MOLECULAR EPIDEMIOLOGY OF FOODBORNE ZOONOTIC PATHOGENS AT THE RODENT-AGRICULTURE INTERFACE

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DEDICATION

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ABSTRACT

Wild mammals are reservoirs for a growing number of emerging infectious zoonotic diseases. In particular, wild rodents are well-known reservoirs and vectors for a wide range of viruses and bacteria that cause illness in humans, including foodborne pathogens. It is critical to understand the role that rodents play in emerging zoonotic disease because they are commonly associated with humans, especially as pests throughout our agricultural production systems. Although numerous studies document a link between rodent pests on farms and outbreaks of foodborne pathogens (e.g., *E. coli* O157, *Salmonella*, etc.) in Europe and Asia, comparatively little research has focused on the rodent-agricultural interface in the United States. Knowing this, the functional role that rodent pests serve in the amplification and transmission of zoonotic foodborne pathogens is poorly understood in the United States. Given this knowledge gap, my dissertation research is novel in that it will provide a new and unexplored dimension of the rodent-Ag interface in one of the most agriculturally productive regions in the United States, the Midwest. My objective is to test the hypothesis that peridomestic rodents, inhabiting food animal farms, are reservoirs and amplifiers of zoonotic foodborne pathogens and antibiotic resistant bacteria that threaten food production systems and public health initiatives.

We used a robust combination of mammalogy field methods, cutting-edge next generation sequencing, and traditional microbiological techniques to discover rodent-borne pathogens circulating on food animal farms. We collected rodents from food animal farms in MN and WI, identified the rodent species, and conduct metagenomic surveillance of putative foodborne pathogens from rodent feces. With the completion of this goal we were be able to describe rodent species diversity in different food animal farms and characterized the metagenomic bacterial community of rodent feces with passive screening for presence of putative zoonotic foodborne pathogens. We then phenotypically characterized rodent fecal isolates including antibiotic resistant *Escherichia coli* and Shiga-toxin producing *E. coli* (STEC) using traditional microbiological and molecular methods. We applied whole genome sequencing (WGS)

to confirm their virulence traits (e.g., virulence genes, sequence types, serotypes, plasmid) and antibiotic resistance. We utilized a robust combination of comparative genomics and phylogenetic methods to analyze the resulting WGS data. Additional phylogenetic analyses were conducted in the context of relevant human and food animal pathogens of clinical origin gathered from publicly available databases. Successful completion of this goal enabled us to describe the molecular epidemiology of rodent associated zoonotic foodborne pathogens and how closely related or distant the rodent isolates were from human or animal origin (i.e., based on phylogenetic evolutionary relationships). Data generated from my dissertation research has filled an important knowledge gap regarding connections between the rodent-Ag interface and food safety in the two Midwestern states. Furthermore, we provided baseline data on the potential foodborne pathogens and AMR carried by rodents, which can be effectively used to inform public health officials, epidemiologists and farmers in order to improve rodent-associated biosecurity and protect our food supply.

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LIST OF ABBREVIATIONS

ARG antimicrobial Resistance Gene

AMR Antimicrobial Resistance

ARB Antimicrobial Resistant Bacteria

BHI Brain Heart Infusion

DNA Deoxyribonucleic acid

EHEC Enterohemorrhagic *Escherichia coli*

EPEC Enteropathogenic *Escherichia coli*

ESBL Extended-Spectrum Beta-lactamase

ETEC Enterotoxigenic *Escherichia coli*

ExPEC Extraintestinal pathogenic *Escherichia coli*

HUS Hemolytic uremic syndrome

IACUC institutional animal care and use committee

MDR Multi drug resistant

MGE Mobile genetic element

MLST Multilocus sequence typing

NARMS National Antimicrobial Resistance Monitoring System

ST Sequence type

NCBI National Center for Biotechnology Information

PAI Pathogenicity island

SNP Single nucleotide polymorphism

STEC Shiga toxin-producing *E. coli*

SRST2 Short read sequence typing for bacterial pathogens program

UPEC Uropathogenic *Escherichia coli*

UTI Urinary tract infection

VAG Virulence-associated gene

VTEC Verotoxigenic *Escherichia coli*

WGS Whole-genome sequencing

CHAPTER 1
Introduction

Introduction

Throughout history, various species of rodents have readily adapted to human dwellings and human-altered environments, becoming synantropic, peridomestic or agrophilic species depending on the environment (Régnier 1928; Khlyap and Warshavsky 2010; Paliy et al., 2021). Given the ever-present connection between rodents and human-related habitats, a number of rodent-borne zoonoses that negatively impact human health have emerged (Han et al. 2015). Indeed, rodents are one of the most important groups of zoonotic disease reservoirs, being associated with more than 80 zoonotic diseases (Han et al. 2016). These commensal species are mostly evaluated as possible carriers and vectors of various zoonotic pathogens including bacteria, viruses and parasites (Hornok et al., 2015; Jahan et al., 2020; Meheretu et al., 2021). Major rodent-borne diseases include hantavirus pulmonary syndrome, hemorrhagic fever with renal syndrome, plague, leptospirosis, and foodborne diseases among others (Meerburg et al. 2009, reviewed in Jahan et al. 2020). Rodent-borne diseases can be spread to humans through various means such as the consumption of contaminated food, the inhalation of the aerosol, rodent bites and arthropod vectors from rodents (Meerburg et al., 2009). Rodent feces are a significant source of foodborne pathogens, especially gastrointestinal diseases in humans and animals (Guenther et al., 2013). For example, house mice (*Mus musculus*) are in frequent contact with humans as they have readily adapted to our homes, restaurants, and food processing facilities. *Mus musculus*, along with other peridomestic

rodents (i.e., Norway rats; *Rattus norvegicus*) have been shown to harbor many pathogenic bacteria (Himsworth et al., 2013), which can easily be transmitted to humans via direct or indirect contact with rodent fecal shedding (e.g., contamination of food, water, utensils). Additionally, foodborne zoonotic pathogens can be transmitted directly to humans through the ingestion of wild rodents, as particular species are routinely consumed and are an important source of protein in some parts of the world (e.g., Africa, Asia, South America) (Gruber 2016).

Rodents are categorized as zoonotic disease reservoir or non-reservoir species based on features such as greater geographic range size and a fast-paced life history strategy that include earlier sexual maturity and higher reproduction rate (Han et al. 2015). Reservoir rodents generally capitalize on faster reproduction thus increasing population size rapidly, which likely reduces their vulnerability to demographic stochasticity (Pimm 1991). Therefore, zoonotic disease reservoir species of rodents are considered to be more resilient to anthropogenic disturbances, such as land-use change and urbanization (Rubio et al. 2014; Guo et al. 2018). For example, habitat conversion such as urbanization may result in favoring peridomestic rodent diversity and an increase in rodent abundance across a specific niche, which is an underlying mechanism for increasing disease risk (Mills 2006; Gottdenker et al. 2014; McCauley et al. 2015). Additionally, urbanization and food production systems (e.g., agricultural land, food animal farms) may also increase the prevalence of rodent-borne zoonotic pathogens regardless of the type of pathogen or its mode of transmission (e.g., direct contact, mediated by vectors; Ostfeld and Holt 2004). Given the well-documented connection between rodents and human disease, especially relating to human food sources, it is

critically important to investigate the bacterial community of rodent pests inhabiting agricultural landscapes (e.g., food animal farms, fresh produce farms). Such research might yield discoveries that can be leveraged to identify rodent reservoir species and prevent outbreaks of zoonotic food-borne pathogens (i.e., Nontyphoidal *Salmonella* spp., *E. coli*, *Campylobacter* spp., *Clostridium* spp., *Listeria monocytogenes* etc.) through proactive species-specific biosecurity plans (Jahan et al. 2020).

Antimicrobial resistance (AMR) is an emerging health concern worldwide for its adverse effects on both humans and animals. It is estimated that approximately 700,000 deaths occur each year due to antimicrobial-resistant bacterial infections globally (O'Neill 2020). Furthermore, over 2.8 million antimicrobial-resistant infections occur each year in the United States, with more than 35,000 fatal cases (CDC 2019). Antimicrobial resistance refers to the capacity of microorganisms to develop physical or biochemical mechanisms and processes that render antimicrobial agents ineffective, including antibiotics (Berry et al., 2013). In general, AMR is classified into two groups such as intrinsic or acquired. Intrinsic AMR results from the ability of the microorganism to survive in the presence of an antimicrobial agent because of an inherent property of the microorganism (e.g. efflux pump, enzyme), whereas acquired AMR stems either from gene mutation or by the microorganism acquiring extra gene coding for a resistance mechanism through horizontal gene transfer (HGT) from other bacteria or surrounding environment (Mathur and Singh 2005). Antimicrobials are commonly used in the treatment of microbial infections in both human and veterinary medicine, and also as growth promoters in livestock and food animal production in developing countries. Such

extensive use and misuse of antimicrobial agents in both human and animal health management put bacteria under selection pressure to develop and acquire resistance against several commonly used antibiotics including those used as a last resort (Mathur and Singh 2005; Singer et al., 2016). The human, animal, and environmental interface acts as a hotspot for the exchange of AMR microorganisms and their resistance genes (Hassell et al., 2019). Previous studies reveal that interaction with livestock can result in the transmission of antimicrobial resistance to wild animals, which is a direct consequence of agricultural pollution on AMR (Furness et al., 2017). However, data on the fate of AMR outside human and food animals is very limited, thus contributing to an inadequate understanding of the epidemiology of AMR (Hassell et al., 2019).

The occurrence of AMR in commensal rodents is a major human health concern, as they might transmit AMR to humans via direct contact or indirectly through food contamination. Moreover, invasive species of rodents can invade agricultural lands and thus they pose a threat to food animals, produce, and ultimately to human consumers. An increasing body of evidence shows that antimicrobial resistance occurs in wildlife, including mice and rats. Because peridomestic animals share the same environment with people, zoonotic disease transmission potential is high including the exchange of the AMR bacteria (Kruse and Sørum 1994; Desvars-Larrive et al., 2019; Zhong et al., 2020) through rodent fecal shedding (Kayihura 1982, Gilliver et al., 1999; Meerburg et al., 2009; Himsworth et al., 2014; and Himsworth et al., 20016). Examples of human disease causing bacteria isolated from rodents include *Yersinia pestis, Salmonella enterica* serovar. *Typhimurium. P*, Nontyphoidal *Salmonella*, *Streptococcus moniliformis*, *E. coli*, *Leptospira interrogans,* and *Yersinia enterocolitica*, some of which were observed to be

resistant to antimicrobials (Kayihura 1982; Wullenweber et al., 1995; Cleri et al., 1997; and Ong et al., 2020). In addition to the pathogenic bacteria, the role of commensal gut bacteria (e.g. Enterobacterales) should be considered as they dually constitute reservoirs and vectors of AMR and pose a threat to immunocompromised people. Several studies have reported bacteria in rodents harboring genes conferring resistance to ampicillin, penicillin, amoxicillin/clavulanic acid, tetracycline, streptomycin, cotrimoxazole, trimethoprim, ciprofloxacin, cefotaxime, gentamicin, apramycin, sulfamethoxazole, chloramphenicol, cephradine, cefuroxime, nalidixic acid, amoxicillin chloramphenicol, and orfloxacin (Gilliver et al., 1999; Hansen and Velschow 2000; Gakuya et al., 2001; Furness et al., 2017; Ong et al., 2020). Therefore, rodents can be considered as multiplication foci and mixing vessels for ARGs and are thus sources of AMR, which can be transmitted to other organisms such as humans, food animals and other wildlife (Gakuya et al., 2001).

The underlying mechanisms of successful transfer and transmission of resistant organisms among rodent, food animal, and human is unclear. One study conducted in New York City on the surveillance of AMR and pathogen carriage showed that house mice (*Mus musculus*) carry several drug resistant bacteria including resistance to fluoroquinolones (qnrB) and beta-lactam drugs (blaSHV and blaACT/MIR) in varying abundance (Williams et al. 2018). Interestingly, male mice exhibited more AMR genes compared to the females in a separate study, a pattern attributed to the comparatively broader exposure and larger home territory of male mice (Mikesic et al., 1992; Williams et al., 2018). In another study examining the potential source of drug resistance in a UK poultry farm, house mice were determined as a risk factor for the occurrence of

ciprofloxacin resistant *E. coli* in turkey flocks (Jones et al., 2013). Furthermore, *Salmonella* is an important foodborne pathogen causing millions of hospitalizations worldwide and increasing drug resistance in such pathogen is a matter of great concern (Skarz˙yn´ska et al. 2020). A study investigating the source of MDR *Salmonella* Infantis and extended spectrum cephalosporin resistant *S.* Heidelberg in a broiler farm suggested the inhabiting rodent population was the putative source of the resistant pathogens (Elhariri et al., 2020).

Transmission cycles of zoonotic pathogens and bacterial dissemination of AMR determinants are complex. Furthermore, the ability of rodents to act as reservoirs and vectors of zoonotic pathogens points to their potential use as sentinels of zoonosis and AMR occurrence. In general, sentinels are considered as bioindicators that are organisms with the potential to be used as an early warning system for infectious zoonotic diseases. In fact, a handful of studies have shown the effective use of small mammals such as mice, voles, and insectivores as sentinels of pathogenic and antimicrobial-resistant bacteria and their ARGs (Furness et al., 2017; Kmet et al., 2018). Hence, the detection of various zoonosis and AMR in rodents might lead to further investigations of zoonosis and AMR in humans and potential public health risks. However, the significance of rodents, as reservoirs, vectors, and sentinels of AMR, is poorly understood in the United States, especially where anthropogenic factors (e.g. human waste, pollution, agriculture) are involved (Kozak et al., 2008; Jardine et al., 2010; Radhouani et al., 2014; and Huijbers et al., 2019), thus warranting additional research.

Instrumental developments in sequencing techniques in recent times have provided important tools that can be leveraged to elucidate the genomic background and

pathways of zoonosis and AMR transmission. Genomic characterization of bacteria enables the correct identification of circulating strains among different environments and detection of possible links to clinically relevant resistant pathogens causing human and animal infections (Gan et al., 2020; Valli et al., 2020). To broaden our knowledge on diverse zoonotic pathogen and antimicrobial resistant bacterial transmission routes, the extensive surveillance of wild rodents, especially with the use of next generation sequencing technologies (e.g., Illumina, Nanopore), is required to reveal the zoonotic potential of bacteria originating from rodent reservoirs. Whole-genome sequencing (WGS) of isolates derived from wild rodents demonstrating diverse virulence and AMR profiles would help resolve their phylogenetic relationships and potential identification of novel genes responsible for pathogenicity (i.e., virulence, serotype, ARG). Furthermore, *in silico* analysis would allow the characterization of plasmids contributing to horizontal transfer of virulence and antibiotic resistance genes, and identification of novel antimicrobial resistance determinants. The application of such modern sequencing techniques might help identify circulating outbreak strains of any pathogen and possible spreading routes, including the direction of these transmissions.

Our goal is to investigate the role of rodent pests on livestock production operations (i.e., dairy and swine production facilities) as reservoirs or carriers of foodborne zoonotic pathogens and antibiotic resistant bacteria (ARB), especially with respect to species-specific patterns (i.e., native vs. invasive species). Three objectives support this goal: 1) Molecular surveillance of foodborne zoonotic pathogens in rodent pests across the agricultural landscape; 2) Phenotypic characterization and quantification of antibiotic resistance of foodborne pathogens isolated from rodent pests; and 3)

Genotypic characterization of rodent associated foodborne pathogens and genomic comparison with historical pathogenic isolates from human and animal sources. To address these objectives, we first collected data from available literatures, including outside the United States, regarding the rodent reservoir status for zoonotic pathogens with a foodborne pathogen focus and antimicrobial resistance. Then we described the rodent fecal metagenomic communities of farm-caught rodents and performed phenotypic and genotypic characterizations of putative foodborne zoonotic pathogens isolated from these rodents.

Through our literature review, we observed that the functional role that peridomestic (living in and around human habitations) rodents serve in the amplification and transmission of foodborne pathogens is underappreciated (**Chapter 2**). Clear links have been identified between rodent pests and outbreaks of foodborne pathogens throughout Europe and Asia, yet, comparatively little research has been devoted to studying this relationship in the United States. In particular, regional studies focused on specific rodent species and their foodborne pathogen reservoir status across the diverse agricultural landscapes of the United States are lacking. We posit that both native and invasive species of rodent pests associated with food-production pipelines are likely sources of seasonal outbreaks of foodborne pathogens throughout the United States.

For objective 1, we live-trapped rodents from food animal farms (e.g., dairy cattle, hog) across Minnesota and Wisconsin, collected both biological (feces) and tissue (colon) samples from each rodent, along with relevant metadata (e.g., species, sex, sampling locality, etc.) (**Chapter 3**). To describe the fecal bacterial community of the rodents circulating in the food animal operations, we used 16S targeted metagenetic

barcoding as a tool for surveillance of putative foodborne zoonotic pathogens important for public health. We found that both native and invasive species of farm-dwelling rodents may carry putative foodborne pathogens of human health concern.

For objective 2 and 3, we isolated putative foodborne pathogens and antibiotic resistant *E. coli* from rodent feces and performed comparative genomics analysis (**Chapter 4**). We used classical microbiological methods and selective enrichment for target foodborne pathogens (*Salmonella* spp., *Escherichia coli* (STEC, non-STEC), generic *E. coli* with MDR). Antibiotic susceptibility testing of isolated *E. coli* (STEC, non-STEC) and generic *E. coli* were performed to assess the phenotypic AMR profile of the rodent isolates. The isolated pathogens from objective 2 were characterized genotypically using Illumina whole genome sequencing (WGS) to describe the virulence profile of the putative foodborne pathogens (Virulence genes, antibiotic resistance genes, serotype, clonal type, sequence type, plasmid etc.). Resulting WGS data were compared with historical human and animal pathogenic strains obtained from publicly available databases to compare the evolutionary relationship and molecular epidemiology of foodborne zoonotic pathogens carried by rodent pests. In our study, we found that farm-dwelling rodent pests carry multidrug resistant *E. coli* and STEC. Taken together, these findings suggest that rodents may contribute to future outbreaks of foodborne and zoonotic diseases, hence farm-level biosecurity efforts must be improved to control rodent pests.

