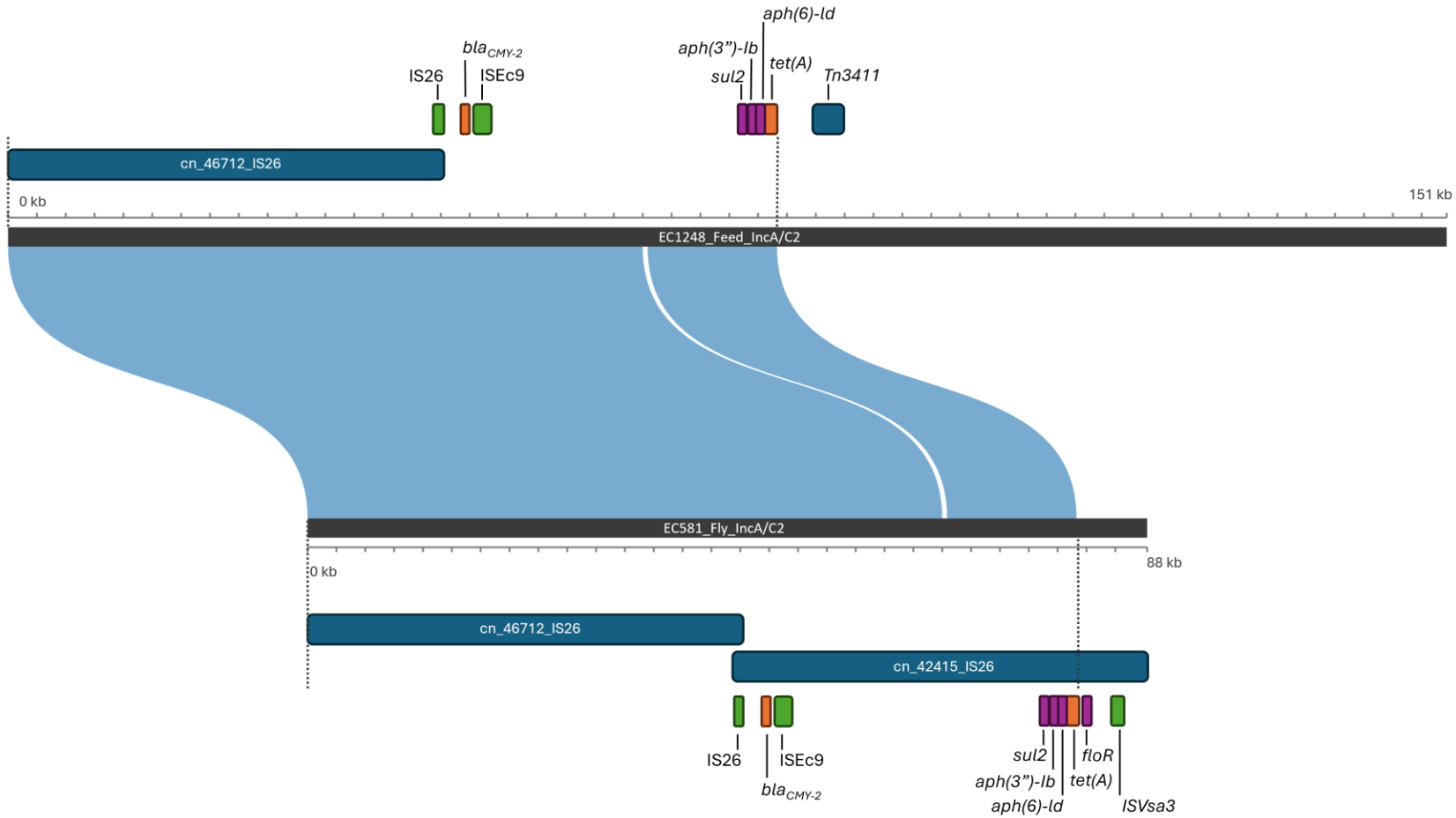


Figure 4.6. Gene synteny map of IncA/C2 plasmids from *E. coli* isolated from a house fly and feed sample

Graphical representation of the synteny of genes from the IncA/C2 plasmids found in isolates EC581 (house fly origin) and EC1248 (feed origin), as well as the approximate position of identified mobile elements and ARGs. Blue boxes indicate transposons, green boxes represent insertion sequences, orange boxes represent the targeted ARGs (with previously demonstrated horizontal gene transfer potential), and purple boxes represent the ARGs additionally identified on each plasmid.

Chapter 5 - Conclusion

This dissertation research investigated the multifaceted role house flies play in harboring bacteria and antimicrobial resistance (AMR) within cattle operations. There were three key objectives: first, to assess the biological and environmental factors influencing the abundance of culturable bacteria and abundance and AMR profiles of suspected coliforms carried by adult house flies from beef and dairy cattle operations; second, to assess the relationship between culturable isolates of Gram-negative bacteria and their AMR profiles carried by adult house flies and the relationship between fly isolates and isolates from co-located cattle manure compost, cattle manure patties, feed, and water samples; and third, to use whole genome sequencing to elucidate the genetic relatedness and shared resistance genotypes between AMR *Escherichia coli* isolated from house flies and environmental sources within a confined beef operation.

Consistent with prior observations (Thomson et al. 2017, Neupane et al. 2020), female house flies carried higher abundances of culturable aerobic bacteria, Gram-negative bacteria, and suspected coliforms (SC) across all locations and collection dates in confined cattle operations (Chapters 2 and 3). These findings are unsurprising since female house flies exhibit distinct food preferences linked to their nutritional and reproductive needs (Neupane et al. 2023). Female house flies likely explore a wider range of resources to acquire nutrients essential for proper egg development (Glaser 1923), and more frequently interact with microbe-rich substrates when seeking potential oviposition sites leading to higher bacterial loads (Shah et al. 2016, Thomson et al. 2017). Abiotic factors including collection date, location, and farm type (beef, dairy) also significantly influenced the bacterial abundance in house flies, though the specific impact varied among aerobic bacteria, SC, and Gram-negative bacteria. As detailed in Chapter 2, our data allowed us to develop predictive models for estimating the risk of higher bacterial abundance in

house flies, thereby indicating the potential for increased bacterial transmission by flies and associated risks to cattle health. However, further research is necessary to enhance model accuracy and explore additional variables that might influence the abundance of bacteria carried by house flies, ultimately assessing the broader implications for human and animal health.

Bacterial taxa and their associated resistance phenotypes appeared to be highly dependent on their origin. This phenomenon was evident for SC isolates from house flies in both beef and dairy operations (Chapter 2) and for Gram-negative bacteria isolated from house flies, manure compost, manure patties, feed, and water (Chapter 3). Notably, SC isolates from house flies from beef operations exhibited a wider range of AMR phenotypes compared to those from dairy house flies, with significantly higher proportions of florfenicol, enrofloxacin, and ceftiofur resistance in SC isolates from beef flies (Chapter 2). We suspect that lower resistance to these antibiotics in dairy fly isolates is due to several factors: 1) florfenicol and enrofloxacin are not approved for use in female dairy cattle over 20 months of age (FDA-HHS 2023), 2) extralabel use of enrofloxacin and ceftiofur is prohibited (FDA-HHS 1997, FDA-HHS 2012), and 3) dairy operations generally experience a lower influx of animals from diverse geographic regions or with varied treatment histories.

We hypothesized that the AMR profiles of Gram-negative bacteria would cluster by sample type (i.e., house flies or environmental samples). However, we observed that while some taxa and resistance phenotypes were unique to fly isolates and environmental samples, the AMR trends for a given sample type were more strongly influenced by location (Chapter 3). This also suggests that AMR profiles in house flies are shaped by their surrounding environment, particularly the management practices (including history of antimicrobial use) and resulting antibiotic selective pressure on microbes in animals and in the environment. Similar conclusions

can be drawn from other studies where AMR bacteria prevalence in animal manure at organic cattle operations was lower compared to non-organic operations (Sato et al. 2005, Sjöström et al. 2020, Ager et al. 2023). Consequently, house flies could serve as valuable sentinels or indicators for AMR trends over time and can help identify areas requiring targeted AMR mitigation.

Chapters 3 and 4 provide compelling evidence that house flies are important carriers and potential reservoirs of AMR bacteria, mirroring their surrounding environments. A significant overlap was observed, with flies carrying 52.4% of unique taxa/AMR phenotype profile combinations shared between isolates from flies, manure compost, manure patties, feed, and water. Within sampled locations, flies carried isolates with over 70% of the AMR phenotype profiles observed in co-located environmental isolates (Chapter 3). Notably, some AMR phenotype profiles were unique to fly isolates, suggesting that house flies may carry newly introduced or evolving AMR that is not yet widespread in the environment, or residual AMR from previous exposures to sources no longer present in the environment. Furthermore, genomic analysis revealed a close genetic relationship between AMR *E. coli* from house flies and isolates from cattle manure patties, feed, and water (Chapter 4). A striking 90% of the unique antimicrobial resistance genes (ARGs) found in environmental isolates were also present in fly isolates, underscoring the role house flies play as carriers of ARGs present in the environmental bacteria they interact with daily. Crucially, conserved regions of IncA/C_2 plasmids, containing multiple ARGs and mobile genetic elements, were identified in both a fly and a feed *E. coli* isolate. These findings strongly suggest a historical horizontal gene transfer event and highlight house flies as key players in the dissemination of evolved AMR within confined cattle environments.

In conclusion, the findings from these three objectives demonstrate that house flies play a significant role in the movement of bacteria and AMR at cattle operations. Factors such as fly sex, climate, and geographic location can influence the abundance of aerobic bacteria carried by house flies and should be carefully considered when assessing the risks associated with house flies as vectors of pathogenic and AMR bacteria. Furthermore, the strong relationship between AMR bacteria and resistance genotypes carried by house flies and those found in environmental substrates highlights the strong influence of historic antibiotic usage and environmental contamination on the AMR profiles carried by adult house flies. Future research expanding on these outcomes would be instrumental in developing and implementing targeted fly pest management programs that could potentially mitigate the evolution, prevalence, and dissemination of pathogenic and AMR bacteria, thereby safeguarding livestock and human health.

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Sjöström K, Hickman RA, Tepper V, et al. 2020. Antimicrobial resistance patterns in organic and conventional dairy herds in Sweden. Antibiotics. 9:834. https://doi.org/10.3390/antibiotics9110834

Thomson JL, Yeater KM, Zurek L, et al. 2017. Abundance and accumulation of *Escherichia coli* and *Salmonella* Typhimurium procured by male and female house flies (Diptera: Muscidae) exposed to cattle manure. Ann. Entomol. Soc. Amer. 110: 37-44. https://doi.org/10.1093/aesa/saw082

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Appendix A - Supplemental Information for Chapter 2

Appendix Table A.1. Climate variables and their descriptions gathered from daily and hourly Kansas Mesonet weather station historical data reports.