CHAPTER 2

The role of peridomestic rodents as reservoirs for zoonotic foodborne pathogens

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Synopsis

Although rodents are well-known reservoirs and vectors for a number of zoonoses, the functional role that peridomestic rodents serve in the amplification and transmission of foodborne pathogens is likely underappreciated. Clear links have been identified between rodent pests and outbreaks of foodborne pathogens throughout Europe and Asia; however, comparatively little research has been devoted to studying this relationship in the United States. In particular, regional studies focused on specific rodent species and their foodborne pathogen reservoir status across the diverse agricultural landscapes of the United States are lacking. We posit that both native and invasive species of rodent pests associated with food-production pipelines are likely sources of seasonal outbreaks of foodborne pathogens throughout the United States. Here, we review the evidence that identifies peridomestic rodents as reservoirs for foodborne pathogens and we call for novel research focused on the metagenomic communities residing at the rodent-agriculture interface. Such data will likely result in the identification of new reservoirs for foodborne pathogens and species-specific demographic traits that might underlie seasonal enteric disease outbreaks. Moreover, we anticipate that a One Health metagenomic research approach will result in the discovery of new strains of zoonotic disease circulating in peridomestic rodents. Data resulting from such research efforts would directly inform and improve upon biosecurity efforts, ultimately serving to protect our food supply.

Keywords MLST, *Streptococcus suis*, multilocus sequence typing, pathogenic, pathotype, porcine, serotyping

Introduction

Foodborne illnesses are a major threat to human health and it is estimated that foodborne pathogens sicken at least 48 million in the United States annually, causing upwards of $70 billion USD in health-related costs (Scallan et al. 2011; Scharff 2012; Hoffman et al. 2015; Hoffmann and Scallan 2017). Moreover, approximately 64% of hospitalization and deaths associated with foodborne pathogens are caused by zoonotic bacteria including nontyphoidal *Salmonella* spp., *Campylobacter* spp., Shiga-toxin producing *Escherichia coli* (STEC) and *Listeria monocytogenes*. The direct environmental point-source for many of the zoonotic enteric pathogens that enter our food supply is typically difficult to determine, however, wildlife distributed throughout local environments associated with a given outbreak are frequently implicated in spreading zoonosis (Atwill et al. 2012; Himsworth et al. 2013; Greig et al. 2015; Sellers et al. 2018; Ayyal et al. 2019). When considering putative reservoirs and vectors for zoonotic foodborne disease, a critically important and uniting feature of the major pathogens listed above is that each is associated with peridomestic rodent hosts (Backhans et al. 2011; Himsworth et al. 2015; Ayyal et al. 2019; Bondo et al. 2019; Tan et al. 2019) (Table 1).

Rodents are the most specious group of mammals in the world with over 2,000 species recognized and they are well known for harboring a plethora of zoonoses of human health concern (e.g., Hantavirus, Lassa fever virus, giardia, plague, Lyme Disease, etc.) (Han et al. 2015). Commensal and peridomestic rodent species are of special interest to the global One Health initiative, as regionally invasive and native species of mice and rats have benefitted from human activities, especially agricultural systems. Rodent pests

are a common component of the agricultural landscape and these animals are known to transmit foodborne pathogens to livestock, poultry, and raw produce by contaminating the overall farm environment (Rodriguez et al. 2006; Meerburg 2010; Backhans and Fellström 2012; Kilonzo et al. 2013). This transmission is largely due to the amplification of foodborne pathogens through the daily deposition of urine and fecal pellets into the production environment and a clear example of this rodent-based amplification is found with *Salmonella* (Figure 1). Fifteen *Salmonella* Enteritidis cells can successfully infect a mouse (*Mus musculus*) and the infection can be maintained for over 10 months (Trampel et al. 2014). During active shedding, one mouse fecal pellet can contain up to 230,000 *Salmonella* cells and the rodent can output more than 100 fecal pellets in a day (Figure 1). Thus, a single rodent within a barn or food-production facility can introduce upwards of 23 million *Salmonella* bacteria into production pipelines within 24 hours (Davies and Wray 1995; Trampel et al. 2014).

Despite a growing list of examples that document strong links between rodent pests on farms and outbreaks of foodborne pathogens in Europe and Asia (Berndtson et al. 1996; Kozak et al. 2009; Burt et al. 2012; Lapuz et al. 2012; Espinosa et al. 2018), comparatively little research on the rodent-Ag interface has occurred in the United States (Henzler and Opitz 1992; Kilonzo et al. 2013). Moreover, studies investigating this interface frequently lack specific knowledge of the rodent species involved and refer to rodents simply as “mice” or “rats”, therefore preventing the identification of species-specific patterns with respect to reservoir status, demographic trends, etc. (Hancock et al. 1998; Hiett et al. 2002). For these reasons, the role of peridomestic rodents as reservoirs or vectors of zoonotic foodborne pathogens in the United States is not well defined and

there is a great need to fill this epidemiological knowledge gap. Here we summarize some of the major rodent species that are at the heart of the rodent-Ag interface in the Midwestern United States (i.e. Iowa, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota and Wisconsin), one of the most agriculturally productive regions of the world. Importantly, the rodents we highlight represent a regional example and are not exhaustive, these are species that are commonly observed on farms throughout the Midwest. We also provide a review of the major bacterial foodborne pathogens linked to rodent reservoirs. Our intent is to bring awareness to the rodent species diversity that must be considered when examining environmental sources of foodborne pathogens, these data can ultimately inform public health policies and species-specific biosecurity measures in food production operations.

Primary species of the rodent-agriculture interface across the Midwestern United States

Native species

White-footed Mouse

The White-footed Mouse (*Peromyscus leucopus*) is a relatively small rodent in the genus *Peromyscus* that has a distribution extending from Canada to Mexico, including the eastern and midwestern United States, as far west as Arizona (Figure 2) (Lackey et al. 1985). White-footed mice are semi-arboreal, but also occur in agricultural areas (Drickamer 1970; Cummings and Vessey 1994); and their diet consists of seeds, green vegetation, insects, and arthropods (Lackey et al. 1985; Wolff et al. 1985). Cummings and Vessey (1994) reported that *P. leucopus* can be found in nearly all areas

trapped across the farmsteads of Ohio and was second in abundance only to the invasive house mouse (*Mus musculus*; see below). White-footed mice are of special concern to One Health studies because they have been positively identified as vectors for not only hantavirus (Cummings and Vessey 1994), but also Lyme disease, human babesiosis, and human granulocytic ehrlichiosis (Stafford et al. 1999). When considering food-borne zoonoses, multiple pathogens have been identified within *Peromyscus* hosts (Table 1).

Deer Mouse

A close relative to the White-footed Mouse is the Deer Mouse (*Peromyscus maniculatus*) from the same genus, *Peromyscus*. The Deer Mouse is a common rodent species that is distributed throughout North America, including Canada, nearly all the United States, and southward into Mexico (American Society of Mammalogists and King 1968). Similar to the White-footed mouse, deer mice feed on seeds, vegetation, and arthropods (Jameson 1952; Wolff et al. 1985). Deer mice are well adapted to different environments including grasslands, prairies, and agricultural lands (Beckwith 1954), and have been reported to prefer indoor granaries when living in a farm setting, especially during winter months (Bovet 1970). *Peromyscus maniculatus* is a known reservoir for many zoonoses including Sin Nombre Virus (Madhav et al. 2007), *Cryptosporidium* and *Giardia* (Kilonzo et al. 2017), *Coxiella burnetti* and *Pasteurella pestis* (Orsborn et al. 1959). This species is also associated with several food-borne pathogens (Table 1).

Meadow Vole

The Meadow Vole (*Microtus pennsylvanicus*) can be distinguished from *Peromyscus* species by a darker pelage and shorter tail (Reich 1981). It has a large range compared to other voles in the genus *Microtus*. The Meadow Vole occurs throughout Canada, the north and eastern parts of the United States, extending southward into Mexico (Reich 1981). This species has been known to frequent farm settings in the Midwest and is associated with dense grassland habitats (American Society of Mammalogists and Tamarin 1985). Meadow voles are mainly herbivores, although they have been found to eat insects and scavenge on animal remains (Zimmerman 1965; Reich 1981). *Microtus pennsylvanicus* has been implicated as a reservoir for Lyme disease (*Borrelia burgdorferi*) (Markowski et al. 1998) and the *Rickettsia* responsible for Rocky Mountain Spotted fever (Gould and Miesse 1954). Multiple species of voles (*Microtus*) have been implicated as reservoirs for *Campylobacter* sp., *E. coli*, and *Listeria* sp. (Table 1).

Thirteen-lined Ground Squirrel

The Thirteen-lined Ground Squirrel (*Ictidomys tridecemlineatus*) is recognizable by its slender body and distinctly alternating light-colored longitudinal stripes and rows of spots (Lawlor 1982). The distribution for the thirteen-lined ground squirrel extends from Canada to Texas, east of the Rocky Mountains to the Great Lakes region (Streubel and Fitzgerald 1978). This species is considered carnivorous, mainly feeding on grasshoppers, although seeds and other vegetation have been found in their stomach contents (Fitzpatrick 1925; Whitaker 1972). Thirteen-lined ground squirrels have

adapted well to farms and urban areas (Streubel and Fitzgerald 1978), thus increasing the risk of zoonotic disease transfer. For instance, Cloud-Hansen et al. (2007) reported multidrug resistant bacteria from wild-caught thirteen-lined ground squirrels (*Morganella morganii* and *Stenotrophomonas maltophilia*). Further, thirteen-lined ground squirrels have been implicated as potential transmitters of avian influenza viruses (Vandalen et al. 2009). Very little research has focused on the putative pathogens that thirteen-lined ground squirrels carry despite the species being a common resident on farms and in urban areas throughout the Midwest.

Eastern Chipmunk

The Eastern Chipmunk (*Tamias striatus*) is heavier-set than the thirteen-lined ground squirrel and has prominent white dorsal stripes flanked by darker stripes and no spots (as seen in the thirteen-lined ground squirrel). The Eastern Chipmunk is distributed throughout eastern North America, including Canada and southward to the Gulf states; but is absent from the coastal plain (Snyder 1982). Eastern chipmunks are omnivorous and their diet includes seeds, nuts, insects, fungi, frogs, snakes, birds, and small mammals (Elliott 1978; Snyder 1982). *Tamias striatus* has been associated with West Nile Virus (Gómez et al. 2008) and was implicated as an amplifier host for La Crosse Virus (Hanson et al. 1975). Further, isolates of *E. coli* have been found in chipmunks (Maldonado et al. 2005). Other datasets showing putative links to foodborne pathogens are lacking.

Eastern Gray and Fox Squirrels

The Eastern Gray Squirrel (*Sciurus carolinensis*) is distributed throughout the Eastern United States and northward into Canada. Several introductions have occurred in the western United States and Europe (Koprowski 1994a). The Fox Squirrel's (*Sciurus niger*) range overlaps with much of the Eastern Gray Squirrel's range, but extends further west in the United States and Canada (Koprowski 1994b). The Eastern Gray Squirrel is distinguishable from the Fox Squirrel by its silver/white hair on the ventral side (belly) compared to the Fox Squirrel's reddish-orange ventral hair. Nuts are the main food source for both species, including acorns, pecans, and walnuts (Koprowski 1994a, 1994b). The Eastern Gray and Fox squirrels are a common component of urban and rural areas. Viruses and bacteria associated with these species include West Nile Virus (Kiupel et al. 2003) and *Leptospira* (Dirsmith et al. 2013) in fox squirrels, and *Salmonella* from eastern gray squirrels (Jijón et al. 2007). Despite co-occurring with humans and agricultural animals at relatively high densities, little to no research investigates the role of tree squirrels in harboring foodborne pathogens or zoonotic diseases.

Invasive species

House Mouse

The ancestral range for the House Mouse (*Mus musculus*) was most likely India, however, this species readily colonized worldwide as an invasive species due to their close proximity to humans (Boursot et al. 1993; Phifer-Rixey and Nachman 2015). Wild *Mus musculus* are distinguishable from white-footed mice and deer mice by their shorter pelage, smaller eyes, and scaly tail (Schmidly and Bradley 2016). House mice are

commonly found in urban and agricultural areas, although feral populations do exist in the wild (Phifer-Rixey and Nachman 2015). Wild *Mus musculus* are heavily studied and this species has been shown to be a vector for many human diseases and are frequently implicated as reservoirs for foodborne pathogens (including multiple pathogenic *Salmonella* serovars, *E. coli, C. difficile*, etc.) and other zoonoses (e.g., avian influenza) of special interest to agricultural production systems (Table 1) (Shimi et al. 1979; Henzler and Opitz 1992; Allen et al. 2011; Burt et al. 2012).

Norway Rat

The Norway Rat (*Rattus norvegicus*), also known as the Brown Rat, is native to China and Mongolia but now has a worldwide distribution as its range expanded alongside humans, facilitated in large part to global trade (Frittelli 2008; Puckett et al. 2016). Like house mice, Norway rats are a common component of both rural and urban environments across the United States, especially on farms. Norway rats have connections to several of zoonotic diseases and foodborne pathogens including Hepatitis E virus (Kanai et al. 2012), *Coxiella burnetti* (Reusken et al. 2011), *Salmonella* (Hilton et al. 2002), and *Clostridium difficile* (Himsworth et al. 2014), among others (Table 1). The ability of these two invasive species, the house mouse and the Norway rat, to adapt to any environment, and their quick reproductive cycles make them especially important to understand and document as reservoirs of foodborne pathogens.

Major bacterial foodborne pathogens linked to rodents

Shiga toxin–producing *Escherichia coli* (STEC) and Multidrug resistant generic *E. coli*

Shiga toxin–producing *Escherichia coli* (STEC), also known as verocytotoxigenic *E. coli* (VTEC), is an important foodborne zoonotic pathogen that causes illness ranging from mild diarrhea to hemorrhagic colitis and life-threatening hemolytic uremic syndrome (HUS) (Shimi et al. 1979; Henzler and Opitz 1992; Allen et al. 2011; Burt et al. 2012). Cattle and other ruminants are considered to be natural reservoirs for STEC, however STEC strains have been isolated from other domestic species as well as wild animals (e.g. goats, sheep, pigs, cats, dogs, deer, wild rabbits, birds, and rodents) (Espinosa et al. 2018). For example, in 2011 a large outbreak of *E. coli* O157:H7 was traced back to fresh strawberries contaminated with deer feces (Laidler et al. 2013). With respect to rodent-specific analyses, several studies have identified STEC strains circulating in peridomestic species on both agricultural and urban landscapes including the Norway Rat (*R. norvegicus*), Black Rat (*R. rattus*) and House Mouse (*Mus musculus*) (Čížek et al. 1999; Blanco Crivelli et al. 2012; Himsworth et al. 2015; Williams et al. 2018). In particular, Nielsen et al. reported the isolation of a STEC strain from a Norway Rat (*R. norvegicus*) identical to a cattle isolate from the corresponding farm with respect to serotype, virulence profile, and pulsed-field gel electrophoresis type (Nielsen et al. 2004). Moreover, in a Czech cattle farm, *E. coli* O157 was isolated from 40% of Norway rats, emphasizing the importance of rodents as potential vectors of pathogenic *E. coli* in cattle productions (Čížek et al. 1999). In pathogen survival studies, it was shown that *E. coli* O157 could survive for up to 9 months in the feces of experimentally infected

rodents, whereas in ruminant feces the pathogen survived for up to 18 weeks (Fukushima et al. 1999; Letunic and Bork 2007; Guenther et al. 2013). This finding suggests that rodent mobility and longer survival periods of the pathogen in rodent feces may make rodents competent vectors of such pathogens particularly when they have access to livestock production farms. In addition, limited or no data exist with respect to STEC screening of populations of native rodent species occurring on farms across the Midwestern states (e.g., *Peromyscus* spp., *Microtus* spp., *Sciurus* spp.; see above), thus additional research is required to investigate the role of these rodents in spreading or harboring strains of STEC.

Non-specific *E. coli* strains with antibiotic resistance or multidrug resistance (MDR) are also of great health concern, as MDR infections are complicated to treat and, in worst case scenarios, are completely without antibiotic treatment options. Moreover, MDR bacteria can greatly contribute to the dissemination of antibiotic resistance genes (ARG) through horizontal gene transfer to other bacteria. MDR bacteria have been found at high prevalence in the intestinal bacteria of wild rodents living in close proximity to livestock. For example, one study reported 71% MDR *E. coli* positive samples (cecum) from 49 wild house mice (*Mus musculus*) captured from swine farms (Allen et al. 2013). Furthermore, Allen et al. (2011) studied antimicrobial resistance in generic *E. coli* from wild small mammals (e.g., mouse, vole, shrew) living in swine farms, residential areas, landfills, and natural environments in Ontario, Canada. They observed the highest (48%) resistant *E. coli* population isolated from the small mammals living in swine farms compared to other areas (Allen et al. 2011). A study by Guenther et al. reported low (5.5%) prevalence of antibiotic resistance among 188 *E. coli* isolated from rodents

captured from rural areas in Germany (Guenther et al. 2010), which also suggests transmission of resistant bacteria from livestock or farm environment to wild rodents. With respect to species-specific behavioral traits, it was suggested by Himsworth et al. (2015) that Black Rats (*R. rattus*) are less likely to carry *E. coli* than Norway Rats (*R. norvegicus*) as the later species is ground dweller and thus experiences more fecal exposure than the upper level dweller. Moreover, Kozak et al. (2009) found that rodents residing in swine farms were five times more likely to carry tetracycline and multidrug resistant *E. coli* than rodents living in natural areas, where 83% of the swine also carried tetracycline resistant *E. coli*, thus reflecting their role as environmental sponge. However, the direction of transmission of these resistant pathogens remains unclear.

Nontyphoidal *Salmonella* spp.