Climate Variable	Description	Units
CollectionMinTemp	Minimum air temperature on collection date	°C
CollectionMaxTemp	Maximum air temperature on collection date	°C
CollectionAvgTemp	Average air temperature on collection date	°C
CollectionRelHumidityAvg	Average relative humidity on collection date	%
CollectionTotalPrec	Total precipitation amount on collection date	mm
CollectionWindAvg	Average wind speed on collection date	m/s
CollectionMaxWind	Maximum wind speed on collection date	m/s
CollectionSoilTempAvg	Average soil temperature at depth of 2 inches on collection date	°C
CollectionMinSoilTemp	Minimum soil temperature at depth of 2 inches on collection date	°C
CollectionMaxSoilTemp	Maximum soil temperature at depth of 2 inches on collection date	°C
3DAvgMinTemp	Average daily minimum air temperature for the collection date and 2 days prior	°C
3DAvgMaxTemp	Average daily maximum air temperature for the collection date and 2 days prior	°C
3DAvgTemp	Average air temperature for the collection date and 2 days prior	°C
3DAvgHumidity	Average of the daily average relative humidity for the collection date and 2 days prior	%

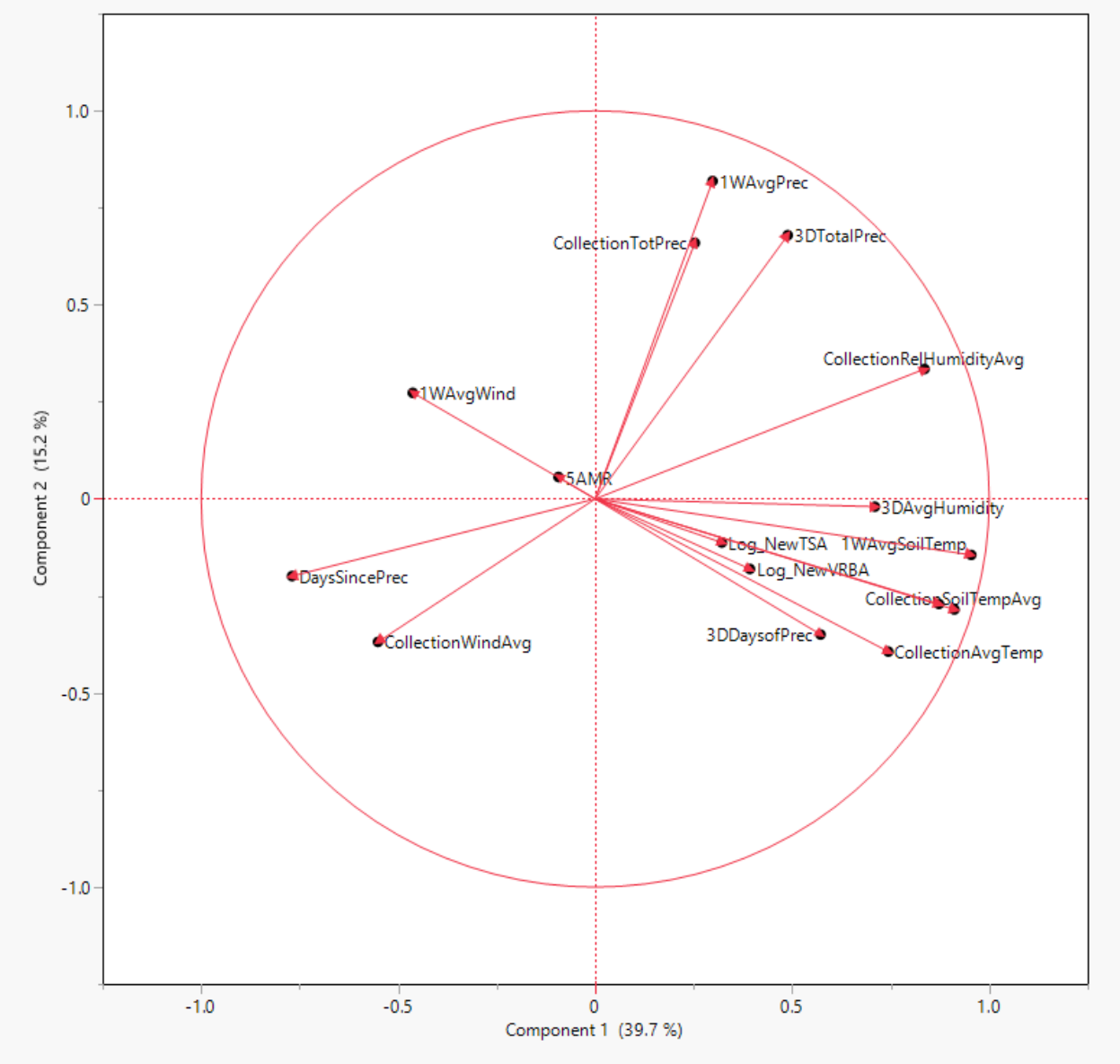
3DTotalPrec	Total precipitation amount for the collection date and 2 days prior	mm
3DDaysofPrec	Count of days with > 0 mm daily total precipitation for the collection date and 2 days prior	Count
3DAvgPrec	Average daily total precipitation for the collection date and 2 days prior	mm
3DAvgWind	Average of the daily average wind speed for the collection date and 2 days prior	m/s
3DAvgMaxWind	Average daily maximum wind speed for the collection date and 2 days prior	m/s
3DAvgSoilTemp	Average daily soil temperature at depth of 2 inches for the collection date and 2 days prior	°C
3DAvgMaxSoilTemp	Average daily maximum soil temperature at depth of 2 inches for the collection date and 2 days prior	°C
3DAvgMinSoilTemp	Average daily minimum soil temperature at depth of 2 inches for the collection date and 2 days prior	°C
1WAvgMinTemp	Average daily minimum air temperature for the collection date and 6 days prior	°C
1WAvgMaxTemp	Average daily maximum air temperature for the collection date and 6 days prior	°C
1WAvgTemp	Average air temperature for the collection date and 6 days prior	°C
1WAvgHumidity	Average of the daily average relative humidity for the collection date and 6 days prior	%
1WTotalPrec	Total precipitation amount for the collection date and 6 days prior	mm
1WDaysofPrec	Count of days with > 0 mm daily total precipitation for the collection date and 6 days prior	Count

1WAvgPrec	Average daily total precipitation for the collection date and 6 days prior	mm
1WAvgWind	Average of the daily average wind speed for the collection date and 6 days prior	m/s
1WAvgMaxWind	Average daily maximum wind speed for the collection date and 6 days prior	m/s
1WAvgSoilTemp	Average daily soil temperature at depth of 2 inches for the collection date and 6 days prior	°C
1WAvgMaxSoilTemp	Average daily maximum soil temperature at depth of 2 inches for the collection date and 6 days prior	°C
1WAvgMinSoilTemp	Average daily minimum soil temperature at depth of 2 inches for the collection date and 6 days prior	°C
DaysSincePrec	Count of days with rain > 0 mm daily total precipitation leading up to the collection date	Count

Appendix Table A.2. Mean abundances of culturable bacteria and suspected coliforms from male and female flies collected at each location. CFU = colony forming units.

Farm Type	County	Sex	*n*	Culturable Bacteria $\log_{10}$(CFU+1) ± SEM/fly	Suspected Coliform $\log_{10}$(CFU+1) ± SEM/fly
Dairy	Riley	F	28	5.84 ± 0.14^{A}	4.92 ± 0.20^{A}
		M	30	5.33 ± 0.13^{AB}	4.14 ± 0.20^{ABC}
	Marion	F	29	5.83 ± 0.13^{A}	4.58 ± 0.20^{AB}
		M	29	4.67 ± 0.13^{C}	3.21 ± 0.20^{CD}
	Washington	F	30	5.65 ± 0.13^{A}	4.36 ± 0.20^{AB}
		M	30	4.85 ± 0.13^{BC}	3.37 ± 0.20^{D}
Beef	Riley	F	29	5.88 ± 0.13^{A}	4.90 ± 0.20^{A}
		M	29	5.54 ± 0.13^{A}	4.37 ± 0.20^{AB}
	Marion	F	29	5.32 ± 0.13^{AB}	4.53 ± 0.20^{AB}
		M	30	4.70 ± 0.13^{C}	3.80 ± 0.20^{BCD}
	Washington	F	29	5.56 ± 0.13^{AB}	4.61 ± 0.20^{AB}
		M	30	4.81 ± 0.13^{BC}	4.04 ± 0.20^{ABCD}

*Differing letters indicate significant differences in pairwise comparisons of least square means (Tukey HSD, $p < 0.05$).



Appendix Figure A.1. Biplot of Loading Coefficients of the First and Second Principal Components, the collected climate variables with culturable bacteria and coliform abundances from house flies.

Appendix Table A.3. Tetracycline-resistant (Tet-R) SC isolates recovered from house flies collected at Kansas dairy and beef operations. Confirmed Tet-R isolates grew on tetracycline-infused MH agar and were interpreted as Tet-R by CLSI standards using disk susceptibility testing.

Farm Type	County	Total Isolates (*n*)	Suspected Tet-R (*n*)	Confirmed Tet-R (*n*)	Tet-R Isolates (%)
Dairy	Riley	93	44	25	26.88%
	Marion	70	30	15	21.43%
	Washington	86	41	15	17.44%
	Total	249	115	55	22.09%
Beef	Riley	146	91	39	26.71%
	Marion	159	100	53	33.33%
	Washington	107	69	40	37.38%
	Total	412	260	132	32.04%

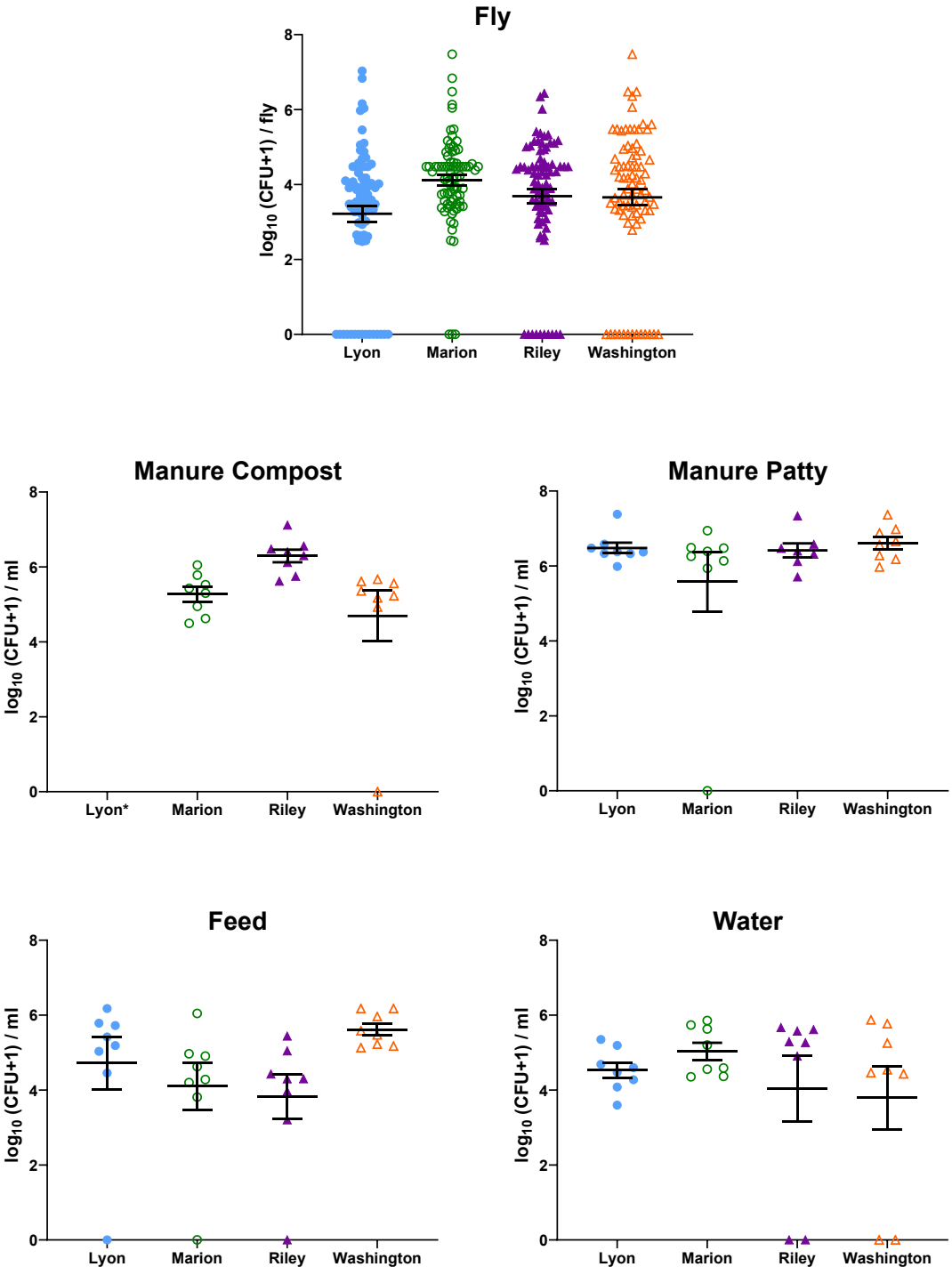
Appendix Table A.4. AMR SC isolates with single resistance recovered from house flies collected at Kansas dairy and beef operations.