Salmonellosis is a common foodborne illness worldwide, caused by the bacteria *Salmonella* that has over 2,500 serotypes making this pathogen highly virulent and very challenging to control. In the United States, nontyphoidal *Salmonella* spp. cause 11% of all foodborne illness, 35% of hospitalization and 28% of all foodborne illness related death (Scallan et al. 2011). Additionally, 95% of all nontyphoidal *Salmonella* cases are foodborne, suggesting an origin from food animals or contaminated food products derived from food animals and fresh produce (Hoelzer et al. 2011). Many studies have isolated *Salmonella* at higher rates from farm environments, indicating the overall farm environment is an important *Salmonella* reservoir (Bondo et al. 2017). Both wildlife and companion animals represent potential sources of human salmonellosis, as *Salmonella* can cause disease in these animals and some animals can be asymptomatic carriers as well (Rodriguez et al. 2006). Rodents are effective amplifiers of *Salmonella* (see above,

Figure 1) with the capacity to output millions of *Salmonella* bacteria into a particular environment. Thus, rodents associated with poultry farms are a serious public health concern (Antunes et al. 2016; Nidaullah et al. 2017). Interestingly, poultry will preferentially feed on rodent fecal pellets if present in feed or housing facilities, which is highly suggestive of rodent pests being a critical risk factor in production operations (Davies and Wray 1995). It is likely that rodent-related *Salmonella* outbreaks occur seasonally, coinciding with key demographic and/or climatic shifts that influence rodent behavior (e.g., reproductive cycles, seeking of indoor shelter with lower autumn nightly temperatures, seeking novel food sources during periods of drought, etc.). Furthermore, Umali et al. (2012) showed an important connection between persistent *Salmonella* infection in layer chicken houses and rodents, where *Rattus rattus* intermittently shed *Salmonella* up to 24 weeks, thus reintroducing the pathogen to replacement flocks after cleaning and disinfection. Several other studies reported the isolation of highly similar strains of *Salmonella* from food animal (chicken, pig) and rodent pests (*Rattus rattus* and undefined rodent species) circulating in the same farm environment (Lapuz et al. 2012; Andrés-Barranco et al. 2014). Moreover, Andrés-Barranco et al. (2014) suggested a greater role of rodents compared to wild birds in maintaining *Salmonella* spp. in a farm environment, where the associated livestock population was a major source of *Salmonella* contamination. There are a growing number of examples documenting the relationship between rodents and *Salmonella* outbreaks. Although not occurring in a wild or agricultural setting, a 2004 multi-state outbreak of *Salmonella Typhimurium* occurred was traced to hamsters, rats and mice sold in pet shops across the United States (Hoelzer et al. 2011). Studies investigating the contamination of chicken and pork sausage by

Salmonella have implicated poor rodent-control measures within processing facilitates (Trimoulinard et al. 2017). Given the strong connection between rodents and *Salmonella* spp., we recommend renewed and proactive monitoring of rodents associated with food animal farms and food production facilities.

Campylobacter spp.

Campylobacter are major pathogens that cause significant episodes of foodborne illness and hospitalization across the global human population (Scallan et al. 2011). Campylobacteriosis typically causes symptoms of gastroenteritis, including vomiting, diarrhea and fever (Viswanathan et al. 2017). The majority of *Campylobacter* infections are self-limiting, however, in the case of immunocompromised individuals, hospitalization and treatment is required. Thus, antibiotic resistant strains of *Campylobacter* pose a great threat to public health. Poultry, contaminated retail meat and dairy products have largely been associated with campylobacteriosis cases (Scallan et al. 2011). Poultry and other food animals are thought to be the primary reservoir of *Campylobacter* spp., however, wildlife surrounding farm environments are a potential source (Wilson et al. 2008). Viswanathan et al. (2017) studied the prevalence of *Campylobacter* in livestock (i.e., beef cattle, dairy cattle and swine) and wildlife including rodents (i.e., meadow voles (*Microtus pennsylvanicus*), house mice (*Mus musculus*), species of *Peromyscus* and Norway rats (*Rattus norvegicus*)), and identified *Campylobacter* across their samples (Table1). Importantly, the presence of rodents in poultry farms has been identified as a significant risk factor for *Campylobacter* colonization in turkeys and broilers (Agunos et al. 2014). Hiett et al. (2002) conducted

molecular subtyping analyses of *Campylobacter* spp. from Arkansas and California poultry operations. They isolated identical strains of *Campylobacter* from broiler feces and from a mouse (undefined species) captured from the same farm (Hiett et al. 2002). Similarly, other studies have reported greater prevalence of antimicrobial resistant strains of *Campylobacter* from wildlife living in or surrounding farms (Agunos et al. 2014). Several studies have investigated the role of rodent pests as potential reservoirs of *Campylobacter* spp., showing an association between the presence of rodents on farms and an increased risk for flocks to become infected with *Campylobacter* (Berndtson et al. 1996; Adhikari et al. 2004; Meerburg and Kijlstra 2007). These studies collectively indicate that rodents are a risk factor for the transmission of *Campylobacter* on food animal operations and additional studies are required to better understand these transmission dynamics.

***Clostridium* spp.**

The CDC estimates that approximately 1 million cases of foodborne illnesses each year are linked to *Clostridium* species (Grass et al. 2013). The most common species causing these foodborne illnesses include *C. perfringens,* and *C. botulinum. Clostridium* is anaerobic, spore-forming bacteria and produces enterotoxin that can cause mild diarrhea to fatal colitis and death of humans (Fredriksson-Ahomaa et al. 2010; Keessen et al. 2011). Over 90% of *Clostridium* outbreaks are associated with meat products (e.g., beef, pork and poultry) (Grass et al. 2013). However, very limited information is known about animal or environmental reservoirs for *Clostridium* spp.*,* information that could be crucial for implementing biological control measures at animal production farms and

processing facilities (McClane 2007). Companion and food animals have been considered as potential sources of *Clostridium* infection (Rodriguez et al. 2017), however only a few studies have investigated the presence of *Clostridium* species in peridomestic rodent pests despite having clear connections (Hensgens et al. 2012; Himsworth et al. 2013). Himsworth et al. (2014) reported an overall prevalence of 13.1% of *C. difficile* across 724 rodents (*Rattus rattus, Rattus norvegicus*) collected in Vancouver, Canada. Another study in the Netherlands stated high prevalence (66%) of *C. difficile* in house mice (*Mus musculus*) from a swine farm, indicating the potential role of these rodent pests in the maintenance and transmission of the pathogen (Burt et al. 2012). Many studies have established rodents as reservoirs for *C. difficile* (Burt et al. 2012, 2018; Himsworth et al. 2014). In addition, Krijger et al. (2019) reported six ribotypes of *C. difficile* associated with human infections that were isolated from rodents captured from swine and dairy farms in the Netherlands. A study by Andres-Lasheras et al. (2017) in Spain observed that the odds of finding *C. difficile* from environmental rodent (*Rattus sp., Mus musculus*) fecal pellets were 10 times higher than from swine fecal samples, suggesting that rodents can transmit the pathogen on farms. Another study reported the isolation of identical clonal types of *C. difficile* from rodents (*Rattus rattus, Mus musculus)* and piglets from two swine farms in Brazil, suggesting rodent pests were introducing the pathogen to the piglets (de Oliveira et al. 2018). Overall, these studies suggest on-farm rodent pests as potential *C. difficile* reservoirs.

Listeria monocytogenes

Another important foodborne pathogen is *Listeria monocytogenes,* which causes

significant hospitalization and is a leading cause of death by foodborne illness (Scallan et al. 2011). Listeriosis is less commonly reported, perhaps due to its long incubation period and/or because the bacteria are not detected by routine stool culture (Smith et al. 2018). However, listeriosis can cause severe conditions including stillbirths, abortions, septicemia, and meningitis in high risk groups such as pregnant women, elderly, young children and immune compromised individuals (Montero et al. 2015). *L. monocytogenes* can be found naturally in the environment (e.g., soil, water), and consequently in fresh produce, livestock and wild animals (Lyautey et al. 2007). Fecal carriage of *L. monocytogenes* is common and widely documented in food animals (cattle, goat, sheep, swine) and wildlife (mammals, birds, reptiles) (Weber et al. 1993, 1995; Arumugaswamy and Gibson 1999; Bauwens et al. 2003). The specific reservoir status of farm-associated rodent pests for *Listeria* spp. is poorly understood, nevertheless a few studies have reported varying levels of *Listeria* presence in rodents. For example, Ayyal et al. (2019) reported 5% prevalence of *Listeria* spp. among 120 black rats within an urban setting in Baghdad, Iraq. Fazira et al. (2016) conducted a study in Kubah national park (Sarawak, Malaysia) to detect the prevalence and antibiotic resistance of *L. monocytogenes* from wild animals (bats, rats and shrews) and water samples. They reported 33% prevalence of *L. monocytogenes* from the samples collected and found the isolates to be uniformly resistant to tetracycline and erythromycin. Another species, *Listeria ivanovii*, also causes listeriosis and Cao et al. (2019) described 3.7% prevalence of the bacteria in wild rodents collected from six regions of China, suggesting wild rodents might be a long-term host of the pathogen. Inoue et al. (1992) captured 254 wild rats including 41 *Rattus rattus* and 126 *R. norvegicus* in buildings from six different areas in Kanto, Japan and they observed

high prevalence of listeria (up to 77.8%). These same authors also reported frequent isolation of *L. monocytogenes* from *R. rattus* inhabiting Tokyo restaurants (Inoue et al. 1991). Trimoulinard et al. (2017) investigated contamination of chicken and pork sausage by *L. monocytogenes* and their findings suggested a positive association with fresh rodent droppings. Additional studies are required to describe the epidemiology of *Listeria spp.* from peridomestic rodent pests inhabiting farms and to assess the public health risk.

Conclusions

At present, many epidemiological studies of zoonotic foodborne pathogen outbreaks in humans focus almost entirely on the role and risk factors of food animals in transmission dynamics. There remains limited published research on the role that individual species of peridomestic rodents play in the transmission of these pathogens to humans, food animals and within overall food production systems. Despite the connections between rodents and zoonotic foodborne pathogens highlighted above, there is a lack of research on this subject across the diverse agricultural landscape of the United States. In light of this paucity of data, we recommend novel research that focuses on the rodent-Ag interface and that leverages modern metagenomic approaches. For example, high-throughput metagenomic analyses are ideally suited for investigating the rodent-Ag interface and such approaches could elucidate the origins of zoonotic foodborne pathogens (Sekse et al. 2017; Andersen and Hoorfar 2018; Carleton et al. 2019). Moreover, mammalogists must be engaged in this research in order to appropriately identify those rodent species that are associated with food production pipelines in the United States. This is important for several reasons, especially when considering that

species-specific behaviors and natural history traits (as described above) can influence pest control measures (e.g., avoidance of poison baits), facilitating large on-farm populations. When considering native rodent species, the local habitat and ecology surrounding farms are important for understating seasonal population densities, as local environmental conditions can directly influence behavior and on-farm population density. Knowing the resident rodent species composition throughout our food production facilities would allow for the development of sophisticated predictive modeling of rodent populations and the implementation of species-specific biosecurity measures.

There is an undeniable connection between rodent pests and agriculture that has existed since the dawn of civilization. This connection is ongoing and should not be ignored when investigating the origin of zoonotic foodborne pathogens. Therefore, we recommend the formation of multi-disciplinary One Health research teams consisting of farmers, veterinarians, epidemiologists, microbiologists and mammalogists to focus on this area of research. It is likely that such efforts will identify novel reservoirs for emerging zoonotic foodborne pathogens and will ultimately help to secure food production systems across the United States.

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Table 1. Zoonotic foodborne pathogens associated with rodent reservoirs.

Foodborne Pathogens	Known Rodent Reservoirs	References
Campylobacter spp.	*Apodemus sylvaticus* (Wood mouse), *Microtus agrestis* (Field vole), *M.longicaudus* (Longtail vole), *M. richardsoni* (Water vole), *Mus musculus* (House mouse), *Myodes glareolus* (Bank vole), *Peromyscus maniculatus* (Deer mouse), *Rattus norvegicus* (Norway rat), *Zapu princeps* (Western jumping mouse)	Fernie and Park (1977), Rosef et al. (1983), Pacha et al. (1987), Meerburg et al. (2006), Viswanathan et al. (2017)
Clostridium spp.	*Apodemus sylvaticus*, *Microtus arvalis* (Common vole), *Micromys minutus* (Eurasian harvest mouse), *Mus musculus*, *Rattus norvegicus*, *R. rattus* (Black rat)	Burt et al. (2012), Jardine et al. (2013), Himsworth et al. (2014), Krijger et al. (2019)
Coxiella burnetii	*Akodon cursor* (Cursor grass mouse), *Allactaga elater* (Small five-toed jerboa), *Apodemus agrarius* (Striped field mouse), *A. flavicollis* (Yellow-necked mouse), *A. speciosus*, *Arvicola terrestris* (European water vole), *Callospermophilus lateralis* (Golden-mantled ground squirrel), *Clethrionomys gapperi* (Southern Red-backed voles), *Cricetulus migratorius* (Gray hamster), *Dipodomys heermanni* (Heermann's kangaroo rat), *D. microps* (Chisel-toothed kangaroo rat), *D. ordii* (Ord's kangaroo rat), *Eliomys quercinus* (Garden dormouse), *Eutamias amoenus* (Yellow pine chipmunk), *E. sibiricus* (Siberian Chipmunk), *Glaucomys sabrinus* (Northern flying squirrel), *Lemniscomys barbarus* (Zebra mouse), *Meriones shawi* (Shaw's jird), *Microtus arvalis*, *M. californicus* (Meadow mouse), *M. fortis* (Reed vole), *Mus musculus*, *Myocastor coypus* (Nutria), *Myodes glareolus*, *M. rutilus* (Northern red-backed vole), *Napaeozapus insignis* (Woodland jumping mice), *Neotoma cinerea* (Bushy-tailed woodrat or packrat), *N. fuscipes* (Dusky-footed woodrat), *Neotamias minimus* (Least chipmunk), *N. sonomae* (Sonoma chipmunk), *Oligoryzomys nigripes* (Black-footed pygmy rice rat), *Ondantra zi bethicus* (Muskrat), *Onychomys leucogaster* (Northern grasshopper mouse), *Otospermophilus beecheyi* (Beechy ground squirrel), *Oxymycterus dasytrichus* (Atlantic Forest hocicudo), *Peromyscus boylii* (Brush mouse), *P. maniculatus*, *P. true i* (Pinyon mouse), *Proechimys cayenne* (Cayenne spiny rat), *P. cuvieri* (Cuvier's spiny rat), *Rattus*	Blanc et al. (1947), Perez Gallordo et al. (1952), Zhmaeva et al. (1955), Blanc and Bruneau (1956), Syrucek and Raska (1956), Orsborn et al. (1959), Stoenner et al. (1959), Yevdoshenko and Proreshnaya (1961), Burgdorfer et al. (1963), Enright et al. (1969), Enright et al. (1971), Riemann et al. (1979), Ejercito et al. (1993), Gardon et al. (2001), Meerburg and Reusken (2011), Reusken et al. (2011), Thompson et al. (2012), Liu et al. (2013), Foronda et al. (2015), Bolaños-Rivero et al. (2017), Rozental et al. (2017)

	norvegicus, R. rattus, Reithrodontomys megalotis (Harvest mouse), *Rhombomys opimus* (Great gerbil), *Sciurus griseus* (Western gray squirrel), *Spermophilus relictus* (Relict ground squirrel), *Tamiasciurius hudsonicus* (North American red squirrels)	
Cryptosporidium spp.	*Apodemus agrarius, A. chejuensis* (Jeju striped field mouse), *Mus musculus, Peromyscus californicus* (California mouse), *P. maniculatus, Rattus argentiventer* (Rice-field rat), *R. norvegicus, R. exulans* (Pacific rat), *R. rattus, R. tanezumi* (Asian house rat), *R. tiomanicus* (Malayan field rat)	Kilonzo et al. (2013), Song et al. (2015), Saki et al. (2016), Tan et al. (2019)
E. coli (STEC and multiple AMR strains)	*Apodemus agrarius, A. flavicollis, A. sylvaticus, Arvicola amphibius* (European water vole), *Micromys minutus, Microtus agrestis, M. arvalis, M. pennsylvanicus* (Meadow vole), *M. subterraneus* (European pine vole), *Mus musculus, Myodes glareolus, Peromyscus californicus, P. maniculatus, Rattus norvegicus, Tamius striatus* (Eastern chipmunk)	Cizek et al. (1999), Nielsen et al. (2004), Ulrich et al. (2008), Guenther et al. (2010), Allen et al. (2011), Allen et al. (2013), Kilonzo et al. (2013)
Francisella tularensis	*Apodemus agrarius, A. flavicollis, A. peninsulae, A. sylvaticus, Arvicola amphibius, A. terrestris, Callithrix jacchus* (Common marmosets), *Castor canadensis* (American beaver), *Cricetus migratorius* (European hamster), *C. triton* (Greater Long-tailed Hamster), *Cynomys ludovicianus* (Black-tailed prairie dog), *Eutamias sibiricus, Meriones lybicus* (Libyan jird), *Microtus agrestis, M. arvalis, M. pennsylvanicus, M. montebelli* (Japanese grass vole), *Mus musculus, Myodes glareolus, M. rufocanus* (Gray-sided vole), *Ondatra zibethicus, Rattus norvegicus, R. rattus, Sciurus carolinensis* (Eastern gray squirrel), *Sciurus griseus, Sciurus niger* (fox squirrel), *Spermophilus dauricus* (Daurian ground squirrels), *Urocitellus richardsonii* (Richardson's ground squirrel)	Bell and Stewart (1975), Bruce (1978), Magee et al. (1989), Gurycova et al. (2001), Avashia et al. (2002), Berrada et al. (2006), Friend (2006), Zhang et al. (2006), Splettstoesser et al. (2007), Kaysser et al. (2008), Williams and Barker (2008), Gyuranecz et al. (2010), Nelson et al. (2014), Rossow et al. (2014), Sharma et al. (2014)
Giardia spp.	*Apodemus agrarius, A. flavicollis, A. sylvaticus, Bandicota indica* (Greater bandicoot rat), *Maxomys surifer* (Red spiny rat), *Microtus agrestis, M. arvalis, Myodes glareolus, Peromyscus californicus, P. maniculatus, R. argentiventer, R. norvegicus, R. exulans, R. rattus, R. tanezumi, R. tiomanicus*	Pacha et al. (1987), Kilonzo et al. (2013), Masakul et al. (2016), Helmy et al. (2018), Tan et al. (2019)

Hepatitis E virus	*Apodemus sylvaticus, Bandicota bengalensis* (Lesser bandicoot rat), *Mus musculus, Peromyscus maniculatus, Rattus exulans, R. norvegicus, R. pyctoris* (Turkestan rat), *R. rattus, R. tanezumi, Sigmodon hispidus* (Cotton rat)	Karetnyĭ et al. (1993), Kabrane-Lazizi et al. (1999), Favorov et al. (2000), Arankalle et al. (2001), He et al. (2002), Hirano et al. (2003), Vitral et al. (2005), Johne et al. (2010), Purcell et al. (2011), Kanai et al. (2012), Lack et al. (2012), Depamede et al. (2013), De Sabato et al. (2020)
Leptospira spp.	*Rattus norvegicus, Sciurus niger*	Bharti et al. (2003), Dirsmith et al. (2013), Allen et al. (2014)
Listeria monocytogenes	*Apodemus agrarius, A. chevrieri* (Chevrier's field mouse), *A. peninsulae* (Korean field mouse), *Callosciurus* (Beautiful squirrels), *Niviventer confucianus* (Chinese white-bellied rat), *Rattus lossea, R. norvegicus, R. rattus, Tscherskia triton* (Greater long-tailed hamster)	Inoue et al. (1992), Lesley et al. (2016), Wang et al. (2017), Cao et al. (2019)
Norovirus (multiple strains)	*Rattus norvegicus*	Wolf et al. (2013), Summa et al. (2018)
Salmonella spp.	*Apodemus sylvaticus, Microtus agrestis, Mus musculus, Myodes glareolus, Peromyscus californicus, P. maniculatus, Rattus norvegicus, Sciurus carolinensis*	Shimi et al. (1979), Singh et al. (1980), Henzler and Opitz (1992), Guard-Petter et al. (1997), Pocock et al. (2001), Hilton et al. (2002), Davies and Breslin (2003), Garber et al. (2003), Meerburg et al. (2006), Jijón et al. (2007), Kilonzo et al. (2013)
Staphylococcus aureus	*Apodemus flavicollis, Microtus agrestis, Mus musculus, Myodes glareolus, Rattus norvegicus, R. rattus*	Giessen et al. (2009), Mrochen et al. (2017), Lee et al. (2019)
Toxoplasma gondii	*Apodemus sylvaticus, Microtus arvalis, Mastomys erythroleucus* (Guinea multimammate mouse), *M. natalensis* (Natal multimammate mouse), *Mus musculus domesticus* (Western European house mouse), *Peromyscus sp?, Rattus norvegicus, R. rattus*	Dubey et al. (1995), Weigel et al. (1995), Kijlstra et al. (2008), Meerburg et al. (2012), Ruffolo et al. (2016), Khademvatan et al. (2017), Brouat et al. (2018)