		Dairy				Beef			
Genus	Phenotype	Riley	Marion	Washington	Total	Riley	Marion	Washington	Total
Citrobacter	Tet-R	2	0	2	**4**	0	0	1	**1**
	Amp-R	1	1	1	**3**	0	0	0	**0**
Enterobacter	Tet-R	1	0	0	**1**	0	0	0	**0**
	Amp-R	0	0	0	**0**	1	3	3	**7**
Escherichia/Shigella	Tet-R	14	7	5	**26**	18	28	23	**69**
	Amp-R	0	0	1	**1**	3	0	0	**3**
Klebsiella	Tet-R	0	0	0	**0**	0	1	0	**1**
	Amp-R	5	2	3	**10**	18	13	8	**39**
Kluyvera	Tet-R	0	0	2	**2**	1	1	1	**3**
	Amp-R	0	0	3	**3**	0	1	0	**1**
Kosakonia	Tet-R	0	0	0	**0**	0	0	0	**0**
	Amp-R	0	0	0	**0**	0	1	0	**1**
Leclercia	Tet-R	0	0	0	**0**	1	0	0	**1**
	Amp-R	0	0	0	**0**	0	0	0	**0**
Morganella	Tet-R	0	0	0	**0**	0	0	0	**0**
	Amp-R	0	1	0	**1**	0	0	0	**0**
Pantoea	Tet-R	0	0	0	**0**	0	0	0	**0**
	Amp-R	0	0	0	**0**	0	1	0	**1**
Proteus	Tet-R	0	0	0	**0**	0	1	1	**2**
	Amp-R	0	0	0	**0**	0	0	0	**0**
Providencia	Tet-R	1	2	0	**3**	5	0	2	**7**
	Amp-R	0	0	0	**0**	0	0	0	**0**
Pseudescherichia	Tet-R	0	0	0	**0**	0	2	0	**2**
	Amp-R	0	0	0	**0**	0	0	0	**0**
Pseudocitrobacter	Tet-R	0	0	0	**0**	0	0	0	**0**
	Amp-R	1	0	0	**1**	0	0	0	**0**
Raoultella	Tet-R	0	0	0	**0**	0	0	0	**0**
	Amp-R	0	0	0	**0**	1	0	0	**1**
Serratia	Tet-R	0	0	0	**0**	1	0	0	**1**
	Amp-R	3	4	3	**10**	2	5	5	**12**
Siccibacter	Tet-R	0	0	0	**0**	1	0	0	**1**
	Amp-R	0	0	0	**0**	0	0	0	**0**

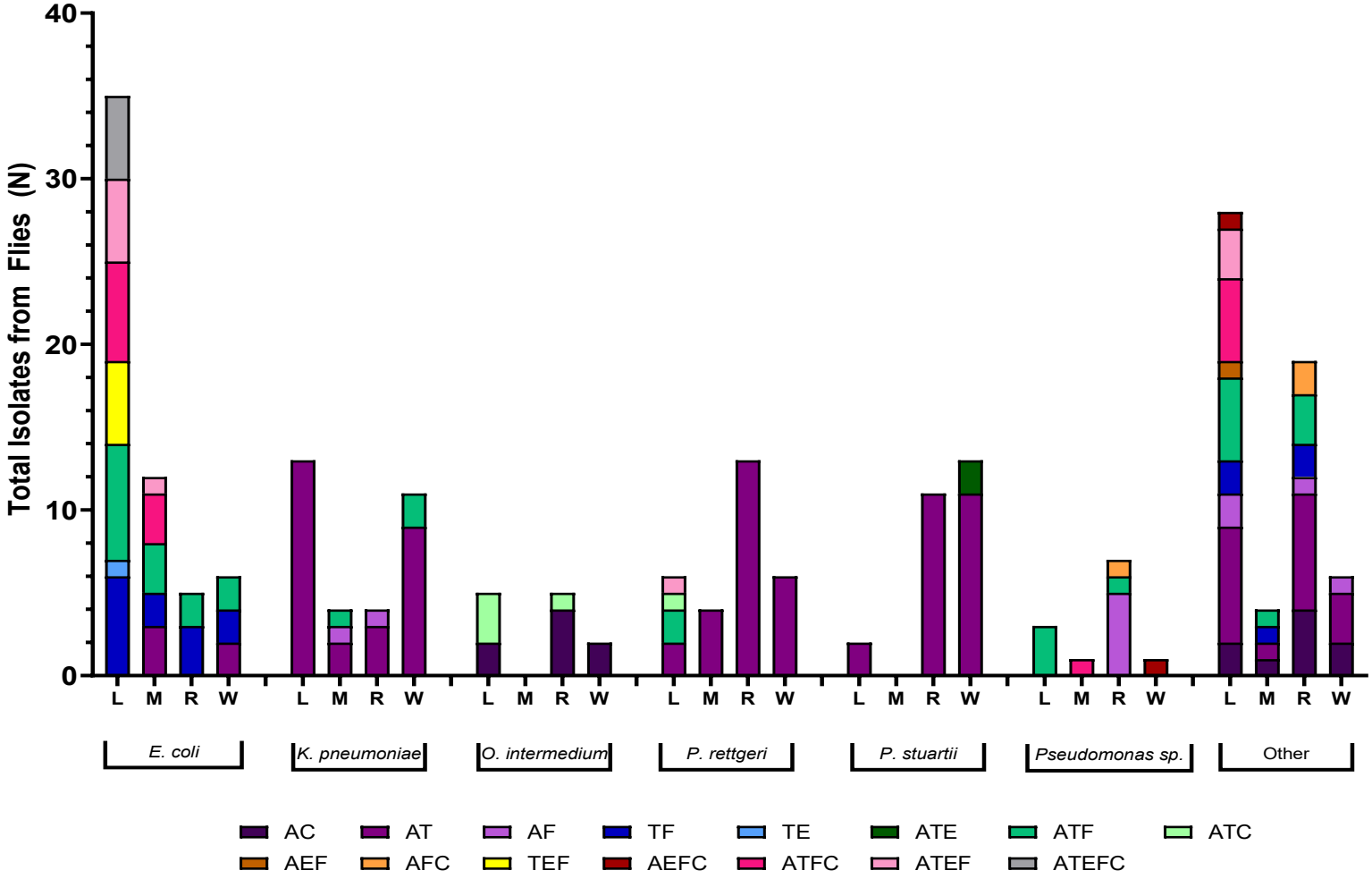
Appendix Table A.5. Multidrug-resistant SC isolates recovered from house flies collected at Kansas dairy and beef operations. Resistance phenotypes: T = tetracycline; F = florfenicol; A = ampicillin; C = ceftiofur; E = enrofloxacin.

			Dairy				Beef			
Phenotype	(N)	Genus	Riley	Marion	Washington	Total	Riley	Marion	Washington	Total
TA	(33)	*Enterobacter*	0	0	0	**0**	1	0	0	**1**
		Escherichia/Shigella	0	2	1	**3**	2	1	1	**4**
		Klebsiella	2	0	0	**2**	1	7	0	**8**
		Kluyvera	0	0	1	**1**	0	0	0	**0**
		Morganella	0	0	2	**2**	1	0	1	**2**
		Proteus	0	0	1	**1**	1	0	0	**1**
		Providencia	0	0	1	**1**	0	0	1	**1**
		Raoultella	1	0	0	**1**	1	0	0	**1**
		Serratia	0	1	0	**1**	0	2	1	**3**
TF	(9)	*Escherichia/Shigella*	1	0	0	**1**	1	3	2	**6**
		Pseudescherichia	0	0	0	**0**	0	2	0	**2**
TE	(1)	*Escherichia/Shigella*	0	0	0	**0**	0	1	0	**1**
FA	(2)	*Klebsiella*	0	0	0	**0**	0	0	1	**1**
		Serratia	0	0	0	**0**	0	0	1	**1**
FC	(1)	*Comamonas*	1	0	0	**1**	0	0	0	**0**
TFA	(18)	*Escherichia/Shigella*	3	2	0	**5**	4	3	4	**11**
		Pseudescherichia	0	1	0	**1**	0	0	1	**1**
TCA	(1)	*Escherichia/Shigella*	0	0	0	**0**	0	0	1	**1**
TFECA	(1)	*Pseudomonas*	0	0	0	**0**	0	1	0	**1**