Trichinella spp.	*Apodemus agrarius*, *A. flavicollis*, *A. sylvaticus*, *Bandicota bengalensis*, *Mastomys natalensis*, *Mus musculus*, *Meriones persicus* (Persian Jird), *Peromyscus leucopus* (White-footed mouse), *P. maniculatus*, *Rattus norvegicus*, *R. rattus*	Martin et al. (1968), Sadighian et al. (1973), Shaikenov and Boev (1983), Loutfy et al. (1999), Dick and Pozio (2001), Larrieu et al. (2004), Stojcevic et al. (2004), Pozio (2005), Kanai et al. (2007), Pozio et al. (2009), Ribicich et al. (2010)
Yersinia spp.	A*podemus agrarius*, *A. argenteus* (Geisha mice), *A. flavicollis*, *A. speciosus* (Woods mice), *Ashizomys andersoni* (Oriental voles), *A. niigatae* (Oriental voles), *Clethrionomys rufocanus bedfordiae* (Large red-backed mice), *C. rutilus* (Redbacked mice), *Eothenomys kageus* (Oriental voles), *Micromys minutus*, *Microtus agrestis*, *M. levis* (Sibling vole), *M. montebelli* (Japanese grass vole), *Mus musculus*, *Myodes glareolus*, *Rattus norvegicu*s, *R. rattus*, *Tamias sibiricus* (Asiatic chipmunks), *T. striatus*	Pokorna and Aldova (1977), Kaneko and Hashimoto (1981), Shayegani et al. (1986), Iinuma et al. (1992), Backhans et al. (2011), Joutsen et al. (2017)

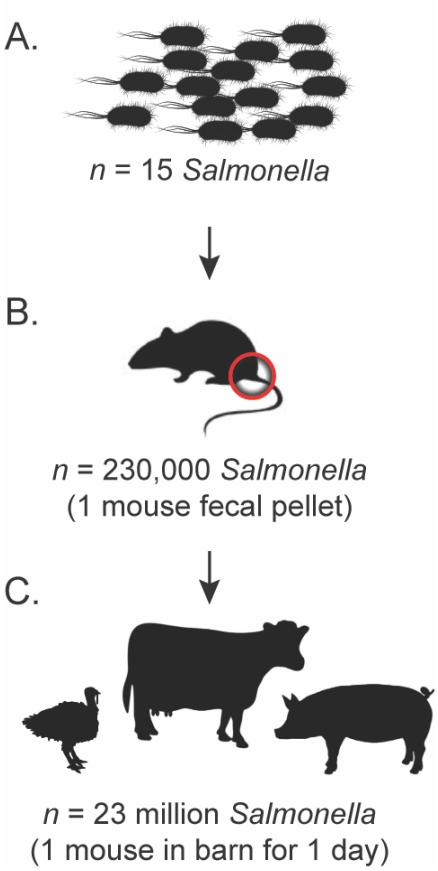

Figure 1. *Salmonella* amplification by a rodent host. (A) Approximately 15 Salmonella bacteria are required to establish infection within a rodent. (B) After colonization, *230,000 Salmonella are shed in a single mouse fecal pellet. (C) One rodent can produce 100 fecal pellets in 24 h. Thus, at least 23 million Salmonella can be introduced into a barn environment by a single rodent pest. This process contributes to outbreaks of enteric disease due to contaminated food or water resources.

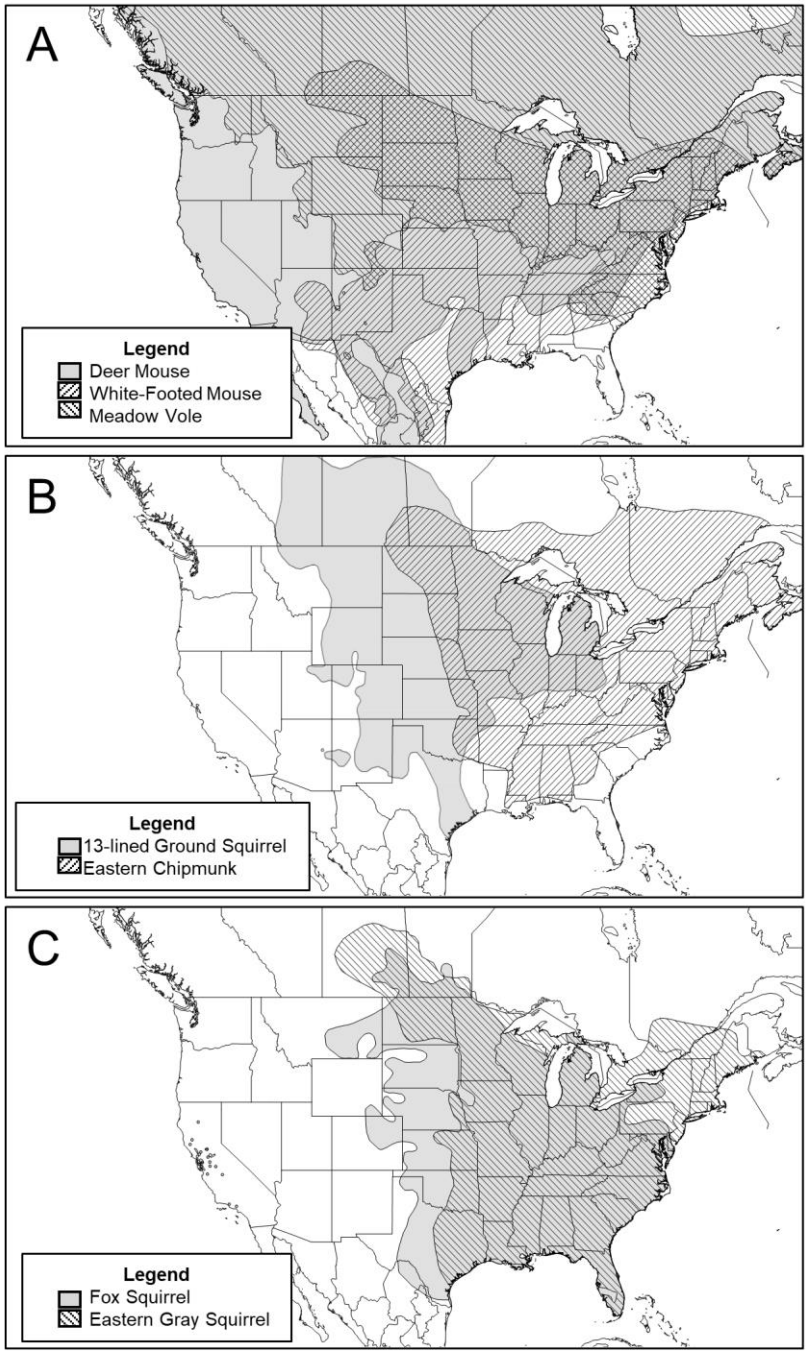

Figure 2. Geographic distributions of native rodent species highlighted herein. (A) Deer Mouse (*Peromyscus maniculatus*), White-footed Mouse (*Peromyscus leucopus*), Meadow Vole (*Microtus pennsylvanicus*). (B) Thirteen- lined Ground Squirrel (*Ictidomys tridecemlineatus*), Eastern Chipmunk (*Tamias striatus*), (C) Fox Squirrel (*Sciurus niger*), Eastern Gray Squirrel (*Sciurus carolinensis*).

CHAPTER 3

Nanopore-based surveillance of zoonotic bacterial pathogens in farm dwelling peridomestic rodents

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Synopsis

The effective control of rodent populations on farms is a critical component of food-safety, as rodents are reservoirs and vectors for many foodborne pathogens in addition to several zoonotic pathogens. The functional role of rodents in the amplification and transmission of pathogens is likely underappreciated. Clear links have been identified between rodents and outbreaks of pathogens throughout Europe and Asia, however, comparatively little research has been devoted to studying this rodent-agricultural interface in the USA, particularly across the Midwest. Here, we address this existing knowledge gap by characterizing the metagenomic communities of rodent pests collected from Minnesota and Wisconsin food animal farms. We leveraged the Oxford Nanopore MinION sequencer to provide a rapid real-time survey of the putative zoonotic food-borne and other human pathogens. Rodents (mice and rats) were live trapped from three dairy and mixed animal farms. Tissues and fecal samples were collected from all rodents. DNA extraction was performed on 90 rodent colons along with 2 shrew colons included as outgroups in the study. Full-length 16S amplicon sequencing was performed with the MinION. Our data suggests the presence of putative foodborne pathogens including *Salmonella spp., Campylobacter spp., Staphylococcus aureus*, and *Clostridium spp.*, along with many important mastitis pathogens. A critically important observation is that we discovered these pathogens within all five species of rodents (*Microtus pennsylvanicus, Mus musculus, Peromyscus leucopus, Peromyscus maniculatus*, and *Rattus norvegicus*) and shrew (*Blarina brevicauda*) in varying abundances. Interestingly, we observed a higher abundance of enteric pathogens (e.g. S*almonella*) in shrew feces compared to the rodents analyzed in our study, however more data is required to establish

that connection. Knowledge gained from our research efforts will directly inform and improve upon farm-level biosecurity efforts and public health interventions to reduce future outbreaks of foodborne and zoonotic disease.

Keywords agriculture; 16S amplicon sequencing; metabarcoding; nanopore sequencing; dairy cattle; One Health; *Peromyscus leucopus; Mus musculus; Blarina brevicauda; Rattus norvegicus*

Introduction

Rodents are the largest taxonomic assemblage of mammals in the world and they are well known for harboring a plethora of zoonotic pathogens of concern for human and animal health (Jahan et al., 2020). Both native and invasive species of mice and rats have benefitted from human activities, especially agricultural systems. Rodents are a common hindrance of food production systems globally and they are known to transmit zoonotic pathogens to food animals and raw produce by contaminating the overall farm environment (Backhans & Fellström, 2012; Kilonzo et al., 2013; Meerburg, 2010; Rodriguez et al., 2006). This transmission is largely due to the amplification of foodborne pathogens through the daily deposition of urine and fecal pellets into the production environment. For example, a single rodent within a barn or food-production facility can introduce upwards of 23 million *Salmonella* bacteria into production pipelines within 24 hours (Davies & Wray, 1995; Trampel et al., 2014). However, the functional role that peridomestic (i.e., living in and around human habitations) rodents serve in the amplification and transmission of various zoonoses is likely underappreciated. For example, clear links have been identified between rodent pests and outbreaks of zoonotic diseases throughout Europe and Asia (Berndtson et al., 1996; Burt et al., 2012; Camba et al., 2020; Espinosa et al., 2018; Lapuz et al., 2012); yet, comparatively little research has been devoted to studying this relationship in the United States (Henzler & Opitz, 1992; Kilonzo et al., 2013). Specifically, regional studies focused on specific rodent species and their pathogen reservoir status across the diverse agricultural landscapes of the United States are lacking. Hence, our overarching research goal was to investigate the role of

rodent pests on food animal farms as reservoirs or carriers of zoonotic pathogens, especially with respect to species-specific patterns.

Emerging genomic technologies are providing exciting new opportunities for the surveillance of zoonotic pathogens in diverse settings and environments. Next-generation sequencing platforms have opened the door to metagenomic profiling, particularly using the 16S rRNA gene. 16S rRNA gene sequence data is particularly useful as molecular marker for bacterial identification, including for pathogens with clinical relevance (Sheahan et al., 2019; Srinivasan et al., 2015). The 16S rRNA gene has nine hypervariable regions (V1-V9) with varying levels, of which the V3 and V4 regions have been the most sequenced for host-associated microbiota and taxonomy assignation of bacteria (Walters et al., 2011). Second-generation sequencing platforms, such as Illumina (e.g., MiSeq, HiSeq) yield very high quality, however the resulting sequence data consist of relatively short (~300bp) reads, often permitting only the analysis of particular subregion of the full length (~1,550bp) 16S rRNA gene (Goodwin et al., 2016). Hence, the taxonomic assignment of reads and identification of bacterial taxa, specifically at the species level may be less reliable. On the other hand, the Oxford Nanopore Technologies (ONT) MinION sequencer is a third-generation sequencing platform based on single molecule synthesis technology with the capacity to sequence large DNA fragments to produce ultra-long sequencing reads (thousands to millions of bases in length)(Jain et al., 2018; Tyler et al., 2018). For this reason, MinION sequencing technology can be used to sequence the full length 16S rRNA gene, thus, providing greater confidence in taxonomic assignments at the species level. This approach is important given that bacterial pathogenicity is typically considered a species or strain level phenomenon (Byrd et al.,

2018). Although per-base accuracy of nanopore sequencing is lower (~98%) than that of more commonly utilized next-generation sequencing platforms (e.g., Illumiina, PacBio) similar or even greater taxonomic resolution has still been achieved with this technology (Kilianski et al., 2015; Nygaard et al., 2020; Oikonomopoulos et al., 2016; J. Shin et al., 2016). Furthermore, with continued product development, technological advances and better bioinformatic tools nanopore DNA sequencing will likely improve per base accuracy in the coming months and years (Calus et al., 2018; C. Li et al., 2016).

The Oxford Nanopore MinION platform has been successfully applied in several studies including the characterization of bacterial mock communities (Benítez-Páez et al., 2016; Brown et al., 2017; C. Li et al., 2016); microbiota profiling of species and tissues such as dog skin (Cuscó et al., 2017), canine feces (Cuscó et al., 2021), equine-gut (Kinoshita et al., 2021), water buffalo milk (Catozzi et al., 2020), sea louse (Gonçalves et al., 2020), microalgae (H. Shin et al., 2018); identification of fungi (D'Andreano et al., 2020), and plastic-associated species in the Mediterranean sea (Davidov et al., 2020). Additionally, metagenomic analyses of environmental samples obtained from glacier regions (Edwards et al., 2019), aquatic environments (e.g., ocean water column (Hamner et al., 2019), river water (Reddington et al., 2020), wastewater (Ma et al., 2017), freshwater (Urban et al., 2021), building-dust (Nygaard et al., 2020), and the International Space Station (Stahl-Rommel et al., 2021), demonstrates the potential and applicability of nanopore sequencing for microorganism detection in diverse environments. Notably, nanopore sequencing has been applied to describe human gut (Matsuo et al., 2021), and nasal microbiota (Heikema et al., 2020), along with the identification of the bacterial community associated with colorectal cancer tumors (Taylor et al., 2020), and thrombus

samples (Vajpeyee et al., 2021). Furthermore, pathogen surveillance in EMS vehicles (Sheahan et al., 2019), analysis of infected prosthetic devices in real-time (Sanderson et al., 2018), *Salmonella* outbreak monitoring in hospitals (Quick et al., 2015), and detection of antibiotic resistance markers in clinical samples (Ashton et al., 2015; Bradley et al., 2015; Judge et al., 2015; Lemon et al., 2017), shows the potential of the MinION platform as a pathogen surveillance tool. However, more studies are required to assess the plausibility of using this platform to analyze the bacterial community composition at the species level. To our knowledge there is only one previous study that analyzed rodent (mouse) fecal microbiota demonstrating the superiority of Nanopore sequencing at characterizing the bacterial community at the species level compared to the usage of short-read technologies for bacterial community characterization (J. Shin et al., 2016).

In the current study, we aim to describe the fecal metagenomic communities of farm-dwelling rodents and identify putative zoonotic pathogens potentially harbored by these rodents using the nanopore full length 16S amplicon sequencing method. Our study area included farms in the Upper Midwest of the United States (i.e., Minnesota and Wisconsin). Research focused on the rodent-farm interface in this geographic region is severely lacking, thus warranting study (Jahan et al., 2020). Our intent was twofold, to 1) better understand the farm-level rodent diversity in our study area and 2) to molecularly characterize rodent biological samples to assess their reservoir status of zoonoses of agricultural concern.

Materials and Methods

Rodent trapping and sample collection on farms

During the summer (2019) and fall (2019, 2020), we collected rodents from one dairy cattle farm (A), one mixed animal (dairy cattle and hog) farm (B) and another mixed animal (cattle and horse) farm (C). Farms A and B are located in Nicollet and Stevens counties of Minnesota, respectively and Farm C is in Sauk county of western Wisconsin. Farm A is a large dairy operation (~20,000 dairy cattle), Farm B is a medium-sized operation (600 dairy cattle and 400 hogs), and Farm C is a small-sized family farm (mixed species, e.g. cattle and horses < 100 animals). Rodent activity was elevated on farms per observations by farm managers, particularly on Farm A where we observed hundreds of Norway rats (*Rattus norvegicus*) actively foraging around compost piles during daylight hours. All farms had poison bait stations, kill traps and cats as rodent control measures during the time of our visits. Four nights of rodent trapping were conducted at each study site using 150 Sherman live traps baited with oats. Decontamination of all traps was performed using a 10% sodium hypochlorite solution (10 min soak) before and after each trapping event. All trapped animals were humanely euthanized following approved UMN IACUC protocols (protocol number 1809-36374A). Standard morphological techniques were used to identify rodents to species-level and metadata (e.g., species, age, weight, sex, body measurements) were collected for each individual animal. Biological samples (e.g., feces, intestine, colon) were collected and preserved (e.g., liquid nitrogen, freezer) for metagenomic analysis and further quantification of pathogens of interest. A schematic workflow of the overall study design is shown in Figure 3.