Appendix B - Supplemental Information for Chapter 3



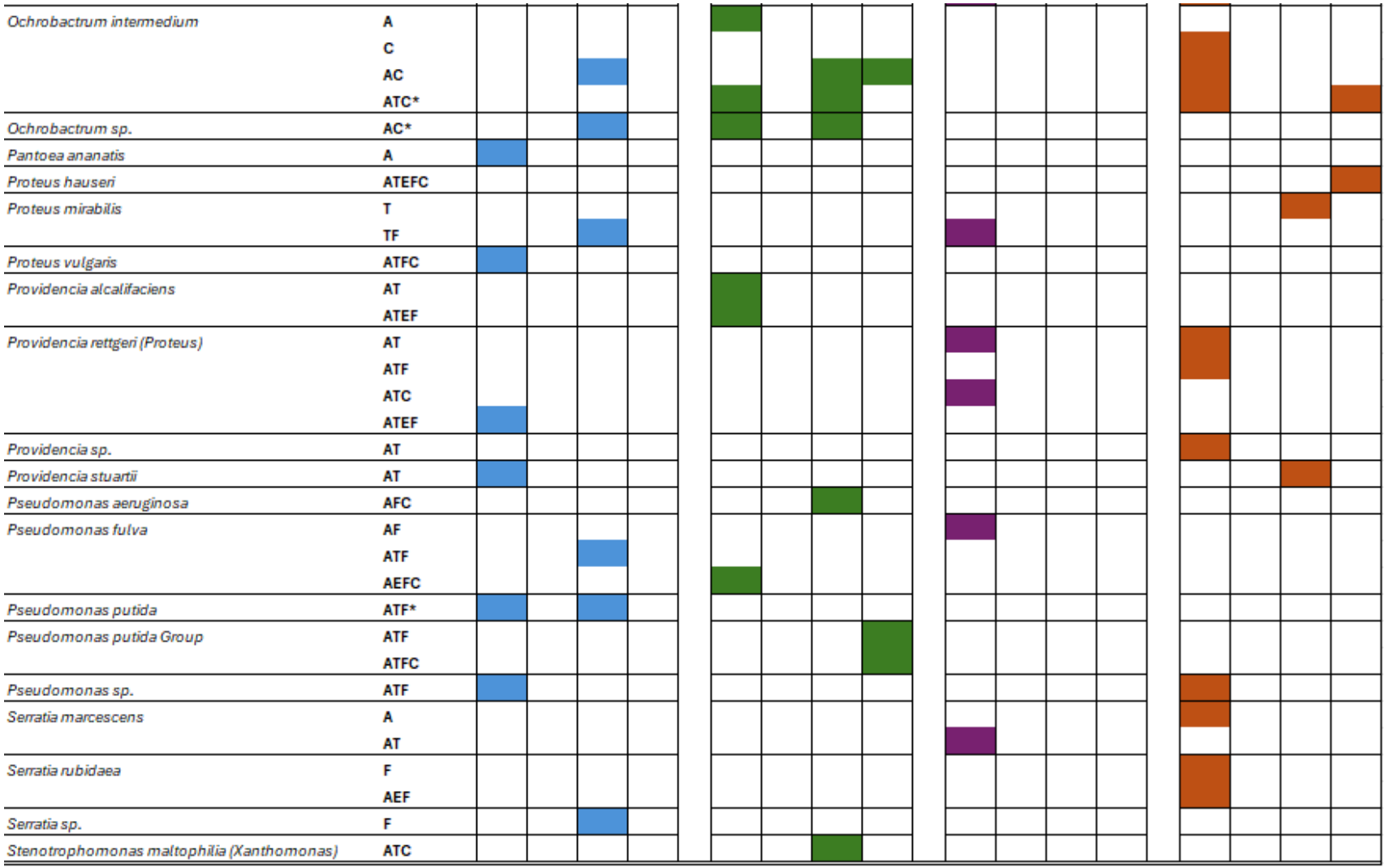
Appendix Figure B.1. Bacterial abundances (mean ± SEM) measured in flies, manure compost, manure patty, feed, and water samples from each location.



Appendix Figure B.2. Total isolates (N) from flies collected at Lyon (L), Marion (M), Riley (R), and Washington (W) locations with MDR profiles and assigned to *Escherichia coli, Klebsiella pneumoniae, Ochromobacter intermedium, Providencia rettgeri, Providencia stuartii*, *Pseudomonas sp.,* or "other" species. A= ampicillin, T= tetracycline, E= enrofloxacin, F= florfenicol, C= ceftiofur.

Appendix Table B.1. Prevalence of isolates from sample types collected at Lyon each week with corresponding taxa and AMR profiles. Shaded boxes indicate > 1 samples with an isolate belonging to the relevant taxon/AMR profile combination. "Fly Coverage" indicates the number of taxa/AMR profile combinations shared between flies and one or more environmental sample types within a collection week.

		Week 1 Fly Coverage = 7/14				Week 2 Fly Coverage = 4/18				Week 3 Fly Coverage = 0/3				Week 4 Fly Coverage = 1/5			
Taxa	**AMR Profile**	**Fly**	**MP**	**Feed**	**Water**	**Fly**	**MP**	**Feed**	**Water**	**Fly**	**MP**	**Feed**	**Water**	**Fly**	**MP**	**Feed**	**Water**
Achromobacter mucicolens	AF																
Acinetobacter radioresistens	T																
	ATF																
Acinetobacter sp.	C																
Alcaligenes faecalis (Pseudomonas odorans)	AC																
Citrobacter freundii	AT																
	ATF																
Comamonas jiangduensis	ATEF																
Enterobacter asburiae	A																
Enterobacter cloacae	A																
	AT*																
	ATF																
Enterobacter hormaechei	AT																
Enterobacter sp.	T																
Escherichia coli	T*																
	TF*																
	TE																
	ATF*																
	TEF																
	ATEF*																
	ATFC*																
	ATEFC																
Klebsiella aerogenes (Enterobacter)	ATFC																
Klebsiella pneumoniae	A																
	AT*																
Leclercia adecarboxylata	TF																
Morganella morganii (Proteus)	ATFC																
	ATEF																



*Isolates cultured from fly and environment on same collection date.

MP= Manure patty

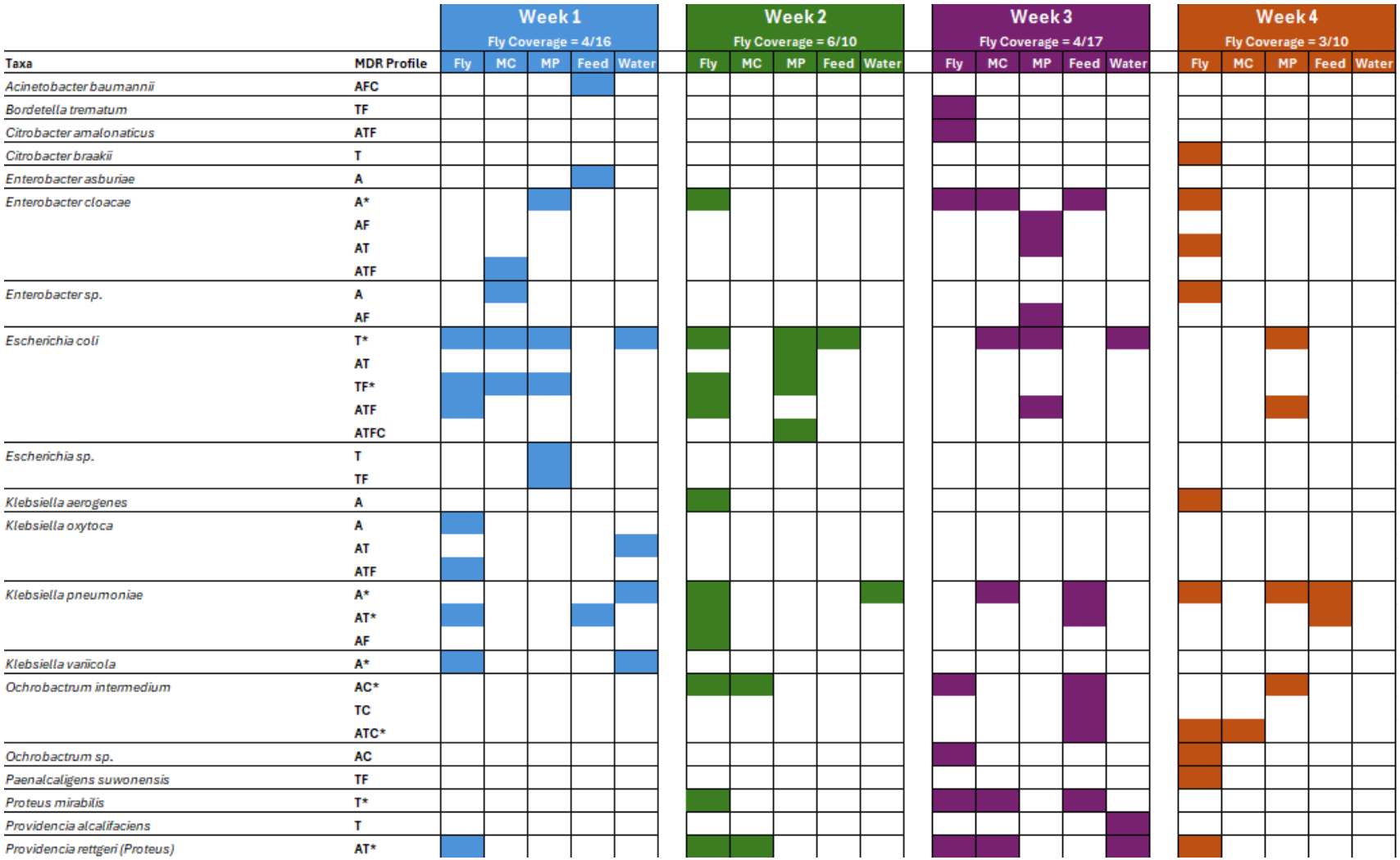
Appendix Table B.2. Prevalence of isolates from sample types collected at Marion each week with corresponding taxa and AMR profiles. Shaded boxes indicate > 1 samples with an isolate belonging to the relevant taxon/AMR profile combination. "Fly Coverage" indicates the number of taxa/AMR profile combinations shared between flies and one or more environmental sample types within a collection week. Fly column for week 2 was colored gray to show loss of samples.

Pseudomonas plecoglossicida	A																					
Pseudomonas putida	AF																					
Pseudomonas sp.	ATFC																					
Raoultella ornithinolytica	A																					
Serratia marcescens	A																					
	AT																					
Serratia nematodiphila	AT																					
Serratia rubidaea	A*																					
	F																					
	AF																					
Stenotrophomonas maltophilia (Xanthomonas)	AC																					
Unknown	AFC																					
	AEFC																					

*Isolates cultured from fly and environment on same collection date.

MC=manure compost; MP= manure patty

Appendix Table B.3. Prevalence of isolates from sample types collected at Riley each week with corresponding taxa and AMR profiles. Shaded boxes indicate > 1 samples with an isolate belonging to the relevant taxon/AMR profile combination. "Fly Coverage" indicates the number of taxa/AMR profile combinations shared between flies and one or more environmental sample types within a collection week.



Providencia sp.	AT*																					
Providencia stuartii	AT*																					
Providencia vermicola	AT																					
Pseudomonas aeruginosa	AFC																					
Pseudomonas fulva	AF																					
Pseudomonas putida	A																					
	AF																					
Pseudomonas sp.	AF																					
	ATF																					
	AFC																					
Raoultella ornithinolytica	A																					
Serratia marcescens	A*																					
	AT																					
Serratia rubidaea	A																					
	F																					
Serratia ureilytica	A																					
Stenotrophomonas maltophilia (Xanthomonas)	AC																					

*Isolates cultured from fly and environment on same collection date.

MC=manure compost; MP=manure patty

Appendix Table B.4. Prevalence of isolates from sample types collected at Washington each week with corresponding taxa and AMR profiles. Shaded boxes indicate > 1 samples with an isolate belonging to the relevant taxon/AMR profile combination. "Fly Coverage" indicates the number of taxa/AMR profile combinations shared between flies and one or more environmental sample types within a collection week.

Serratia marcescens	A																					
	AT																					
Serratia rubidaea	A																					
	AF																					
Serratia sp.	A																					
	AF																					
Stenotrophomonas maltophilia (Xanthomonas)	AC																					

*Isolates cultured from fly and environment on same collection date.

MC=manure compost; MP=manure patty

Appendix C - Supplemental Information for Chapter 4

Appendix Table C.1. Metadata summary for each *Escherichia coli* isolate and values from quality check of DNA extracts in preparation for whole genome sequencing.

Isolate ID	Collection Date	Sample Type	Fly Sex	Sample ID	DNA Concentration (ng/ul)	Total Volume (ul)	Total DNA (ug)	A260/280
EC452	8/6/2020	Fly	Female	F59-1.5-R1	25.07	20	0.50144	1.97
EC454	8/6/2020	Fly	Female	F59-1.5-R1	23.3	20	0.466	2.00
EC455	8/6/2020	Fly	Female	F59-1.5-R1	33.38	21	0.70094	1.96
EC456	8/6/2020	Fly	Female	F59-1.5-R1	31.57	21	0.66287	1.82
EC457	8/6/2020	Fly	Female	F59-1.5-R1	9.94	20	0.19886	1.78
EC461	8/6/2020	Fly	Female	F59-1.5-R2	36.21	20	0.72426	2.01
EC465	8/6/2020	Fly	Female	F56-0.5-R2	23.4	20	0.468	1.97
EC467	8/6/2020	Fly	Female	F56-0.5-R2	23.36	20	0.46716	1.99
EC468	8/6/2020	Fly	Female	F56-0.5-R2	26.63	20	0.5326	1.89
EC471	8/6/2020	Fly	Female	F55-H-R1	21.83	20	0.43654	1.95
EC472	8/6/2020	Fly	Female	F55-H-R1	27.55	20	0.551	1.96
EC473	8/6/2020	Fly	Female	F55-H-R1	23.6	20	0.472	2.00
EC474	8/6/2020	Fly	Female	F55-H-R1	14.1	20	0.282	1.94
EC475	8/6/2020	Fly	Female	F55-H-R2	36	22	0.792	1.88
EC485	8/6/2020	Fly	Male	F43-1-R1	19.6	20	0.392	1.90
EC486	8/6/2020	Fly	Male	F43-1-R1	28.2	20	0.564	1.92
EC487	8/6/2020	Fly	Male	F43-1-R1	22.6	20	0.452	2.03
EC491	8/6/2020	Fly	Male	F43-1-R1	15.7	20	0.314	1.94
EC496	8/6/2020	Fly	Male	F45-H-R2	23.2	20	0.46406	2.01
EC497	8/6/2020	Fly	Male	F45-H-R1	16.65	20	0.33298	1.99
EC498	8/6/2020	Fly	Male	F45-H-R1	28.64	20	0.57276	1.81
EC509	8/6/2020	Fly	Male	F48-1-R1	24.51	20	0.4901	1.86

EC511	8/6/2020	Fly	Male	F48-1-R1	23.45	20	0.46898	1.93
EC516	8/6/2020	Fly	Male	F50-1-R2	31.03	20	0.62064	1.80
EC523	8/6/2020	Fly	Female	F51-1-R2	31.4	20	0.62808	1.91
EC526	8/6/2020	Fly	Female	F51-1-R1	18.73	20	0.37464	1.90
EC528	8/6/2020	Fly	Female	F51-1-R1	32.73	20	0.65454	1.96
EC529	8/6/2020	Fly	Female	F51-1-R1	31.25	20	0.62502	1.99
EC535	8/6/2020	Fly	Female	F52-1-R2	28.14	21	0.591	1.82
EC561	8/6/2020	Feed		B5B-1.5-R1	31.7	20	0.63402	1.95
EC570	8/6/2020	Feed		B6B-2-R2	67.91	20	1.35822	1.91
EC571	8/6/2020	Feed		B6B-2-R2	37.43	20	0.74856	1.91
EC572	8/6/2020	Feed		B6B-2-R2	13.36	20	0.2672	1.82
EC573	8/6/2020	Feed		B6B-2-R2	31.63	20	0.63256	1.86
EC576	8/6/2020	Feed		B6B-2-R1	23.1	20	0.46198	2.01
EC580	8/6/2020	Feed		B6B-2-R1	19.36	20	0.38726	1.83
EC581	8/6/2020	Feed		B6B-2-R1	62.35	20	1.24692	1.97
EC656	8/6/2020	Patty		P5A-2.5-R1	36.95	20	0.73894	2.02
EC657	8/6/2020	Patty		P5B-2.5-R1	16.62	21	0.349	2.06
EC658	8/6/2020	Patty		P5B-2.5-R1	27.41	20	0.54818	1.99
EC659	8/6/2020	Patty		P5B-2.5-R2	18.33	21	0.38499	2.04
EC663	8/6/2020	Patty		P6B-2.5-R2	40.66	20	0.81316	2.05
EC664	8/6/2020	Patty		P6B-2.5-R2	36.1	20	0.72196	2.02
EC665	8/6/2020	Patty		P6B-2.5-R2	27.7	22	0.60929	2.01
EC668	8/6/2020	Patty		P6B-2.5-R2	34.31	20	0.68624	1.98
EC669	8/6/2020	Patty		P6B-2.5-R1	28.3	20	0.56598	1.97
EC670	8/6/2020	Patty		P6B-2.5-R1	21.59	20	0.43172	1.91
EC1034	8/20/2020	Patty		P6A-2.5-R1	24.22	20	0.48436	1.92
EC1037	8/20/2020	Patty		P6A-2.5-R1	28.56	20	0.57122	1.86
EC1039	8/20/2020	Patty		P6A-2.5-R1	27.22	21	0.57166	1.89

EC1042	8/20/2020	Patty		P6B-2.5-R1	27.44	20	0.5488	1.93
EC1073	8/20/2020	Water		W6A-2-R2	12.63	20	0.25266	1.91
EC1076	8/20/2020	Water		W6A-2-R2	18.44	20	0.3688	1.86
EC1078	8/20/2020	Water		W6A-2-R2	23.02	20	0.46034	1.90
EC1080	8/20/2020	Water		W6A-2-R2	23.74	20	0.47488	1.98
EC1242	8/20/2020	Fly	Male	F46-0.5-R1	42.14	20	0.84274	2.00
EC1246	8/20/2020	Fly	Male	F46-0.5-R1	22.19	20	0.44384	2.02
EC1248	8/20/2020	Fly	Male	F46-0.5-R1	45.45	20	0.909	1.97
EC1598	9/17/2020	Water		W6B-2.5-R1	41.34	20	0.82684	1.98
EC1679	9/17/2020	Fly	Male	F49-H-R1	18.3	21	0.3843	2.07
EC1686	9/17/2020	Fly	Male	F45-H-R2	45.72	20	0.91444	1.95
EC1690	9/17/2020	Fly	Male	F45-H-R2	29.55	20	0.591	1.97
EC1737	9/17/2020	Fly	Female	F51-4-R1	38.51	20	0.77024	1.95
EC2044	10/1/2020	Water		W5B-1.5-R2	21.14	20	0.4227	2.02
EC2246	10/1/2020	Fly	Male	F50-0.5-R2	30.39	20	0.60778	2.05
EC2566	8/20/2020	Patty		P6A-2.5-R1	25.21	21	0.52937	2.00

Appendix Table C.2. Sequencing statistics for each *Escherichia coli* isolate following whole genome sequencing, adapter removal, and trimming.

Isolate ID	No. Raw Reads	Total Raw Data (Gb)	Q20 (%)	Q30 (%)	GC (%)	No. Reads Passed Trimming	Proportion of Reads Trimmed(%)
EC1034	15063744	2.3	99.27	96.68	50.49	14993126	0.468794478
EC1037	16339054	2.5	99.24	96.57	50.74	16255968	0.508511692
EC1039	13709630	2.1	99.22	96.46	50.7	13640006	0.507847404
EC1042	16381616	2.5	99.21	96.42	50.51	16296282	0.520913199
EC1073	16272690	2.4	99.25	96.5	50.51	16197962	0.459223398
EC1076	17719354	2.7	99.25	96.56	50.77	17633340	0.485424017
EC1078	16657058	2.5	99.27	96.67	50.77	16577260	0.47906419
EC1080	16511012	2.5	99.21	96.33	50.9	16428182	0.501665192
EC1242	14782378	2.2	97.59	91.03	50.77	14468586	2.122743716
EC1246	15642568	2.3	99.26	96.59	50.67	15566710	0.48494595
EC1248	18639368	2.8	99.31	96.79	50.8	18558046	0.436291617
EC1598	17467864	2.6	99.3	96.72	50.67	17389614	0.447965475
EC1679	14768280	2.2	99.27	96.63	50.43	14698620	0.471686615
EC1686	10914384	1.6	99.18	96.29	50.54	10852764	0.564576068
EC1690	16021836	2.4	99.27	96.69	50.53	15945066	0.479158568
EC1737	13875648	2.1	99.09	95.97	50.61	13785606	0.648921045
EC2044	17953176	2.7	99.28	96.7	50.7	17867492	0.477263744
EC2246	17003350	2.6	99.24	96.58	50.71	16911568	0.539787748
EC2566	14673284	2.2	99.26	96.51	50.25	14606994	0.451773441
EC452	9634492	1.4	99.22	97.65	50.38	9581648	0.548487663
EC454	8398448	1.3	99.37	98.02	50.72	8368990	0.350755282

EC455	25654166	3.8	99.22	97.61	50.87	25533912	0.468750378
EC456	10615282	1.6	99.36	97.97	50.81	10576090	0.369203569
EC457	14069254	2.1	99.36	97.98	51.02	14016034	0.378271655
EC461	10341120	1.6	99.26	97.73	50.87	10293658	0.458963826
EC465	12227894	1.8	99.34	97.93	50.52	12181954	0.375698383
EC467	11629742	1.7	99.37	98.01	50.45	11588948	0.350773044
EC468	16013794	2.4	99.34	97.95	50.55	15950058	0.398006868
EC471	9995136	1.5	98.98	96.93	50.38	9946206	0.489538111
EC472	13413562	2	99.37	98	49.88	13365688	0.356907434
EC473	12827018	1.9	99.37	98	50.71	12783612	0.338395097
EC474	17539602	2.6	99.39	98.06	50.86	17453734	0.489566411
EC475	13344268	2	99.34	97.94	50.76	13292182	0.390324895
EC485	22286114	3.3	99.36	97.99	50.78	22200778	0.382911081
EC486	12872922	1.9	99.38	98.04	50.99	12827962	0.349260253
EC487	11741500	1.8	99.33	97.9	50.46	11696718	0.38139931
EC491	14860626	2.2	99.36	98	50.58	14806168	0.366458317
EC496	12302754	1.8	99.38	98.04	50.72	12259676	0.350149243
EC497	18373072	2.8	99.39	98.07	50.5	18312530	0.329514847
EC498	30675554	4.6	99.37	98.01	50.54	30570072	0.34386339
EC509	12026486	1.8	99.37	98.02	50.3	11980908	0.378980194
EC511	24750180	3.7	99.36	97.97	50.53	24665008	0.34412679
EC516	11425230	1.7	99.37	98.02	50.74	11385700	0.345988658
EC523	13913866	2.1	99.35	97.91	50.36	13865520	0.347466333
EC526	11940242	1.8	99.34	97.93	51.03	11893940	0.387781085

EC528	13486552	2	99.37	98.02	50.42	13440418	0.34207409
EC529	28255330	4.2	99.38	98.02	50.36	28158326	0.343312218
EC535	28440416	4.3	99.39	98.05	50.78	28343598	0.340423994
EC561	12041860	1.8	99.37	98	51	11998760	0.357918129
EC570	20360390	3.1	99.34	97.9	50.9	20284570	0.372389723
EC571	10812576	1.6	99.34	97.93	50.59	10772488	0.370753463
EC572	12835810	1.9	99.35	97.92	50.85	12789552	0.360382399
EC573	6535876	1	99.17	97.6	50.69	6482072	0.823210232
EC576	44344070	6.7	99.35	97.95	50.76	44176034	0.3789368
EC580	11111924	1.7	99.34	97.91	50.88	11069206	0.384433875
EC581	12867940	1.9	99.33	97.89	50.92	12818530	0.383977544
EC656	13688330	2.1	99.35	97.95	50.62	13636802	0.376437447
EC657	10196312	1.5	99.33	97.88	50.77	10156482	0.390631436
EC658	14030914	2.1	99.31	97.86	50.86	13971076	0.426472573
EC659	12392330	1.9	99.35	97.95	50.64	12349590	0.344890751
EC663	13425654	2	99.37	98	50.79	13379628	0.342821288
EC664	20299952	3	99.36	98	50.66	20226916	0.359784102
EC665	16418958	2.5	99.35	97.94	50.62	16360898	0.353615619
EC668	15062382	2.3	99.33	97.91	50.49	15005206	0.379594675
EC669	15432850	2.3	99.44	97.25	50.67	15376012	0.368292312
EC670	13659190	2	99.3	96.81	50.62	13597832	0.449206725

Appendix Table C.3. Summary of QUAST quality assessments of genome assemblies for each *Escherichia coli* isolate.