DNA extraction

Microbial metagenomic DNA was extracted with a QIAamp PowerFecal Pro DNA Kit (QIAGEN, Hilden, Germany). Snap-frozen rodent colon contents stored at -80°C were scraped and used for DNA extraction. Briefly, 250 mg of rodent colon contents were added to PowerBead Pro tubes and 800 µl of solution CD1 was mixed by vortexing. A bench top PowerLyzer 24 Homogenizer (QIAGEN, Hilden, Germany) was used for homogenizing the samples at 2000 rpm for 30 s, pausing for 30 s, then homogenizing again at 2000 rpm for 30 s to enhance cell lysis. PowerBead Pro tubes were centrifuged at 15,000 x g for 1 min and the resulting supernatant was transferred to clean microcentrifuge tubes. We used fully automated QIAcube connect instruments (QIAGEN, Hilden, Germany) for DNA extraction following manufacturer's instructions. DNA concentrations were measured by fluorescence in a Qubit 4 fluorometer (ThermoScientific, USA) using the Qubit dsDNA BR Assay Kit (ThermoScientific, USA) following the manufacturer's instructions.

Nanopore library construction and sequencing

The 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies, Oxford, UK) was used to prepare the amplicon library, following the manufacturer's instructions for 1D sequencing strategy. The 16S region (1.5kb) of bacteria was amplified using specific primers (27F-1492R) and subsequently barcoded. This approach enables targeted sequencing of multiple samples and provides genus level resolution. Five sequencing runs were performed with a total of 70 samples, including 12 (run 1-4) and 22 (run 5) barcoded samples from individual rodents and shrews (colon contents). Briefly, genomic

DNA samples were diluted to 100 ng/uL and amplification of the full-16S rRNA gene was performed by PCR with a reaction volume of 50 uL, using the primers 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3', and Taq DNA polymerase LongAmp (NewEngland Biolabs, Ipswich, MA, USA). Amplification was performed using Bio-Rad Laboratories PCR Thermal Cycler T100™ (Bio-Rad Laboratories, CA, USA) with the following PCR conditions: initial denaturation at 95 °C for 1 min, 25 cycles of 95 °C for 20 s, 55 °C for 30 s, and 65 °C for 2 min, followed by a final extension at 65 °C for 5 min.

The PCR products (50 µl each) were purified with 30 µl Agencourt AMPure XP beads and incubated in a HulaMixer for 5 min at room temperature. After the magnetic beads washing step, purified products were eluted in 10 uL of elution buffer (10 mM Tris-HCl pH8.0 with 50 mM NaCl). The amount and purity of the library was quantified using a Qubit 4 fluorometer (Thermoscientific, USA) following the manufacturer's instructions. A single library was synthesized from DNA (100 or 50 fmol) of a pool of rodent colon contents. Libraries were pooled in multiplex mode following the addition of 1 µl of rapid adapter (Oxford Nanopore Technologies) and incubated at room temperature for 5 minutes. The amplicon library (11 µl) was diluted with a running buffer (35 µl) containing 3.5 µl of nuclease-free water and 25.5 µl of loading beads. Five nanopore sequencing libraries were separately run on FLO-MIN106 R9.4 (run 1, 2, 4, 5) and FLO-MIN111 R10.3 (run 3) flow cells (Oxford Nanopore Technologies) periodically after performing a platform quality control analysis. Sequencing runs were performed for 48 hrs. using the MinION control software, MinKNOW 4.0.20 (Oxford Nanopore Technologies).

Bioinformatics analysis

After the completion of the sequencing run, raw signals in nanopore fast5 files were base-called (i.e. converting the electrical signals generated by a DNA or RNA strand passing through the nanopore into the corresponding base sequence of the strand) using Guppy (version3.2.2, Oxford Nanopore Technologies, UK), and a quality filter step was applied to retain only sequences with a mean Q-score ≥ 7. De-multiplexing of the barcoded samples was conducted using Porechop (Wick R., 2017). Adapter trimming and a second round of de-multiplexing were performed using Cutadapt 1.91 (Martin, 2011). Only reads between 1,200bp and 1,800bp were selected for further analysis using Cutadapt, since the desired product size is ~1,550 bp. Read statistics for each sequencing run were obtained using Nanostat and NanoPlot (De Coster et al., 2018). For taxonomic assignments Kraken2 (Wood & Salzberg, 2014) and Bracken (Lu et al., 2017) were used with the Greengenes (GG) database (https://benlangmead.github.io/aws-indexes/k2). While generating the Bracken classification report, a threshold of >100 reads were applied for higher confidence at the genus and species level. For visualization Krona tools and Pavian interactive applications were used to generate taxonomic charts and flow diagrams (Breitwieser & Salzberg, 2020; Ondov et al., 2011). The ggplot2 package (version 3.2.1) in RStudio software (version 3.3.3) was used to create a heatmap (RStudio Team, 2020). BioRender was used for illustrations and diagrams (Created with BioRender.com). Base-called data were uploaded to the EPI2ME interface, a platform for cloud-based analysis of MinION data, and WIMP (What's in my pot) analysis was performed in parallel to compare results.

Results

Rodent trapping on farms

In total we trapped 90 rodents, 29 from Farm A, 43 from Farm B and 18 from Farm C (Table 2). From Farm C we also captured two shrews (*Blarina brevicauda)* and included those in the study as an outgroup. We identified five rodent species across our study sites, including three native (*Peromyscus maniculatus, P. leucopus*, and *Microtus pennsylvanicus*) and two invasive species (*Mus musculus* and *Rattus norvegicus*). The captured shrew species, *Blarina brevicauda,* is native to the Midwest. The majority of rodent captures were centered around feed bunks, grain storage sites, and within cow barns.

Nanopore sequencing workflow of full-length 16S rRNA for rodent microbiome analysis

We utilized the Oxford Nanopore MinION sequencer for rapid surveillance of rodent fecal microbiota. Full-length 16S rRNA (~1,500bp) amplicon sequencing was conducted for metagenomic surveillance of foodborne zoonotic pathogens at the genus and species level. Colon contents were collected and used for DNA extraction (see details in the methods and materials section). We successfully generated full-length 16S amplicon sequencing data comprising more than 33 million DNA bases from 92 farm-caught rodent and shrew colon extracts. Run 1 and Run 3 included 24 rodent colon samples from the large conventional Farm (A), Run 2 and Run 4 included 24 samples from med-sized Farm (B), while Run 5 included all 20 samples from the small family Farm (C). Each of the sequencing runs included 12 molecularly barcoded samples except

Run 5, which included 20 barcoded samples that were collected from individual rodent and shrew colons. The raw reads generated for the five runs from nanopore sequencing ranged from 3,531,607 to 9,445,273 with 500 to 509 average active pores and consisted of one dimensional (1D) forward, 1D reverse reads (Table 3). The mean quality score of the filtered reads ranged from 8.2 to 10.1 (Table 3), which indicated that the error rate of our sequencing was approximately 1 in 10 or 11 bases (~10-11%), a result that is consistent with similar studies using nanopore sequencing (Ip et al., 2015; J. Shin et al., 2016). However, read depth of full-length 16S amplicons of 16X coverage, resulting in high-quality consensus, reduces concerns of per-base sequencing error (Mikheyev & Tin, 2014). To sort high-quality reads, the pass fast5 reads were sorted specifically from raw data using the Guppy base calling program. Although, Run 3 data (3,531,607 reads) showed fewer sequencing reads than other runs, the mean Q score (8.2), mean read length (1,625.40 bp) and read length N50 (1,593 bp) were comparable to the other runs (Table 3). For all the sequencing runs, the read length had a narrow length distribution, and the mean read length ranged from 1,132.70 to 1625.40 bp, which was close to the full-length of the 16s rRNA gene (about 1,550 bp). However, for obtaining higher quality reads, a filtering program (Cutadapt) was applied to set the read length between 1200-1800 bp to discard any shorter or longer reads outside that specified range. After Guppy basecalling, demultiplexing, Porechop adapter trimming, and Cutadapt length trimming steps we filtered out around 20% of the initial raw reads from all runs. These polishing steps are necessary to ensure the presence of enough sequence similarity between the reads generated and precise matching against the 16S rRNA gene reference database. The longer reads that were removed during the filtering steps seemed to be the products of

concatemers formed at the hairpin adapter ligation step, and the corresponding generation of long chimeric reads (J. Shin et al., 2016).

Rodent fecal core microbiome and microbial diversity

The fecal microbiota composition was determined based on the nanopore sequencing data obtained with the taxonomy-supervised approach that allocates sequences directly into taxonomic bins based on their similarity. After the polishing step, all the long-read amplicons sequenced by Nanopore MinION were taxonomically assigned against the GreenGene (GG) reference (13_8 version) using Kraken2. After taxonomic classification, we obtained 96.25% of classified reads and 3.75% of unclassified reads. Total reads corresponding to Bacteria were at 96.25% and 0% reads were assigned to virus, fungi and protozoa. The microbial classifications were obtained at different taxonomic levels (e.g., division, phylum, class, order, family, genus, and species) for all of the 92 colon extract samples. Overall, the most abundant phylum for all five rodent species was Firmicutes (~75% of total reads), followed in abundance by Bacteroidetes (12.5%), Proteobacteria (~ 10%), and other phylum comprised of less than ~ 2.5% from the total classified 80 phylum (Figure 4). To define microbial diversity and observe any specific patterns related to the different rodent species, we compared all five rodent species data at the genus and species level.

At the rodent species level, house mouse (*Mus musculus*) was the most captured species with a total of 48 animals caught from Farm A and B. After taxonomic classification of all the combined house mouse reads, 77 phyla, 886 genera, and 473 species were obtained. At the genus and species level, a filtering step was applied that

included a threshold of 100 reads binned to the respective taxonomic level, which retained 261 genera and 181 species passing the threshold. For *M. musculus,* the most abundant genera were *Lactobacillus* (27.7%), *Ruminococcus* (10%), *Helicobacter* (8%), *Bacteroides* (7.6%), and *Blautia* (6.8%). Other abundant genus included fecal or mammalian gut microbiota (e.g., *Coprococcus, Faecalibacterium, Dorea, Roseburia, Oscillospira*) and potential human pathogens and bovine mastitis causing pathogens such as *Staphylococcus, Streptococcus, Bacillus*, and *Enterococcus,* each constituting more than 1% of the total classified reads.

The second most dominant rodent species was *Peromyscus* spp. with a total of 29 animals captured from Farm A, B, and C. After combining all *Peromyscus* reads and taxonomic classification, 58 phyla, 619 genera, and 344 species were obtained. After threshold filtering 139 genera and 89 species were retained. The most abundant genus was *Lactobacillus* (37.6%), followed in abundance by *Ruminococcus* (16.5%), *Blautia* (10.6%), *Dorea* (4.6%), *Helicobacter* (4%), *Streptococcus* (3.4%), and other fecal related genera forming less than ~ 3% of the total classified reads.

Brown rats (*Rattus norvegicus*) were captured from Farm A and C with a total of 7 animals in the category. Although we observed hundreds of *R. norvegicus* within Farm A, these rats are notoriously difficult to live trap using Sherman Traps during limited trapping durations. Future collections for this species will utilize a variety of trapping methods. All the combined nanopore reads were taxonomically classified into 47 phyla, 583 genera and 357 species. Threshold filtering retained 119 genera and 88 species, where *Lactobacillus* (22%), *Blautia* (17.3%), *Ruminococcus* (11.5%), *Streptococcus* (7.8%), and *Dorea* (6.5%) were most abundant. Other mammalian gut microbiota (e.g.,

Oscillospira, Coprococcus, Faecalibacterium, Roseburia) and potential human pathogenic genera (*Helicobacter, Prevotella, Staphylococcus*, *Clostridium, Bacteroides*) compiled more than 23% of the bacterial genera.

We captured a total of 6 meadow voles (*Microtus pennsylvanicus*) from Farm B and C. Taxonomic classification revealed 52 phyla, 542 genera and 302 species. Subsequent threshold filtering retained 121 genera and 61 species for all combined reads. The most prominent genera in the meadow vole feces were *Lactobacillus* (24%), *Ruminococcus* (19.6%), *Blautia* (11.7%), *Oscillospira* (7.6%), and *Coprococcus* (5.3%).

While comparing species level composition, overall abundance of a few species was observed for all five rodent species (Figure 5). For example, most abundant *Lactobacillus* species included *L. reuteri, L. zeae, L. salivarius, L. delbrueckii, L. brevis, L. helveticus, L. ruminis,* and *L. iners.* Another dominant genus *Ruminococcus* represented *R. gnavus, R. torques, R. flavefaciens, R. bromii, and R. callidus. Blautia* species included *B. producta* and *B. obeum.* Whereas *Dorea formicigenerans, Roseburia faecis, Prevotella copri, Faecalibacterium prausnitzii, Oscillospira guilliermondii, Clostridium perfringens, Helicobacter pylori,* and *Coprococcus eutactus* represented single dominant species across all rodents. Furthermore, *Staphylococcus* species included *S. aureus, S. epidermidis, S. haemolyticus, S. sciuri* and *Streptococcus* species included *S. luteciae, S. anginosus, S. alactolyticus, S. infantis.*

Shrew fecal core microbiome and microbial diversity

Two shrews from the same species *Blarina brevicauda* were captured from the small family farm (Farm C). Taxonomic classification analysis showed 28 phyla, 281

genera, and 178 species from the shrew sequencing reads. The most abundant phylum for both shrews was Proteobacteria (~ 91% of total reads), followed in abundance by Firmicutes (8%), and other phyla comprised less than ~ 1% from the total classified 28 phylum (Figure 6).

At the genus level, a total of 281 genera were identified and 36 genera retained after applying a threshold of 100 reads binned to each genus. The shrew fecal microbiome was rich in *Klebsiella* (18.8%), followed in abundance by *Salmonella* (16.8%), *Serratia* (15.7%), *Erwinia* (12.6%), and *Citrobacter* (6.2%). Moreover, it also contained other fecal-related and potential pathogenic genera including *Providencia, Enterococcus, Morganella, Yersinia, Enterobacter, Proteus, Clostridium, Plesiomonas, Vibrio, Bacillus, Pseudomonas, Staphylococcus,* and *Streptococcus,* representing less than 5% each of the total bacterial composition. The relative abundance of the 18 most abundant taxa determined at genus level is shown using bar graphs in Figure 7.

Furthermore, analysis at the species level revealed a total of 178 species and 29 were retained after applying a threshold of 100 reads binned to each species. *Salmonella enterica* was the most abundant species with 21.5% reads assigned, followed in abundance by *Serratia marcescens* (17.3%), *Klebsiella oxytoca* (16%), *Erwinia soli* (7.4%), *Staphylococcus sciuri* (6.2%), and *Trabulsiella farmeri* (6%). Other abundant species included putative human and plant pathogens such as *Morganella morganii, Providencia stuartii, Enterobacter cowanii, Staphylococcus aureus,* and *Brenneria quercina,* each > 1% of the total bacterial species composition.

Discussion

We utilized the MinION sequencing technology to characterize fecal microbial communities in peridomestic small mammals (i.e., rodents, shrews). DNA samples recovered from animal colon extracts were obtained from different dairy and mixed animal farms over a two-year period, and subjected to full-length 16S ribosomal RNA (rRNA) sequencing of amplicons for microbial identification. The 16S rRNA gene has been shown previously to be a useful molecular marker for bacterial identification, including for pathogens with clinical relevance (Srinivasan et al., 2015). This sequencing method and analysis pipeline demonstrates the convenience of this technology being highly portable, relatively inexpensive, small, and scalable for the rapid surveillance of pathogenic organisms in peridomestic pests (Castro-Wallace et al., 2017; Goordial et al., 2017; Johnson et al., 2017; Pomerantz et al., 2018; Quick et al., 2016). Additionally, we demonstrate the ability to identify important rodent species in the dairy farms to monitor, inform biosecurity practices and suggest the potential role of rodent pests on the spread of pathogenic microorganisms and describe the rodent microbiome in general.

Substantial compositional differences between animal species microbiome

To the best of our knowledge, this is the first study to reveal and compare the composition of bacterial communities in gastrointestinal tracts of wild caught rodents (e.g., *M. musculus, Peromyscus spp., M. pennsylvanicus, R. norvegicus*) using the third-generation sequencing technology. Moreover, our study is the very first published study revealing the gut microbiota of the Northern short-tailed shrew (*B. brevicauda*). Rodents are considered to be the most commensal synanthropic animals living in close proximity

to humans and feeding on/foraging our food systems. Whereas, shrews (*B. brevicauda*) naturally live in woodlands, cultivated fields, vegetable gardens and mainly feed on invertebrates (Hamilton & Whitaker, 1998; Rue, 1967). However, they often retreat into barns, cellars and sheds during winter, which provides more opportunities for human–animal contact, and therefore higher rates of bacterial transmission are likely (Rahman et al., 2018). Therefore, commensalism and food-habit (omnivore vs. insectivore) might explain the difference in gut microbial structure between rodents and shrews. In our study, we observed that the bacterial communities of rodents and shrews (insectivore) were considerably different, even though they were captured from the same habitats. However, all the rodent species from different habitats (Farm A, B and C) had similar core microbiota indicating host tropism, also reported by a previous study investigating gastrointestinal helminths diversity of free ranging Asian house shrews (Rahman et al., 2018). Hence, the difference between the animal species (i.e., rodents and shrews) was greater than the difference within species (i.e., all rodents).

For all five rodent species, phylum level gut microbiome was similar to that of the human gut microbiome with Firmicutes, Bacteroidetes and Proteobacteria comprising more than 97% of the gut microbiota (Everard et al., 2014; Zhang et al., 2012). Firmicutes was the most abundant phylum in all rodent species, ranging from 64% to 91.5%. In shrews, the most abundant phylum was Proteobacteria, covering 91.7% of the total shrew microbiota. This suggests a greater similarity in core gut microbial composition between rodents and humans than between shrews and humans. At the genus level composition, *Lactobacillus* was the top genus for all the rodent species in our study, which is in line with the findings of a previously reported study on laboratory rodent

microbiomes (D. Li et al., 2017). This finding implies that the core fecal bacterial composition of wild and laboratory rodents share a degree of similarity. Additionally, *Lactobacillus* constitutes a significant component of the human gut microbiome as well (Goldstein et al., 2015), reiterating the similarity between the composition of gut bacteria in humans and rodents. Furthermore, *Ruminococcus* was the second most prominent genus in all mouse species (*M. musculus, Peromyscus spp., M. pennsylvanicus*), whereas *Blautia* was prominent in rats (*R. norvegicus*). On the other hand, *Klebsiella* was the most prominent genus in shrew feces followed by *Salmonella*, *Serratia* and *Erwinia*. Whereas, *Clostridium* was the most abundant genus in the Asian house shrew (*Suncus murinus*) (He et al., 2020). This might be explained by geographical location, habitat, feed, shrew species variation and limited sample size.