Isolate ID	Total Length (Mb)	N50 (Kb)	L90 (N)	Total Contigs (N)	Largest Contig (Kb)
EC1034	5.442563	91.374	64	553	230.655
EC1037	5.193094	67.446	97	886	212.019
EC1039	5.189522	67.466	95	861	211.938
EC1042	5.499454	209.842	37	472	327.628
EC1073	5.169546	273.885	25	433	828.557
EC1076	5.047124	235.214	28	429	671.755
EC1078	4.922648	236.463	26	313	614.517
EC1080	4.959026	323.35	15	247	512.418
EC1242	5.24139	239.739	24	523	571.685
EC1246	5.222505	295.989	21	401	670.453
EC1248	5.226455	295.989	21	424	670.353
EC1598	4.935582	175.439	27	351	382.579
EC1679	4.752244	107.963	50	295	250.728
EC1686	5.34535	240.126	30	558	618.237
EC1690	5.339174	240.101	28	546	417.513
EC1737	5.068916	108.63	46	339	292.392
EC2044	5.026494	125.097	41	445	624.707
EC2246	5.216444	172.62	36	467	625.842
EC2566	5.253479	108.071	52	449	294.228
EC452	4.711226	147.595	41	238	352.149
EC454	5.113407	185.958	31	351	539.059
EC455	4.784367	260.71	23	302	441.282
EC456	5.281932	157.481	41	425	365.178
EC457	5.03036	111.964	49	604	345.292
EC461	5.096815	193.653	32	296	538.982
EC465	5.60999	131.47	63	1056	255.669
EC467	5.247263	154.219	43	510	546.324
EC468	5.113038	209.423	26	325	649.577
EC471	5.205897	96.478	55	397	368.308
EC472	4.849537	210.655	22	267	675.276

EC473	4.822842	195.151	25	310	720.592
EC474	5.00007	233.039	20	323	821.426
EC475	5.345691	88.287	70	758	261.702
EC485	5.287454	164.872	35	1246	542.892
EC486	5.060799	226.385	21	406	411.659
EC487	5.263896	188.427	29	314	364.746
EC491	5.515523	200.446	32	1378	373.419
EC496	5.534907	69.062	81	700	308.048
EC497	5.483825	202.056	39	459	416.768
EC498	5.398162	101.695	55	710	379.882
EC509	4.867238	123.97	46	345	270.734
EC511	4.846398	118.44	46	323	255.291
EC516	4.72687	146.532	43	304	351.802
EC523	5.324471	160.816	32	339	478.117
EC526	4.9476	363.311	13	203	761.072
EC528	5.307162	182.922	31	294	478.313
EC529	5.327904	182.922	29	357	478.213
EC535	5.17601	238.251	29	332	613.273
EC561	4.840801	175.777	30	259	835.662
EC570	5.073905	297.063	18	337	599.814
EC571	5.145572	260.391	21	189	420.554
EC572	4.641151	94.473	54	243	217.713
EC573	5.065298	672.534	9	182	1598.611
EC576	5.087713	321.678	18	401	599.814
EC580	4.947332	157.482	38	420	388.492
EC581	5.048803	362.809	15	204	810.108
EC656	5.314576	176.539	35	311	397.105
EC657	5.530176	133.838	55	1009	285.711
EC658	5.092588	203.188	28	311	471.054
EC659	5.305611	176.539	35	272	579.255
EC663	5.059526	321.678	17	280	599.814
EC664	5.400717	75.065	82	911	289.403
EC665	5.529308	75.882	90	1293	289.307

EC668	5.396694	69.693	85	827	289.342
EC669	5.455425	75.058	83	1029	289.403
EC670	5.401586	86.67	80	811	251.163

Appendix Table C.4. Summary of BUSCO quality assessments of genome assemblies for each *Escherichia coli* isolate using the Enterobacteriaceae_odb12 lineage dataset.

Isolate ID	No. Complete BUSCOs	No. Single-Copy BUSCOs	No. Duplicated BUSCOs	No. Fragmented BUSCOs	No. Missing BUSCOs	Total BUSCOs
EC1034	871	869	2	2	1	874
EC1037	872	871	1	1	1	874
EC1039	872	871	1	1	1	874
EC1042	872	870	2	1	1	874
EC1073	872	871	1	1	1	874
EC1076	872	871	1	1	1	874
EC1078	872	870	2	1	1	874
EC1080	872	870	2	1	1	874
EC1242	872	871	1	1	1	874
EC1246	872	871	1	1	1	874
EC1248	872	871	1	1	1	874
EC1598	872	871	1	1	1	874
EC1679	871	870	1	1	2	874
EC1686	871	869	2	2	1	874
EC1690	871	869	2	2	1	874
EC1737	871	870	1	2	1	874
EC2044	872	871	1	1	1	874
EC2246	871	870	1	2	1	874
EC2566	872	871	1	1	1	874
EC452	872	870	2	1	1	874
EC454	872	871	1	1	1	874

EC455	872	871	1	1	1	874
EC456	872	871	1	1	1	874
EC457	872	871	1	1	1	874
EC461	872	871	1	1	1	874
EC465	872	870	2	1	1	874
EC467	871	869	2	2	1	874
EC468	872	871	1	1	1	874
EC471	871	869	2	2	1	874
EC472	872	871	1	1	1	874
EC473	871	870	1	2	1	874
EC474	871	869	2	2	1	874
EC475	870	869	1	3	1	874
EC485	871	870	1	2	1	874
EC486	872	871	1	1	1	874
EC487	871	870	1	2	1	874
EC491	871	870	1	2	1	874
EC496	871	870	1	2	1	874
EC497	872	870	2	1	1	874
EC498	872	871	1	1	1	874
EC509	872	872	0	1	1	874
EC511	872	872	0	1	1	874
EC516	872	870	2	1	1	874
EC523	871	869	2	2	1	874
EC526	872	870	2	1	1	874

EC528	871	869	2	2	1	874
EC529	871	869	2	2	1	874
EC535	871	870	1	1	2	874
EC561	872	871	1	1	1	874
EC570	872	870	2	1	1	874
EC571	871	870	1	2	1	874
EC572	872	871	1	1	1	874
EC573	872	871	1	1	1	874
EC576	872	870	2	1	1	874
EC580	872	870	2	1	1	874
EC581	872	870	2	1	1	874
EC656	872	871	1	1	1	874
EC657	872	871	1	1	1	874
EC658	872	871	1	1	1	874
EC659	872	871	1	1	1	874
EC663	872	870	2	1	1	874
EC664	871	869	2	2	1	874
EC665	872	870	2	1	1	874
EC668	872	870	2	1	1	874
EC669	872	870	2	1	1	874
EC670	872	870	2	1	1	874

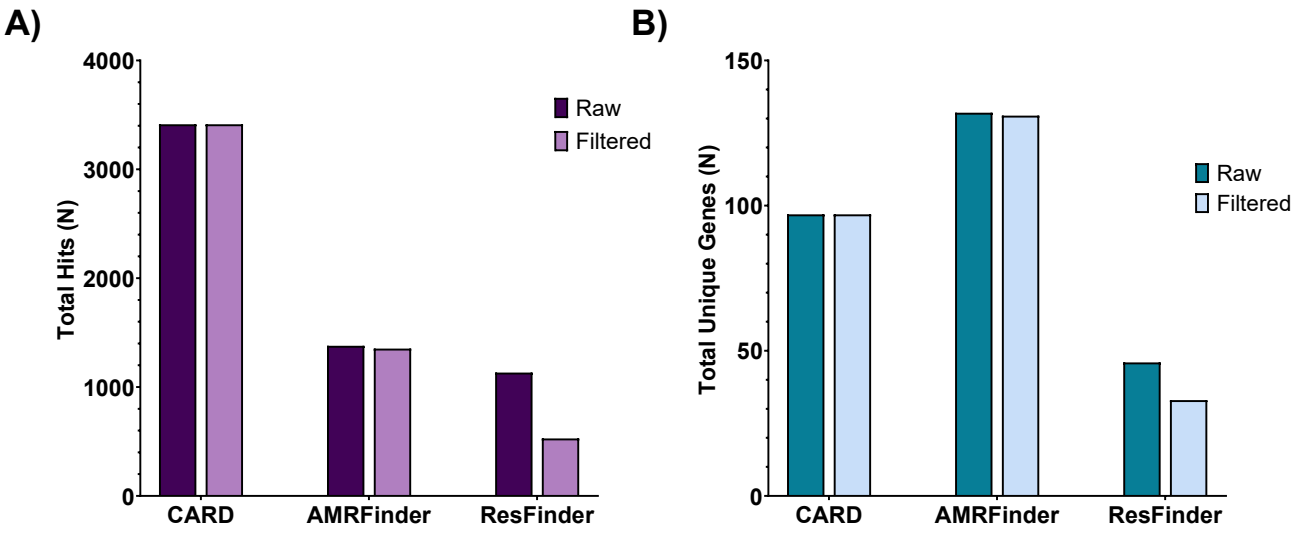
Appendix Table C.5. Summary of assignments for O- and H-antigens, serotype, and pathotypes, as well as pathotype genes identified from whole genomes of *Escherichia coli* isolated from different sample types.

Isolate ID	Sample Type	Sample ID	O-type	H-type	Serotype	Pathotype*	Pathotype Genes
EC1034	Patty	P6A-2.5-R1	O109	H16	O109:H16	ND	*hlyE*
EC1037	Patty	P6A-2.5-R1	O49	H10	O49:H10	EPEC/ETEC	*eae,ehxA,hlyE,sta1*
EC1039	Patty	P6A-2.5-R1	O49	H10	O49:H10	EPEC/ETEC	*eae,ehxA,hlyE,sta1*
EC1042	Patty	P6B-2.5-R1	O128	H2	O128:H2	EPEC	*eae,hlyE*
EC1073	Water	W6A-2-R2	O91	H28	O91:H28	ND	*hlyE*
EC1076	Water	W6A-2-R2	-	H8	-:H8	ND	*hlyE*
EC1078	Water	W6A-2-R2	O36	H5	O36:H5	ND	*hlyE*
EC1080	Water	W6A-2-R2	O91	H23	O91:H23	ND	*hlyE*
EC1242	Fly	F46-0.5-R1	O8	H23	O8:H23	ND	*hlyE*
EC1246	Fly	F46-0.5-R1	O8	H23	O8:H23	ND	*hlyE*
EC1248	Fly	F46-0.5-R1	O8	H23	O8:H23	ND	*hlyE*
EC1598	Water	W6B-2.5-R1	O8/O46/O134	H53	O8/O46/O134:H53	ND	*hlyE*
EC1679	Fly	F49-H-R1	O9	H30	O9:H30	ND	*hlyE*
EC1686	Fly	F45-H-R2	O87	H25	O87:H25	ND	*hlyE*
EC1690	Fly	F45-H-R2	O87	H25	O87:H25	ND	*hlyE*
EC1737	Fly	F51-4-R1	O8	H20	O8:H20	ND	*hlyE*
EC2044	Water	W5B-1.5-R2	O28/O42	H8	O28/O42:H8	ND	*hlyE*
EC2246	Fly	F50-0.5-R2	O8	H32	O8:H32	ND	*hlyE*
EC2566	Patty	P6A-2.5-R1	O4	H16	O4:H16	ND	*hlyA,hlyE*
EC452	Fly	F59-1.5-R1	-	H9	-:H9	ND	*hlyE*
EC454	Fly	F59-1.5-R1	O100	H25	O100:H25	ND	*hlyE*
EC455	Fly	F59-1.5-R1	O99	H9	O99:H9	ND	*hlyE*

EC456	Fly	F59-1.5-R1	O8	H19	O8:H19	ND	*hlyE*
EC457	Fly	F59-1.5-R1	O9	H25	O9:H25	ND	*hlyE*
EC461	Fly	F59-1.5-R2	O100	H25	O100:H25	ND	*hlyE*
EC465	Fly	F56-0.5-R2	O103	H2	O103:H2	EHEC-STEC	*eae,ehxA,hlyE,stx1*
EC467	Fly	F56-0.5-R2	O156	H8	O156:H8	EPEC	*eae,hlyE*
EC468	Fly	F56-0.5-R2	O83	H7	O83:H7	ND	*hlyE*
EC471	Fly	F55-H-R1	O13/O135	H11	O13/O135:H11	ND	*hlyE*
EC472	Fly	F55-H-R1	O108	H34	O108:H34	ND	*hlyE*
EC473	Fly	F55-H-R1	O128	H47	O128:H47	ND	*hlyE*
EC474	Fly	F55-H-R1	O83	H23	O83:H23	ND	*hlyE*
EC475	Fly	F55-H-R2	O109	H16	O109:H16	ND	*hlyE*
EC485	Fly	F43-1-R1	O18	H49	O18:H49	ND	*hlyE*
EC486	Fly	F43-1-R1	O114	H23	O114:H23	ND	*hlyE*
EC487	Fly	F43-1-R1	O9	H7	O9:H7	ND	*hlyE*
EC491	Fly	F43-1-R1	O8	H7	O8:H7	ND	*hlyE*
EC496	Fly	F45-H-R2	O159	H28	O159:H28	ETEC	*ehxA,hlyE,sta1*
EC497	Fly	F45-H-R1	O128	H2	O128:H2	EPEC	*eae,hlyE*
EC498	Fly	F45-H-R1	O2	H45	O2:H45	ETEC/STEC	*ehxA,hlyE,sta1,stx1*
EC509	Fly	F48-1-R1	O88	H38	O88:H38	ND	*hlyE*
EC511	Fly	F48-1-R1	O88	H38	O88:H38	ND	*hlyE*
EC516	Fly	F50-1-R2	-	H9	-:H9	ND	*hlyE*
EC523	Fly	F51-1-R2	O8	H7	O8:H7	ND	*hlyE*
EC526	Fly	F51-1-R1	O91	H23	O91:H23	ND	*hlyE*
EC528	Fly	F51-1-R1	O8	H7	O8:H7	ND	*hlyE*

EC529	Fly	F51-1-R1	O8	H7	O8:H7	ND	*hlyE*
EC535	Fly	F52-1-R2	O81	H10	O81:H10	ND	*hlyE*
EC561	Feed	B5B-1.5-R1	-	H10	-:H10	ND	*hlyE*
EC570	Feed	B6B-2-R2	O8	H23	O8:H23	ND	*hlyE*
EC571	Feed	B6B-2-R2	O83	H7	O83:H7	ND	*hlyE*
EC572	Feed	B6B-2-R2	O13/O135	H2	O13/O135:H2	ND	*hlyE*
EC573	Feed	B6B-2-R2	O27	H9	O27:H9	ND	*hlyE*
EC576	Feed	B6B-2-R1	O8	H23	O8:H23	ND	*hlyE*
EC580	Feed	B6B-2-R1	-	H37	-:H37	ND	*hlyE*
EC581	Feed	B6B-2-R1	O8	H23	O8:H23	ND	*hlyE*
EC656	Patty	P5A-2.5-R1	O9/O30	H19	O9/O30:H19	ND	*hlyE*
EC657	Patty	P5B-2.5-R1	O145	-	O145:-	EPEC	*eae,ehxA,hlyE*
EC658	Patty	P5B-2.5-R1	O83	H7	O83:H7	ND	*hlyE*
EC659	Patty	P5B-2.5-R2	O9/O30	H19	O9/O30:H19	ND	*hlyE*
EC663	Patty	P6B-2.5-R2	O8	H23	O8:H23	ND	*hlyE*
EC664	Patty	P6B-2.5-R2	O103	H2	O103:H2	EPEC	*eae,ehxA,hlyE*
EC665	Patty	P6B-2.5-R2	O103	H2	O103:H2	EPEC	*eae,ehxA,hlyE*
EC668	Patty	P6B-2.5-R2	O103	H2	O103:H2	EPEC	*eae,ehxA,hlyE*
EC669	Patty	P6B-2.5-R1	O103	H2	O103:H2	EPEC	*eae,ehxA,hlyE*
EC670	Patty	P6B-2.5-R1	O103	H2	O103:H2	EPEC	*eae,ehxA,hlyE*

**E. coli* pathotypes: ND = not determinable; EHEC = Enterohemorrhagic; EPEC = Enteropathogenic; ETEC = Enterotoxigenic; STEC = Shiga toxin-producing.



Appendix Figure C.2. Total hits (A) and unique genes (B) identified for virulence and resistance genes from each ARG database across all isolates both before and after pseudogene filtering.

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CLUSTAL O(1.2.4) multiple sequence alignment


EC456_Fly                       -TLALVGEKIDRNRFTGEKVENSTFFNCDFSGADLSGTEFIGCQFYDRESQKGCNFSRAM  59
EC455_Fly                       -TLALVGEKIDRNRFTGEKVENSTFFNCDFSGADLSGTEFIGCQFYDRESQKGCNFSRAM  59
EC1039_Patty                    -TLALVGEKIDRNRFTGEKVENSTFFNCDFSGADLSGTEFIGCQFYDRESQKGCNFSRAM  59
NCBIReference_WP_012954666.1    MTLALVGEKIDRNRFTGEKVENSTFFNCDFSGADLSGTEFIGCQFYDRESQKGCNFSRAM  60
EC1037_Patty                    MTLALVGEKIDRNRFTGEKVENSTFFNCDFSGADLSGTEFIGCQFYDRESQKGCNFSRAM  60
EC573_Feed                      MTLALVGEKIDRNRFTGEKVENSTFFNCDFSGADLSGTEFIGCQFYDRESQKGCNFSRAM  60
                                 ************************************************************

EC456_Fly                       LKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGADFRGASFMNMITTRTWFCSAYITNTN  119
EC455_Fly                       LKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGADFRGASFMNMITTRTWFCSAYITNTN  119
EC1039_Patty                    LKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGADFRGASFMNMITTRTWFCSAYITNTN  119
NCBIReference_WP_012954666.1    LKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGADFRGASFMNMITTRTWFCSAYITNTN  120
EC1037_Patty                    LKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGADFRGASFMNMITTRTWFCSAYITNTN  120
EC573_Feed                      LKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGADFRGASFMNMITTRTWFCSAYITNTN  120
                                ************************************************************

EC456_Fly                       LSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSTFDWRAANFTHCDLTNSE  179
EC455_Fly                       LSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSTFDWRAANFTHCDLTNSE  179
EC1039_Patty                    LSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSTFDWRAANFTHCDLTNSE  179
NCBIReference_WP_012954666.1    LSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSTFDWRAANFTHCDLTNSE  180
EC1037_Patty                    LSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSTFDWRAANFTHCDLTNSE  180
EC573_Feed                      LSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSTFDWRAANFTHCDLTNSE  180
                                ************************************************************

EC456_Fly                       LGDLDIRGVDLQGVKLDSYQASLLMERLGIAVIG    213
EC455_Fly                       LGDLDIRGVDLQGVKLDSYQASLLMERLGIAVIG    213
EC1039_Patty                    LGDLDIRGVDLQGVKLDSYQASLLMERLGIAVIG    213
NCBIReference_WP_012954666.1    LGDLDIRGVDLQGVKLDSYQASLLMERLGIAVIG    214
EC1037_Patty                    LGDLDIRGVDLQGVKLDSYQASLLMERLGIAVI-    213
EC573_Feed                      LGDLDIRGVDLQGVKLDSYQASLLMERLGIAVI-    213
                                *********************************
```

Appendix Figure C.3. Alignment with Clustal Omega (Madeira et al. 2024). Full alignment for qnrB19 protein sequences from the NCBI reference gene and those found on Col400I plasmid in E. coli isolates from manure patty (EC1037, EC1039), fly (EC455, EC456), and feed (EC573).

```
EC658       -TGATGAAAAAATCGTTATGCTGCGCTCTGCTGCTGACAGCCTCTTTCTCCACATTTGCT        59
EC1679      ATGATGAAAAAATCGTTATGCTGCGCTCTGCTGCTGACAGCCTCTTTCTCCACATTTGCT        60
             ************************************************************

EC658       CAA 1142
EC1679      CA- 1142
            **
```

Appendix Figure C.4. Alignment with Clustal Omega (Madeira et al. 2024). Misalignment of head and tail end for blaCMY-2 sequences found on Incl1 plasmid in E. coli isolate EC1679 (top) from a fly versus E. coli isolate EC658 (bottom) from a manure patty. Used NCBI tool for sequence/ARG results to align.

```
EC581       TGCCTGCCGGCGCTGCGTCGCGGGCTTTGGAGAAATTCTTCAAATTCCCGTTGCAC    1196
EC1248      TGCCTGCCGGCGCTGCGTCGCGGGCTTTGGAGCGGCGCAGGGCAACGAGCCGATCG    1196
            **********************************     *     *
```

Appendix Figure C.5. Alignment with Clustal Omega (Madeira et al. 2024). Misalignment of tail end for tet(A) sequences found on IncA/C2 plasmid in E. coli isolate EC581 (top) from a fly versus E. coli isolate EC1248 (bottom) from feed.