Potential foodborne, bovine mastitis and other pathogens

We identified a higher relative abundance of potential human and animal pathogens in shrew fecal samples than in rodent fecal samples. In brief, the resulting data indicate the presence of multiple putative foodborne pathogens from the Enterobacteriales order including *Salmonella* spp., *Shigella* spp., *Plesiomonas shigelloides, Yersinia* spp., and *Escherichia coli,* in all small mammal species (Figure 8). Other top foodborne pathogens observed in our analysis included *Listeria monocytogenes, Campylobacter* spp., *Clostridium perfringens*, *Vibrio* spp., and *Staphylococcus aureus*. These are mentioned as major bacterial pathogens that cause foodborne illness and hospitalization in the USA and all over the world each year (Scallan et al., 2011). Further, each of these bacterial foodborne pathogens were shown to

be carried by different native and invasive rodent species all over the world (Jahan et al., 2020).

Interestingly, *Salmonella enterica* was the most abundant species (~21.5%) in the shrew feces and much lower abundance was observed for the rodents (Figure 9). On the contrary, *Clostridium perfringens*, *Bacillus* spp., and *Staphylococcus aureus* seemed to be carried by all rodents in higher abundance compared to shrews. Additionally, *Vibrio* spp. and *Campylobacter* spp. were observed in comparatively higher abundance in *R. norvegicus* and *M. musculus*, respectively. These bacteria can be transmitted by contaminated food and water, and they had a greater relative abundance in fecal samples of shrews than in those of rodents. These observations indicate that *B. brevicauda* may pose a high potential risk for spreading enteric pathogens via water or food contaminated with feces. Furthermore, farm workers can easily get infected by these pathogens carried by the small mammals through direct and indirect contact with fecal droppings.

Mastitis is a leading cause of cow culling and causes a great economic loss to the dairy industry (Halasa et al., 2007). Since a majority of the samples were collected from a large-sized (A) and medium-sized (B) dairy farm, we investigated the potential presence and abundance of pathogens causing bovine mastitis in the fecal samples of the farm dwelling rodents. Many important mastitis-causing pathogens including *Streptococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., *Enterococcus* spp., *Enterobacter* spp., *Mycoplasma* spp., and *Corynebacterium* spp. were observed in varying abundance in the six species of small mammals (rodents and shrews) collected from each farm. *E. coli*, *Streptococcus* spp., *Staphylococcus* spp., and *Corynebacterium* spp. are well known

environmental mastitis pathogens (Jahan et al., 2021), and the presence of these pathogens in the resident rodent population of each dairy farm is a putative health risk to the resident cattle. Our metagenomic data indicate that the rodents sampled during our trapping events are possible reservoirs of mastitis pathogens and have the potential to continuously introduce these pathogens into the dairy farm environments. Moreover, they can amplify and mechanically vector these pathogens from sick to susceptible animals through pathogen amplification as described in (Jahan et al., 2020). Additionally, while comparing the relative abundance of mastitis pathogens from rodent colons from Farm A, B, and C (Fig 9B), the number of reads from house mouse (*M. musculus*) is higher compared to other rodent species for most mastitis pathogens, despite a similar depth of sequencing coverage. We hypothesize that because house mice cohabitate with cows inside the barns, they are exposed to a greater overall amount of mastitis pathogens by direct and indirect interactions with the large dairy herd.

Bacterial pathogens detected in fecal samples of rodents and shrews can cause human diseases by various routes, including saliva, urine, feces and contaminated water and food. In our study, fecal samples from all rodents also contained a variety of human pathogens in high abundance including *Helicobacter pylori* that causes chronic gastritis, gastric ulcers, and stomach cancer (Kusters, 2006); *Prevotella copri* that is associated with the pathogenesis of Rheumatoid Arthritis (Drago, 2019); pathogens related to nosocomial infections such as *Morganella morganii, Serratia marcescens, Providencia stuartii* (Donkor, 2019; Singla et al., 2010); and opportunistic pathogens associated with splenic abscess (*Parabacteroides distasonis*) and anaerobic peritoneal infections (*Bacteroides fragilis*) (Brook, 1989; Gunalan et al., 2020). Additionally, rodents are the

known reservoirs of some severe infectious zoonotic diseases, such as bartonellosis, leptospirosis, plague, and rat-bite fever (Jahan et al., 2020). However, in our study, none of the fecal samples were positive for *Bartonella, Leptospira, Yersinia pestis*, *Streptobacillus moniliformis* and *Spirillum minus*. Similar findings were reported by He et al., 2020 investigating fecal and throat microbiota of Norway rats (*R. norvegicus*) and Asian house shrews (*S. murinus*) (He et al., 2020). Hence, results from our study indicate that these infectious pathogens might not be shed in feces of the rodents, thus not detected in our study because of the sample type.

Both rodents and shrews are commensal animals that live and feed in closer proximity to human populations than most other mammals and may serve as potential sources of infectious zoonotic diseases to humans via pathogen amplification and cross-species transmission. However, less attention has been paid to these small mammals inhabiting our food processing system including food animal farms, fresh produce lands, and processing facilities. In our study, more sequences from the Northern short tailed shrew were annotated as potential foodborne pathogens than those from rodents. Therefore, *B. brevicauda* might be a more important reservoir of bacterial foodborne pathogens than rodents, suggesting that more attention should be paid to *B. brevicauda* in the prevention of foodborne zoonotic diseases in the future. However, more rigorous studies with larger sample sizes focusing on small mammal's gut microbiota, specifically on *B. brevicauda* are required to support and confirm these findings.

Rodent trapping is unpredictable and dependent upon local conditions; hence it is difficult to determine species diversity. Additionally, we were not as successful trapping rats (*R. norvegicus*) compared to mice, despite visual observation of their

abundant presence and activity in Farm A. Enhanced trapping efforts with pre-baiting measures to acclimate the rats and use of repeater live traps instead of Sherman traps might prove as useful strategies to overcome trap avoidance behaviors exhibited by *R. norvegicus* (Himsworth et al., 2015). Nevertheless, our personal observation during trapping and subsequent results from data collected indicate that rodents are a problem in any agricultural setup and are potential reservoirs of many putative zoonotic foodborne and mastitis-associated pathogens.

The long read length, depth of coverage, and rapid results make the Nanopore sequencing method attractive for a molecular surveillance tool. Although Nanopore sequencing has a higher per-base accuracy error rate than other next-generation sequencing methods (Ip et al., 2015), we are basing our initial taxonomic classification on read depths of over 30 million full-length 16S (1.5 kb) reads. Moreover, the flow cell chemistry for the Oxford Nanopore MinION sequencer is constantly being improved upon and we used the latest R 9.4 and R10 Flow Cell sequencing technology that has improved per base accuracy compared to earlier flow cell versions (www.nanoporetech.com). However, a high number of sequencing reads for putative pathogens does not necessarily indicate the absolute presence of the organism. Standard culturing techniques and molecular methods are still required to confirm the presence of putative pathogens. Nevertheless, for our purpose of a primary screening tool, this cost effective and rapid method achieved our surveillance goals.

Acknowledgements

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Table 2: Index of all captured animals from Farm A, B & C.

Rodent Species	Farm A	Farm B	Farm C
House mouse (*Mus musculus*)	27	21	0
White-footed mouse (*Peromyscus leucopus*)	0	13	11
Deer mouse (*Peromyscus maniculatus*)	1	4	0
Meadow vole (*Microtus pennsylvanicus*)	0	5	1
Norway rat (*Rattus norvegicus*)	1	0	6
Outgroup			
Shrew (*Blarina brevicauda*)	0	0	2
Total = 92	**29**	**43**	**20**

Table 3: Statistics of nanopore sequencing (MinION) data

Run	Farm	Active Pores avg.	Read count	Mean read length	Mean Q score	Read length N50	Total BP	QC > Q7
1	A	509	5,011,566	1,132.70	9.3	1,564	5,676,494,189	85.80%
2	B	500	4,460,963	1,178.30	8.3	1,587	5,256,288,918	85.50%
3	A	509	3,531,607	1,625.40	8.2	1,593	5,740,290,501	68.50%
4	B	500	9,445,273	1,477.10	10.1	1,576	13,952,018,041	85.40%
5	C	504	5,829,408	1,472.30	8.4	1,564	8,582,667,914	77.70%

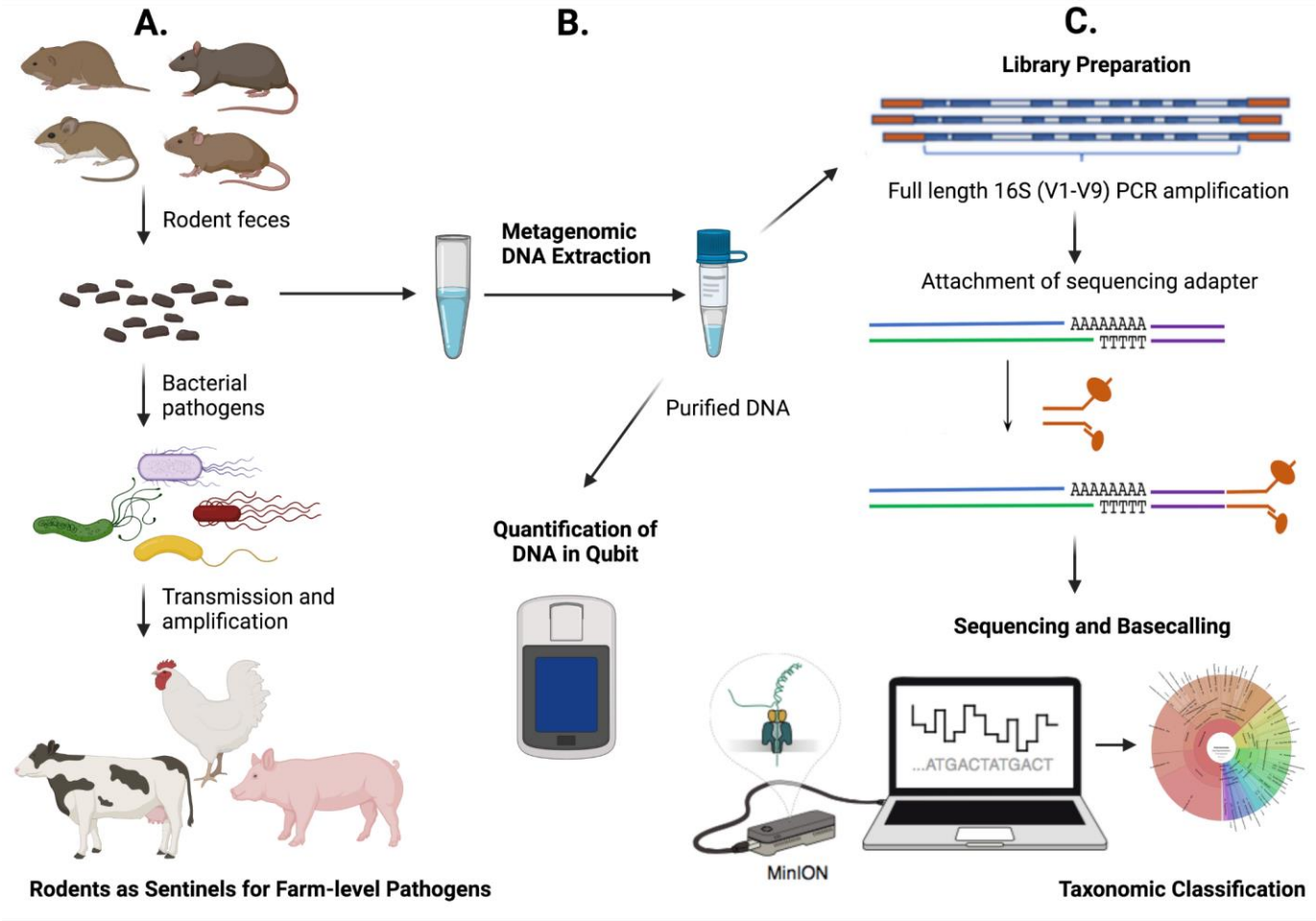

Figure 3: Workflow of the overall study design. **A:** showing rodents as amplifiers of bacterial pathogens through fecal deposition in the farm environment and possible transmission to farm animals. **B:** DNA extraction from rodent colon contents (i.e., feces) and quantification with Qubit fluorometer. **C:** Laboratory workflow to monitor bacterial communities from rodent fecal samples using Nanopore sequencing (Details in Materials and Methods).

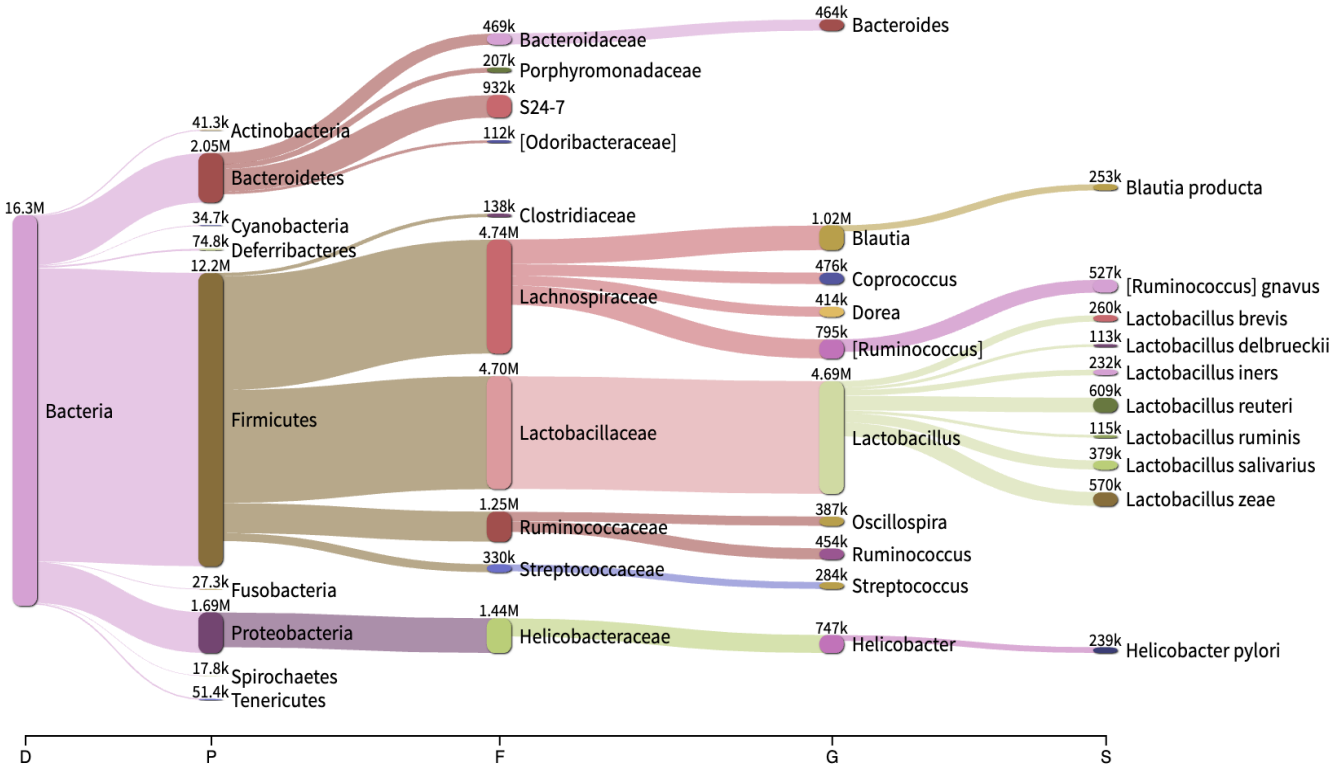

Figure 4: Core rodent fecal microbiome observed herein (n=90)

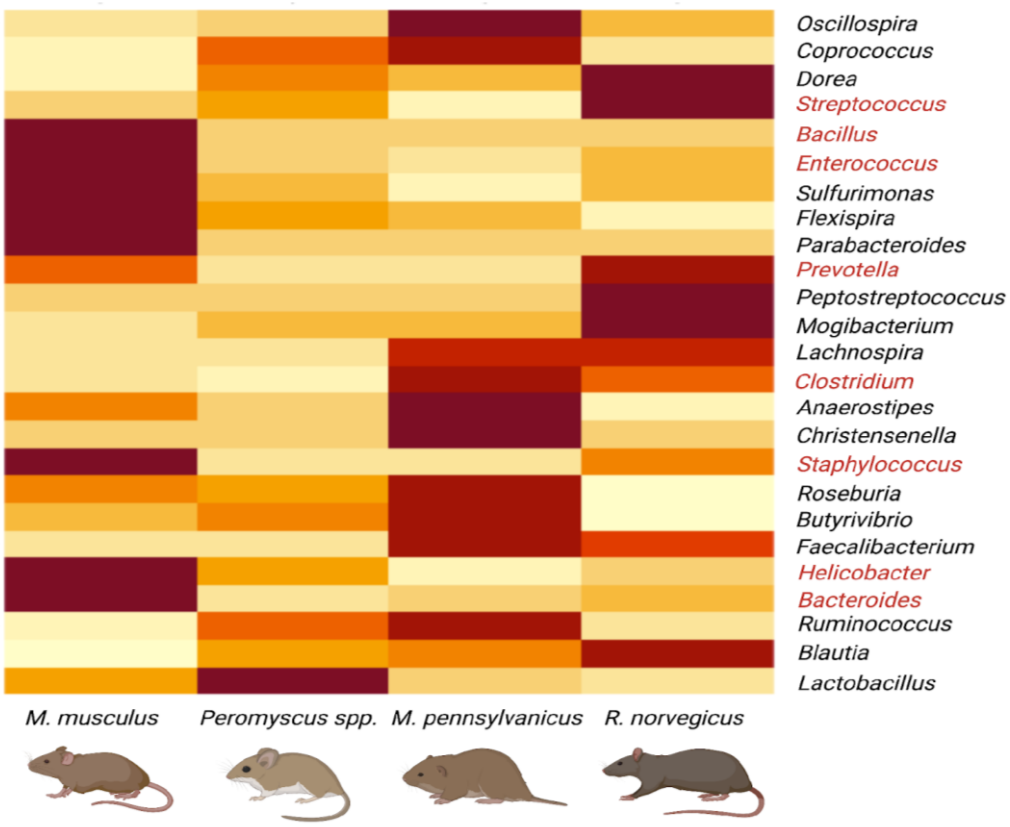

Figure 5: Heatmap of the most abundant (> 1%) bacterial genera identified by mapping 16S rRNA gene amplicons sequenced on Nanopore MinION against the GG reference database. Potential human pathogenic genera are labelled red in the legend.