```
EC581       ASITTWNGWAWIAGAALYLLCLPALRRGLWRNSSNSRC      398
EC1248      ASITTWNGWAWIAGAALYLLCLPALRRGLWSGAGQRAD      398
            ****************************** .:.:
```

Appendix Figure C.6. Alignment with Clustal Omega (Madeira et al. 2024). Misalignment of tail end for tet(A) protein sequences found on IncA/C2 plasmid in E. coli isolate EC581 (top) from a fly versus E. coli isolate EC1248 (bottom) from feed.

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CLUSTAL O(1.2.4) multiple sequence alignment


EC581       -TGATGAAAAAATCGTTATGCTGCGCTCTGCTGCTGACAGCCTCTTTCTCCACATTTGCT        59
EC658       -TGATGAAAAAATCGTTATGCTGCGCTCTGCTGCTGACAGCCTCTTTCTCCACATTTGCT        59
EC1248      -TGATGAAAAAATCGTTATGCTGCGCTCTGCTGCTGACAGCCTCTTTCTCCACATTTGCT        59
EC1679      ATGATGAAAAAATCGTTATGCTGCGCTCTGCTGCTGACAGCCTCTTTCTCCACATTTGCT        60
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EC581       GCCGCAAAAACAGAACAACAGATTGCCGATATCGTTAATCGCACCATCACCCCGTTGATG        119
EC658       GCCGCAAAAACAGAACAACAGATTGCCGATATCGTTAATCGCACCATCACCCCGTTGATG        119
EC1248      GCCGCAAAAACAGAACAACAGATTGCCGATATCGTTAATCGCACCATCACCCCGTTGATG        119
EC1679      GCCGCAAAAACAGAACAACAGATTGCCGATATCGTTAATCGCACCATCACCCCGTTGATG        120
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EC581       CAGGAGCAGGCTATTCCGGGTATGGCCGTTGCCGTTATCTACCAGGGAAAACCCTATTAT        179
EC658       CAGGAGCAGGCTATTCCGGGTATGGCCGTTGCCGTTATCTACCAGGGAAAACCCTATTAT        179
EC1248      CAGGAGCAGGCTATTCCGGGTATGGCCGTTGCCGTTATCTACCAGGGAAAACCCTATTAT        179
EC1679      CAGGAGCAGGCTATTCCGGGTATGGCCGTTGCCGTTATCTACCAGGGAAAACCCTATTAT        180
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EC581       TTCACCTGGGGTAAAGCCGATATCGCCAATAACCACCCAGTCACGCAGCAAACGCTGTTT        239
EC658       TTCACCTGGGGTAAAGCCGATATCGCCAATAACCACCCAGTCACGCAGCAAACGCTGTTT        239
EC1248      TTCACCTGGGGTAAAGCCGATATCGCCAATAACCACCCAGTCACGCAGCAAACGCTGTTT        239
EC1679      TTCACCTGGGGTAAAGCCGATATCGCCAATAACCACCCAGTCACGCAGCAAACGCTGTTT        240
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EC581       GAGCTAGGATCGGTTAGTAAGACGTTTAACGGCGTGTTGGGCGGCGATGCTATCGCCCGC        299
EC658       GAGCTAGGATCGGTTAGTAAGACGTTTAACGGCGTGTTGGGCGGCGATGCTATCGCCCGC        299
EC1248      GAGCTAGGATCGGTTAGTAAGACGTTTAACGGCGTGTTGGGCGGCGATGCTATCGCCCGC        299
EC1679      GAGCTAGGATCGGTTAGTAAGACGTTTAACGGCGTGTTGGGCGGCGATGCTATCGCCCGC        300
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EC581       GGCGAAATTAAGCTCAGCGATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAG        359
EC658       GGCGAAATTAAGCTCAGCGATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAG        359
EC1248      GGCGAAATTAAGCTCAGCGATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAG        359
EC1679      GGCGAAATTAAGCTCAGCGATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAG        360
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EC581       TGGCAGGGTATCCGCCTGCTGCACTTAGCCACCTATACGGCAGGCGGCCTACCGCTGCAG        419
EC658       TGGCAGGGTATCCGCCTGCTGCACTTAGCCACCTATACGGCAGGCGGCCTACCGCTGCAG        419
EC1248      TGGCAGGGTATCCGCCTGCTGCACTTAGCCACCTATACGGCAGGCGGCCTACCGCTGCAG        419
EC1679      TGGCAGGGTATCCGCCTGCTGCACTTAGCCACCTATACGGCAGGCGGCCTACCGCTGCAG        420
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EC581       ATCCCCGATGACGTTAGGGATAAAGCCGCATTACTGCATTTTTATCAAAACTGGCAGCCG        479
EC658       ATCCCCGATGACGTTAGGGATAAAGCCGCATTACTGCATTTTTATCAAAACTGGCAGCCG        479
EC1248      ATCCCCGATGACGTTAGGGATAAAGCCGCATTACTGCATTTTTATCAAAACTGGCAGCCG        479
EC1679      ATCCCCGATGACGTTAGGGATAAAGCCGCATTACTGCATTTTTATCAAAACTGGCAGCCG        480
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EC581       CAATGGACTCCGGGCGCTAAGCGACTTTACGCTAACTCCAGCATTGGTCTGTTTGGCGCG        539
EC658       CAATGGACTCCGGGCGCTAAGCGACTTTACGCTAACTCCAGCATTGGTCTGTTTGGCGCG        539
EC1248      CAATGGACTCCGGGCGCTAAGCGACTTTACGCTAACTCCAGCATTGGTCTGTTTGGCGCG        539
EC1679      CAATGGACTCCGGGCGCTAAGCGACTTTACGCTAACTCCAGCATTGGTCTGTTTGGCGCG        540
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EC581       CTGGCGGTGAAACCCTCAGGAATGAGTTACGAAGAGGCAATGACCAGACGCGTCCTGCAA        599
EC658       CTGGCGGTGAAACCCTCAGGAATGAGTTACGAAGAGGCAATGACCAGACGCGTCCTGCAA        599
EC1248      CTGGCGGTGAAACCCTCAGGAATGAGTTACGAAGAGGCAATGACCAGACGCGTCCTGCAA        599
EC1679      CTGGCGGTGAAACCCTCAGGAATGAGTTACGAAGAGGCAATGACCAGACGCGTCCTGCAA        600
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EC581       CCATTAAAACTGGCGCATACCTGGATTACGGTTCCGCAGAACGAACAAAAAGATTATGCC        659
EC658       CCATTAAAACTGGCGCATACCTGGATTACGGTTCCGCAGAACGAACAAAAAGATTATGCC        659
EC1248      CCATTAAAACTGGCGCATACCTGGATTACGGTTCCGCAGAACGAACAAAAAGATTATGCC        659
EC1679      CCATTAAAACTGGCGCATACCTGGATTACGGTTCCGCAGAACGAACAAAAAGATTATGCC        660
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EC581       TGGGGCTATCGCGAAGGGAAGCCCGTACACGTTTCTCCGGGACAACTTGACGCCGAAGCC        719
EC658       TGGGGCTATCGCGAAGGGAAGCCCGTACACGTTTCTCCGGGACAACTTGACGCCGAAGCC        719
EC1248      TGGGGCTATCGCGAAGGGAAGCCCGTACACGTTTCTCCGGGACAACTTGACGCCGAAGCC        719
EC1679      TGGGGCTATCGCGAAGGGAAGCCCGTACACGTTTCTCCGGGACAACTTGACGCCGAAGCC        720
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EC581       TATGGCGTGAAATCCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCC        779
EC658       TATGGCGTGAAATCCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCC        779
EC1248      TATGGCGTGAAATCCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCC        779
EC1679      TATGGCGTGAAATCCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCC        780
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EC581       AGCCACGTTCAGGAGAAAACGCTCCAGCAGGGCATTGCGCTTGCGCAGTCTCGCTACTGG        839
EC658       AGCCACGTTCAGGAGAAAACGCTCCAGCAGGGCATTGCGCTTGCGCAGTCTCGCTACTGG        839
EC1248      AGCCACGTTCAGGAGAAAACGCTCCAGCAGGGCATTGCGCTTGCGCAGTCTCGCTACTGG        839
EC1679      AGCCACGTTCAGGAGAAAACGCTCCAGCAGGGCATTGCGCTTGCGCAGTCTCGCTACTGG        840
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EC581       CGTATTGGCGATATGTACCAGGGATTAGGCTGGGAGATGCTGAACTGGCCGCTGAAAGCT        899
EC658       CGTATTGGCGATATGTACCAGGGATTAGGCTGGGAGATGCTGAACTGGCCGCTGAAAGCT        899
EC1248      CGTATTGGCGATATGTACCAGGGATTAGGCTGGGAGATGCTGAACTGGCCGCTGAAAGCT        899
EC1679      CGTATTGGCGATATGTACCAGGGATTAGGCTGGGAGATGCTGAACTGGCCGCTGAAAGCT        900
            ************************************************************

EC581       GATTCGATCATCAACGGCAGCGACAGCAAAGTGGCATTGGCAGCGCTTCCCGCCGTTGAG        959
EC658       GATTCGATCATCAACGGCAGCGACAGCAAAGTGGCATTGGCAGCGCTTCCCGCCGTTGAG        959
EC1248      GATTCGATCATCAACGGCAGCGACAGCAAAGTGGCATTGGCAGCGCTTCCCGCCGTTGAG        959
EC1679      GATTCGATCATCAACGGCAGCGACAGCAAAGTGGCATTGGCAGCGCTTCCCGCCGTTGAG        960
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EC581       GTAAACCCGCCCGCCCCCGCAGTGAAAGCCTCATGGGTGCATAAAACGGGCTCCACTGGT        1019
EC658       GTAAACCCGCCCGCCCCCGCAGTGAAAGCCTCATGGGTGCATAAAACGGGCTCCACTGGT        1019
EC1248      GTAAACCCGCCCGCCCCCGCAGTGAAAGCCTCATGGGTGCATAAAACGGGCTCCACTGGT        1019
EC1679      GTAAACCCGCCCGCCCCCGCAGTGAAAGCCTCATGGGTGCATAAAACGGGCTCCACTGGT        1020
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EC581       GGATTTGGCAGCTACGTAGCCTTCGTTCCAGAAAAAAACCTTGGCATCGTGATGCTGGCA        1079
EC658       GGATTTGGCAGCTACGTAGCCTTCGTTCCAGAAAAAAACCTTGGCATCGTGATGCTGGCA        1079
EC1248      GGATTTGGCAGCTACGTAGCCTTCGTTCCAGAAAAAAACCTTGGCATCGTGATGCTGGCA        1079
EC1679      GGATTTGGCAGCTACGTAGCCTTCGTTCCAGAAAAAAACCTTGGCATCGTGATGCTGGCA        1080
            ************************************************************

EC581       AACAAAAGCTATCCTAACCCTGTCCGTGTCGAGGCGGCCTGGCGCATTCTTGAAAAGCTG        1139
EC658       AACAAAAGCTATCCTAACCCTGTCCGTGTCGAGGCGGCCTGGCGCATTCTTGAAAAGCTG        1139
EC1248      AACAAAAGCTATCCTAACCCTGTCCGTGTCGAGGCGGCCTGGCGCATTCTTGAAAAGCTG        1139
EC1679      AACAAAAGCTATCCTAACCCTGTCCGTGTCGAGGCGGCCTGGCGCATTCTTGAAAAGCTG        1140
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EC581       CAA 1142
EC658       CAA 1142
EC1248      CAA 1142
EC1679      CA- 1142
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Appendix Figure C.7. Alignment with Clustal Omega (Madeira et al. 2024). Multi-alignment of blaCMY-2 nucleotide sequences of predicted plasmid origin in E. coli isolates from flies (EC581 and EC1679) versus E. coli isolates from feed (EC1248) and manure patty (EC658).