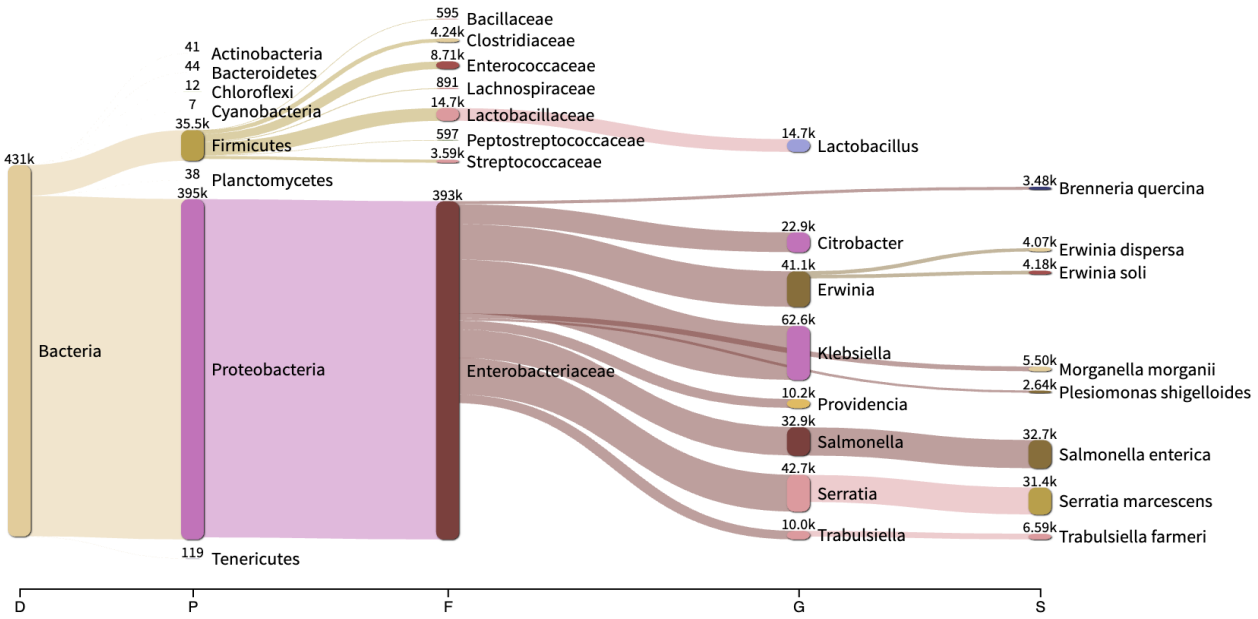

Figure 6: Shrew (*B. brevicauda*) fecal microbiome observed herein (n=2)

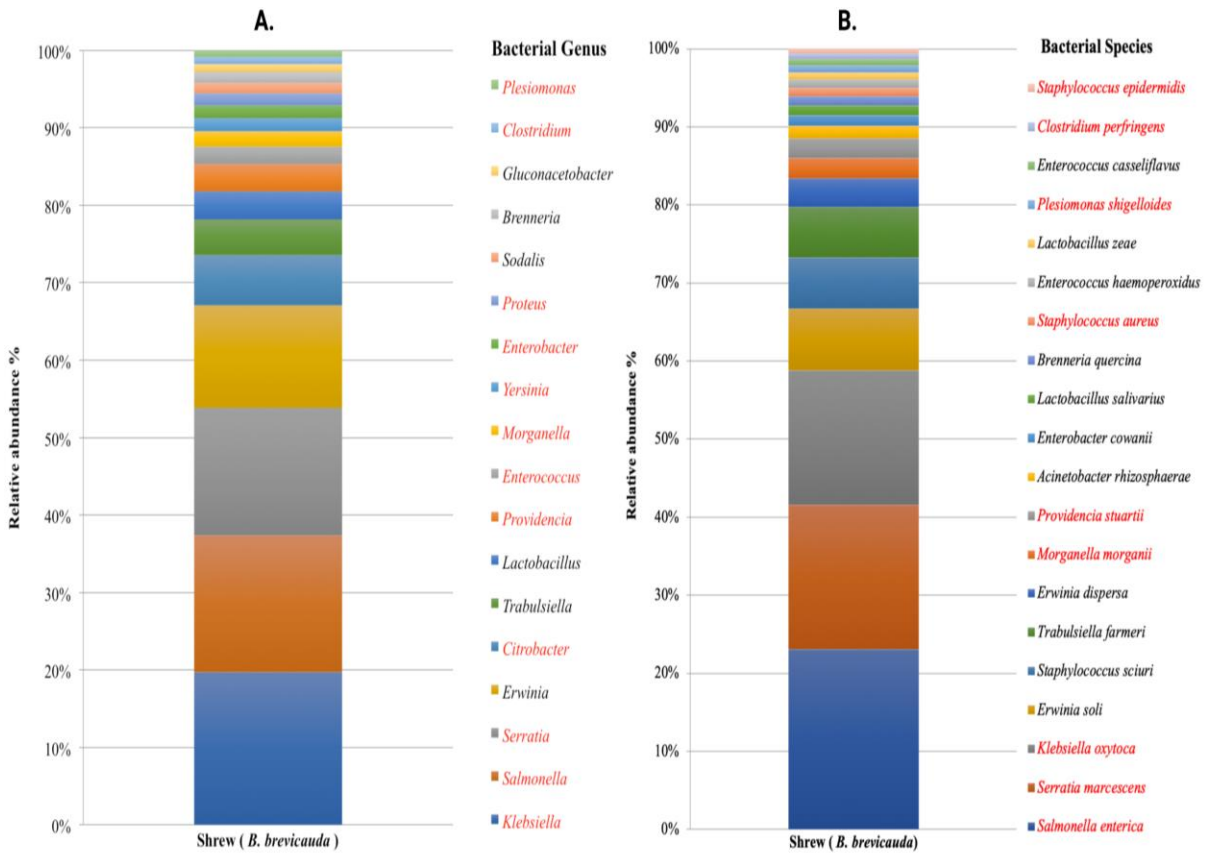

Figure 7: Shrew (*B. brevicauda*) microbiota representing > 1% relative abundance at the genus (A) and species (B) level.

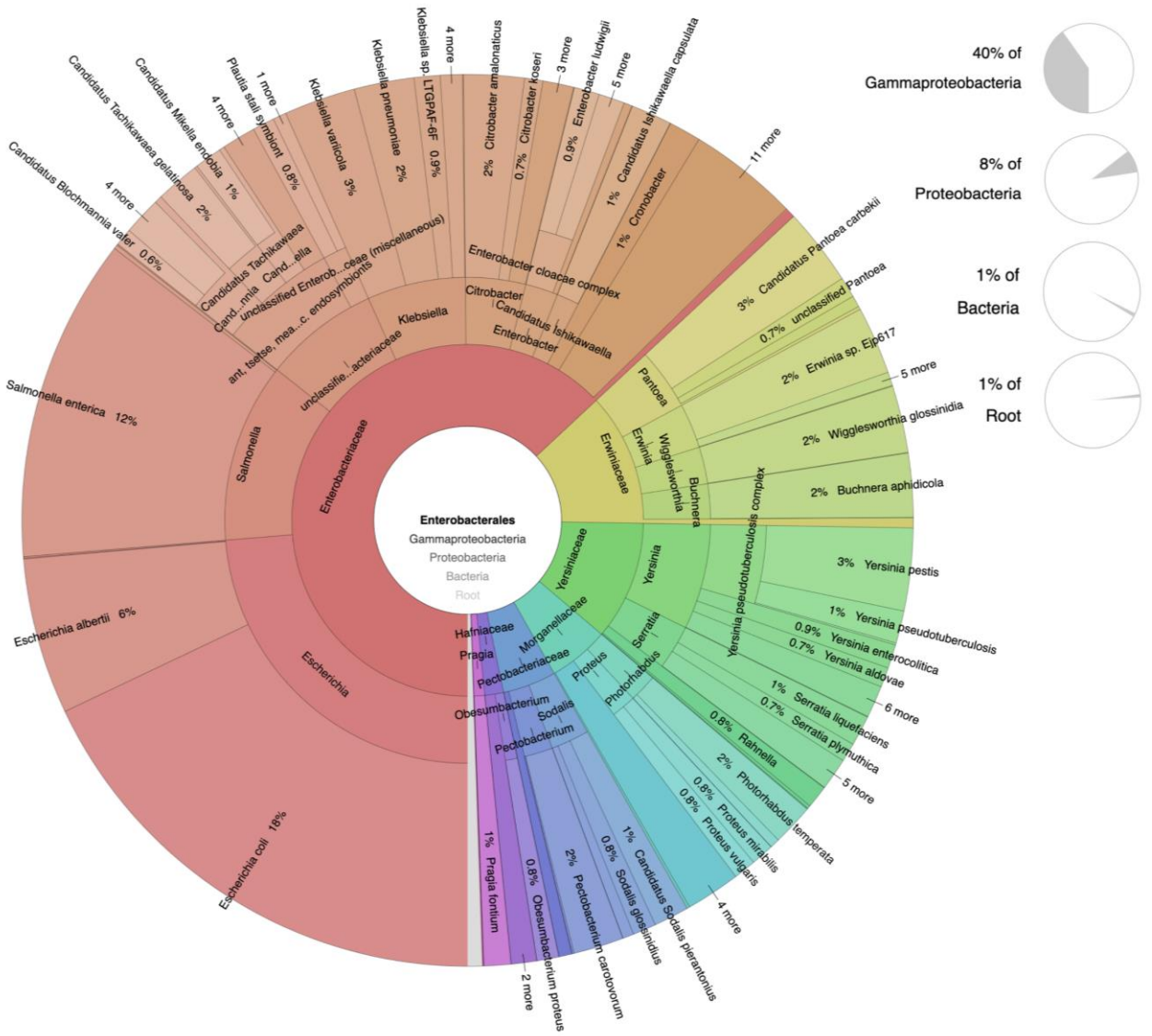

Figure 8: Krona plot showing overall (n=92) bacterial species abundance in the Enterobacteriales order.

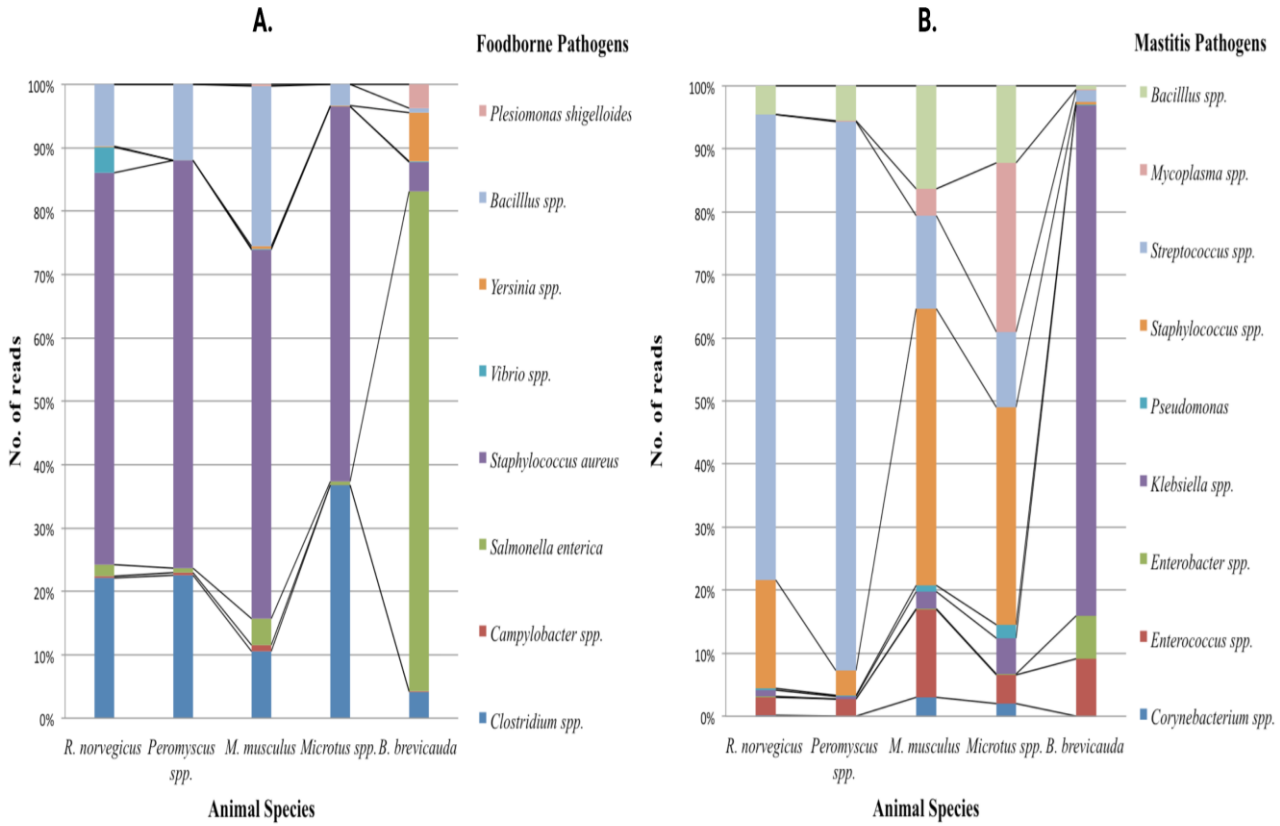

Figure 9: Abundance of Foodborne (A) and Mastitis (B) pathogens in all small mammal species.

CHAPTER 4

Molecular characterization and comparative genomics analysis of antibiotic resistant *Escherichia coli* and STEC isolated from farm dwelling rodent pests in upper Midwest, USA

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Synopsis

The effective control of rodent populations on farms is a critical component of food-safety, as rodents are reservoirs and vectors for many foodborne pathogens (e.g., *Salmonella* spp., *E. coli* O157, etc.). The functional role of rodents in the amplification and transmission of foodborne and AMR pathogens is likely underappreciated in the United States. Here, we address this existing knowledge gap by characterizing antibiotic resistant *E. coli* and STEC from rodent pests that were collected from Minnesota and Wisconsin food animal farms. We leveraged Whole genome sequencing (WGS) with Illumina NovaSeq platform to conduct a comprehensive genomic analysis of the putative food-borne pathogens. Rodents (mice and rats) (n=90) were live trapped from three food animal farms and fecal samples were collected. Antibiotic resistant generic *E. coli* and STEC (n=80) were isolated from rodent feces samples using traditional culture and selective enrichment methods. We observed that the most common phenotypic drug resistance was obtained for Tetracycline (49%), followed by Streptomycin (42%), and Ampicillin (30%). Furthermore, multidrug resistance (MDR) was obtained for 45% of the isolates. Comparative genomics analysis revealed *E. coli* isolates belonged to 35 different O:H serotypes, 34 different Sequence types (ST), and 57 different virulence associated genes. The most frequently found sequence types were ST75 (15.4 %), ST10 (7.7 %), and ST58 (7.7 %), which are often associated with ExPEC isolates. The most frequent serotypes were O45:H8 (15%) and O32:H9 (4.5 %), where O45 is from the well-known big six STEC group. Two isolates belonged to the highly virulent STs associated with human disease ST131 and ST69. In silico resistome analysis revealed highest resistance to tetracycline (56.3%), beta-lactam (46.3%) and aminoglycoside (43.8%) drug classes.

None of the presumptive STEC isolates contained any shiga toxin associated genes (*stx, eae, saa*). Furthermore, phylogenetic analysis did not represent any rodent specific clade for rodentborne *E. coli* isolates, rather clustering was observed mostly based on sequence types. Our findings reveal a diverse genetic background and high virulence and resistance potentials for rodent *E. coli* isolates from dairy cattle farms in Midwest. Knowledge gained from our research efforts will directly inform and improve upon farm-level biosecurity efforts and public health interventions to reduce future outbreaks of foodborne disease.

Keywords agriculture, rodents, STEC, ARG, *E. coli*, whole genome sequencing, dairy cattle

Introduction

Zoonotic pathogens are a major source of emerging infectious diseases including foodborne illness. Approximately 72% of zoonotic emerging infectious diseases were caused by pathogens with a wildlife origin in the last decade, with a recent increasing trend (Jones et al., 2008). Of these, zoonotic foodborne pathogens are an ever-present risk to food production pipelines all over the world. Furthermore, urbanization and adaptation of wild animals such as rodents (e.g. peridomestic rats and mice) in both urban and agricultural areas has resulted in increased contact of these small mammals with both humans and food animals (Jahan et al. 2020). Such interactions increase the potential transmission of zoonotic foodborne pathogens via fecal contamination of the environment, such as animal feed, water supplies or the food processing chain (Rothenburger et al. 2017; Jahan et al. 2020). Several studies have documented the roles of rodents as reservoirs and/or vectors of foodborne pathogens (e.g. *Escherichia coli, Salmonella, Listeria*) with links to foodborne illness outbreaks globally, including the United States (Kilonzo et la. 2013, Himsworth et al. 2015, Himsworth et al. 2013, Greig et al. 2015).

Escherichia coli is a common natural gut flora of mammals, however some pathotypes cause a variety of illnesses in both humans and animals. The most noteworthy is the Shiga toxin–producing *Escherichia coli* (STEC), also known as verocytotoxigenic *E. coli* (VTEC). STEC is a foodborne pathogen of significant public health concern causing human gastrointestinal illness and is estimated to cause over 265,100 cases each year in the United States (Scallan et al., 2011). Moreover, an estimated 96,500 O157 STEC and 168,690 non-O157 STEC infections occur annually in the United States,

resulting in over 3600 hospitalizations and 30 deaths each year (Scallan et al., 2011). The majority of STEC infections may present as a mild gastroenteritis, diarrhea, bloody diarrhea and hemolytic uremic syndrome (HUS), which can be fatal (Noris et al. 2012). While ruminants are recognized as principal reservoirs for STEC, wildlife such as rodents, deer, wild birds, and wild boars have also been associated with the carriage and dissemination of STEC (Espinosa et al. 2018). However more comprehensive data on the prevalence of STEC in wild animal species, particularly rodent pests, are required to better assess the role that these animals have in the transmission of STEC.

Bacterial resistance to antimicrobials is one of the most pressing public health challenges of our time. Among the drug-resistant bacteria of great concern, *E. coli* is a worldwide leading foodborne pathogen, and it is also an essential biomarker for monitoring antimicrobial resistance (AMR) by public health surveillance programs (Brisola et al., 2019, Gerner-Smidt et al. 2019). The frequency of resistance in *E. coli* is considered to be a good indicator of the selection pressure exerted by antimicrobial use in the host population (e.g. human, agricultural animal) and resistant *E. coli* may act as reservoirs of resistance genes for other pathogenic bacteria (van den Bogaard and Stobberingh, 2000). Free-living animals can be exposed to ARB through the environment, an observation shown as a key factor contributing to differences in AMR in particular resident wildlife populations (i.e., farm versus residential areas) (Allen, 2010; Allen et al., 2011). It should be emphasized that rodent pests (e.g., rats, mice) seem to pose a significant source of AMR dissemination. Invasive rodent species (i.e., *Rattus norveigicus*, *Mus musculus*) migrate seasonally, often over long distances and between continents through international transports (e.g. ship, train, aircraft, cargo), which may

contribute to greater exposure of animals and their intestinal microbiome to various AMR drivers (Song et al., 2003, Arnold et al., 2016). In this context, the role of rodent pests as AMR carriers is noteworthy and requires more attention as described by other researchers (Saengthongpinit C. et al. 2019, Greig et al., 2015).

The advent of bacterial whole genome sequencing (WGS) has enabled the scientific community to predict bacterial virulence profiles and antimicrobial resistance with an unprecedented level of confidence (Tyson et al. 2015, Gerner-Smidt et al. 2019). Additionally, WGS tools have matured to the point that they can provide meaningful, low-cost, and timely data for the characterization of bacterial pathogens, including genotypic monitoring of antimicrobial resistance. Furthermore, public health laboratories have recently adopted WGS as a surveillance tool for monitoring foodborne outbreaks and circulating pathogenic strains (Vaughn et al. 2018, Lang et al., 2019; Lindsey et al., 2016). In the current study, we leveraged the Illumina NovaSeq platform to generate whole genome sequences from pre-selected antibiotic resistant *E. coli* and STEC isolates. WGS data enabled large-scale comparison of the strains isolated from rodent to human and animal strains of clinical origin gathered from publicly available database (e.g., NCBI, Enterobase). Here, we used a customized comparative genomics pipeline (Thomsen et al. 2016) to detect virulence genes, antibiotic resistance genes, serotypes, plasmids and clonal type of all selected isolates. Phylogenetic analyses were performed to determine the epidemiological relationships and virulence potential of *E. coli* isolated from rodent pests.

Methods and Materials:

Sample Collection

Rodent fecal samples were collected from three farms including one large dairy cattle and two mixed animal (dairy cattle, hog, equine) farms located in Minnesota and Wisconsin (described in Jahan et al. 2021). Rodent activity was elevated on farms per observations by farm managers, particularly observed on the large conventional farm with hundreds of Norway rats actively foraging around compost piles during daylight hours. All farms had poison bait stations, kill traps and cats as rodent control measures during the time of our visits. At each study site, four nights of rodent trapping was conducted using 150 Sherman live traps baited with oats. Decontamination of all traps was performed using a 10% sodium hypochlorite solution (10 min soak) before and after each trapping event.

Ethical Considerations

All trapped animals were humanely euthanized following approved UMN IACUC protocols (IACUC #: 1809-36374A). Standard morphological techniques were used to identify rodents to species-level and metadata (e.g., species, age, weight, sex, body measurements) were collected for each individual animal. Biological samples (e.g., feces, intestine, colon) were collected and preserved (e.g., liquid nitrogen, freezer) until further processing for bacterial culture work.

Antibiotic resistant *E. coli* and STEC isolation

For the selective enrichment of *E. coli*, all the fecal samples were enriched into 3 ml GN broth for 18-24 hr at 37°C. For the isolation of antibiotic resistant *E. coli* and Shiga-toxin producing *E. coli* (STEC), enriched samples were streaked on both CHROMagar STEC (CHROMagar, Paris, France) and MacConkey agar (BD Diagnostics) supplemented with tetracycline (≥16 μg/ml); ampicillin (≥ 32 μg/ml), and streptomycin (≥ 64 μg/ml) using sterile 10ul loops as previously described (Gunther et al., 2010). Presumptive colonies from both plates (Mauve on CHROMagar STEC and pink colonies on MacConkey agar) were further isolated on sorbitol-MacConkey (Oxoid) plate for secondary confirmation. Colorless (STEC) and pink (Non-STEC) colonies were tested for final confirmation by detecting the presence of Shiga-toxin associated genes (*eaeA, stx1* and *stx2*) and *E. coli* specific genes (*uidA, uspA*) (Godambe et al., 2017) using multiplex PCR as described previously (Meng et al., 1997). Once confirmed, isolates were preserved in 2 ml of brain heart infusion (BHI) broth (Becton Dickinson) with 40% glycerol and stored at -80°C for further analysis. Multidrug resistant (MDR) isolates were further selected by processing only those, which showed phenotypic resistance against two or more antimicrobial substances as determined.

DNA extraction and Sequencing

Genomic DNA was extracted using the protocol for cultured cells from the QIAamp PowerFecal Pro DNA Kit (Qiagen Inc., Germantown, MD, USA) with an automated DNA extraction system called QIAcube Connect (Qiagen Inc., Germantown, MD, USA). Purified DNA were then submitted to the University of Minnesota Genomic

Center (UMGC, St. Paul, MN, USA) for library preparation using DNA Nextera Flex kit (Illumina, San Diego, CA), and whole genome sequencing was performed on a NovaSeq 6000 instrument (Illumina) with 150-bp paired-end reads and a minimum of 30x coverage per isolate. The resulting sequences were uploaded and stored on the Minnesota Supercomputing Institute (MSI) High Performance Computing system. Sequence read quality was assessed using FastQC version 0.11.5 (Andrews, S. 2010) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic v.0.33 was used to cut Nextera adapter sequences, remove nucleotide sequences with an average quality per base cutoff of less than 20, and drop reads fewer than 36bp in length (Bolger et al., 2014). Genomes were assembled de novo with SPAdes v. 3.10 using parameters recommended for bacterial genome assembly (Bankevich et al., 2012).

Genotyping and phylogenetic classification

Assembled contigs were analyzed for the presence of antibiotic resistance genes (ARG), virulence genes, and sequence types using a publicly available database. VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/ VirulenceFinder/) (Kleinheinz et al., 2014) was used to identify virulence genes. Resistance genes were identified using ResFinder3.2 (https://cge.cbs.dtu.dk/services/ResFinder/) (Zankari et al., 2012) and ABRicate (v.0.8.13) (https://github.com/tseemann/abricate/). These analyses were performed with a default setting of a 90% of identity threshold and 60% minimum gene length overlap, and the presence of these genes was confirmed when a coverage and identity >90% was identified. *In silico* serotype analysis was performed using the Short Read Sequence Typing for Bacterial Pathogens (SRST2) program

(http://katholt.github.io/srst2), which maps reads to *E. coli* serotype references (Inouye et al., 2014). The serotype (i.e. O-type and H-type) allele sequences and profiles were obtained from the EcOH database for O serogroup and H flagellar antigen detection (Ingle et al. 2016). Single nucleotide polymorphism (SNP) analysis was performed using snippy version 4.1.0 (https://github.com/tseemann/snippy). A published *E. coli* K12 strain (Accession No. KU00096.3) served as the outgroup to root the tree enabling the easy configuration of the phylogenetic distance between the isolates on the branches. Also, Gubbins version 2.3.4 (Croucher et al. 2014) was used for phylogeny reconstruction. The phylogenetic SNP trees were generated using Snippy and core SNP's were aligned using Snippy core. For maximum-likelihood phylogenetic tree analyses RaxML version 8.2.11 was used and 1000 bootstraps were performed to determine clade confidence level (Stamatakis 2014). A maximum-likelihood phylogenetic tree with 1,000 bootstrap replicates based on core genomes of isolates was created using RaxML-NG (Kozlov et al. 2019). The phylogeny was visualized with annotations for isolate information and in-silico metadata (e.g., ST, serotype) to provide insights into the generated tree.

Additionally, WGS of *E. coli* isolates from various countries and sources (e.g., human, swine, bovine, poultry, rodent) curated at the Enterobase website (https://enterobase.warwick.ac.uk/) were downloaded and used alongside the isolates of this study for the whole-genome phylogeny analysis to ensure a current epidemiological and evolutionary analysis (Enetrobase data table). Phylogenetic trees were visualized, annotated, and edited using iTOL (https://itol.embl.de/) and Figtree (http://tree. bio.ed.ac.uk/software/figtree/).

Results

In total, ninety-two animals were captured including ninety rodents and two shrews from all three farms. Five rodent species were identified across our study sites, including three native (*Peromyscus maniculatus, P. leucopus,* and *Microtus pennsylvanicus*) and two invasive species (*Mus musculus* and *Rattus norvegicus*). The captured shrew species, *Blarina brevicauda,* is native to Minnesota and the upper Midwest. The majority of rodent captures were centered around feed bunks, grain storage sites, compost, and within cow barns. Of these, 48 (52%) corresponds to house mouse (*Mus musculus*) samples, followed by 24 (26%) White-footed mouse (*Peromyscus leucopus*), 7 (7.6%) Norway rat (*Rattus norvegicus*), 6 (6.5%) Meadow vole (*Microtus pennsylvanicus*), 5 (5.4%) Deer mouse (*Peromyscus maniculatus*), and 2 (2.2%) samples belonging to shrews (*Blarina brevicauda*). Species genotyping analysis of isolates revealed 15 presumptive *E. coli* isolate to be unknown. Hence, the isolates were sent to Veterinary diagnostic lab (VDL, UMN) for further characterization with MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time Of Flight). All of the unknown isolates (n = 15) were identified as *Citrobacter* spp. by MALDI-TOF.

STEC and Antibiotic resistant *E. coli*

Phenotypic characterization of STEC with selective enrichment media obtained 19 putative STEC isolates that represented blue-green pigmentation, typical for STEC on the CHROMagar STEC agar medium. All the isolates were further confirmed as *E. coli* with conventional PCR and were also tested for the Shiga-toxin specific genes *stx1, stx2* and *eae* (Table 4). None of the putative STEC isolates had the *stx1, stx2* or *eae* genes

present that are typical of Shiga-toxin producing *E. coli* O157:H7, the most commonly identified STEC strain in the USA.

Primary screening of antibiotic resistance with tetracycline, ampicillin and streptomycin obtained a total of 60 drug resistant *E. coli* isolates from rodent feces, where each isolate represented an individual animal (Figure 10). Using traditional culture techniques, we identified drug resistant *E. coli* in rodent feces from three upper Midwest dairy and mixed animal farms, where 65% isolates (60/92) were resistant to at least one or more antibiotics. Multidrug (≥ 2) resistance profile was observed for 41/92 (45%) isolates. Most common drug resistance was detected for tetracycline 49% (45/92), followed by streptomycin (42%), and ampicillin (30%).

Acquired antimicrobial resistance genes (ARGs)

To detect the genotypic basis for phenotypic antibiotic resistance, following Illumina whole-genome sequencing, we applied *in silico* analysis to identify acquired ARGs in the assembled genomes using the Resfinder database. Genomic analysis of *E. coli* isolates revealed a mosaic of antibiotic resistant determinants and virulence genes within our samples. In total, 50 acquired antimicrobial resistance genes belonging to 9 antimicrobial classes were detected. We found that 18/50 of the ARGs were predicted to have activity against beta-lactams, 8/50 against quinolone, 7/50 against aminoglycosides, 7/50 against trimethoprim, 4/50 against tetracycline, 2/50 against phenicols, and 2/50 against sulphonamide. In addition, we identified a single ARG for colistin and macrolide drug class such as *mcr*-9 and *mph*(A), respectively (Figure 11). Among all isolates, genes

predicted to confer antimicrobial resistance against tetracyclines, beta-lactams, aminoglycosides, and sulfonamides were most common.

Forty-five isolates (56.3 %) were found harboring a total of 4 different genes predicted to encode for resistance against tetracycline. Of these genes, the most common were *tet*(A) and *tet*(B) alone or in combination with other tetracycline resistance genes (i.e. *tet*(D), *tet*(M)). Similarly, thirty-seven isolates (46.3%) had one or more of the 18 genes predicted to confer resistance against the beta-lactam drug class. Furthermore, predicted resistance against aminoglycosides was found in 35 isolates (43.8%) harboring one or more of the 7 genes, where the most common were *strA* and *strB* (Figure 11). Additionally, two genes including *sul1* and *sul2*, conferring resistance against sulfonamides were found among 20 isolates (25%). Phenicol, quinolone, and trimethoprim predicted resistance genes were present in 15, 12, and 11 isolates respectively. Only three isolates had predicated resistance against macrolide with *mph*(A) gene and one isolate with *mcr-9* for colistin drug class. Interestingly, all the isolates belonging to STEC serogroups (O25, O26, O45, and O121) and sequence types (ST) had no acquired resistance genes, except one isolate harboring the *tet*(B) gene which confers tetracycline resistance. However, all the STEC isolates had efflux pump associated gene *mdf*(A) that provides natural or intrinsic resistance against multiple drugs. Moreover, one isolate was predicted to be extensively drug resistant, harboring 13 antimicrobial resistance genes against aminoglycosides, beta-lactams, colistin, quinolones, sulfonamides, tetracyclines and trimethoprim (Figure 11). Furthermore, three isolates had 12 ARGs against aminoglycosides, beta-lactams, macrolide, phenicols, sulfonamides, tetracyclines and trimethoprim.

Virulence associated genes (VAGs)

All isolates (65/66; 98.5 %) except one were found harboring tellurium ion resistance (*terC*), a factor that helps catalyze efflux of tellurium ions in *E. coli*. Other prevalent VFs included acid tolerance encoded by the glutamate decarboxylase (*gad*) gene (61/66; 92.4 %) followed by outer membrane protein complement resistance (*traT*) gene (47/66; 71.2 %), long polar fimbrial adhesion (*lpfA*) gene (39/66, 59.1%), outer membrane protease (*ompT*) gene (34/66, 51.5%), increased serum survival (*iss*) gene (30/66, 45.5%) among 49 other VAGs identified by VirulenceFinder database (Table 5). Interestingly, one isolate belonging to historically known uropathogenic strain ST131-H22 was found to be carrying 18 virulence-associated genes simultaneously. Likewise, another isolate belonging to ST69 also harbored 16 virulence-associated genes.

Other prevalent genes were iron acquisition genes encoding *sitA* (30.3 %), heat-resistant agglutinin gene *hra* (24.2%), serine protease autotransporter gene *pic* (21.2%) and the aggregative heat stable toxin encoding gene *astA* (21.2 %). Shiga-toxin associated VAGs included *terC, lpfA, astA, fyuA, espP*, and *katP*. All of the eight isolates belonging to STEC associated ST75 and O45:H8 serotype had the same VAGs profile including *IpfA, gad, sitA, terC, traT, pic,* and *celb* genes.

MLST, serotyping, and plasmid replicon typing

MLST analysis revealed, 63 (78.8 %) *E. coli* isolates were classified into 34 different sequence types (STs) and 17 isolates (21.2 %) were non-typeable or unknown. Among the known STs, the most frequently found were ST75 (15.4 %), ST10 (7.7 %), and ST58 (7.7 %) followed by ST101 (6.2 %), ST4086 (4.6 %) and ST744 (4.6 %).

ST1704, ST6009, ST2008, ST278 and ST2169 each were represented by 2 (3.1%) isolates. The remaining 22 STs; ST131, ST69, ST542, ST349, ST181, ST201, ST515, ST117, ST48, ST1643, ST46, ST681, ST2485, ST442, ST2614, ST398, ST164, ST533, ST2526, ST388, ST34, and ST56 were represented by only 1 isolate.

Based on in silico serotyping analysis, a total of 35 different serotypes were distributed among the isolates. The most predominant serotype was O45:H8 (15 %) followed by O32:H9 (4.5 %). Two isolates each were found in the serogroups O21:H21, O87:H7, O9:H10, O26:H2, O8:H19, O108: H28 and O159:H8. The serogroups O25:H4, O8:H10, O15:H45, O173:H40, O121:H14, O17:H18, O10:H42, O76:H14, O8:H30, O3:H21, O45:H2, O88:H30, O25:H16, O117:H40, O166:H15, O129:H48, O55:H19, O32:H36, O114:H21, O153:H21, O2:H9, O85:H18, O30:H11, O1:H45, O8:H9, and O9:H12 were least prevalent containing only one isolate each. Additionally, 11 isolates had only H type (i.e. H25, H10, H32, H28, H14 and H7) and 3 isolates had only O type (i.e. O25, O32, O106) identified. STEC associated ST75 isolates were serotypically uniform with O45 O-antigen and were found possessing H8 flagellar antigen. A historically known foodborne uropathogenic strain, *E. coli* ST131 was represented with the typical serotype of O25:H4 and H22 fimtype (i.e. fimbriae).

In silico plasmid replicon typing revealed a total of 14 plasmid type where the IncF type plasmid was the most prominent (82.5 %) followed by ColRNAI (42.8%) among the isolates included in this study. Other replicon types included Col440I, Col8282, Col MG828, Col156, RepA, IncI, IncX, IncHI2, IncB/O/K/Z, IncY, IncQ2, and IncR. Out of the 52 isolates with an IncF replicon type, IncFIB (49/52; 94 %) was most prevalent followed by IncFIA, IncFII and IncFIC. All the STEC related ST75 isolates had

IncFII, IncFIB, ColRNAI, and Col156 plasmid type. However, ST131 represented only IncFIB plasmid type.

Phylogenetic analysis

The phylogenetic relationships among the 65 *E. coli* isolates from this study determined by single nucleotide polymorphism (SNP) analysis is shown in Figure 12. The genome of *E. coli* K-12, a well-known lab strain was used as the reference for the SNP analysis. The initial phylogenetic analysis consisted of a Maximum likelihood tree based on core SNPs (n = 3,829) among these 65 isolates (Figure 12) revealed a complex evolutionary history with the existence of multiple, diverse genomic variants of strains isolated from rodents. Some of these genomes formed discrete groups and clustering was consistent with their ST (ex. all ST75, ST58 strains clustered together). The geographic location of the farms (Minnesota vs. Wisconsin) where the isolates were obtained did not influence clustering. Phylogenetic analysis result of STEC, ExPEC isolates from our study alongside with historical clinical isolates from different sources and region were shown in Figure 13. Similar observations of isolates clustering based on sequence type was observed, which lends confidence to the underlying SNP data.

Discussion

The impact of zoonotic pathogens and antibiotic resistant bacteria on global public health and environmental issues is unquestionable. Thus, the identification of potential wildlife reservoirs, such as particular rodent species, is critical to elucidate sources of zoonotic ARB and to take appropriate and prompt actions against possible

emerging AMR mechanisms (Davies and Davies, 2010). Cattle are considered a major reservoir of STEC and molecular risk assessment studies on STEC isolates from cattle and other food animals have contributed to a better understanding of the virulence potential that STEC present to humans. However, most reports on virulence, antimicrobial resistance, and molecular epidemiology of STEC isolates are lacking data from wildlife and environmental sources. Rodent pests are present in almost every cattle farm regardless of the size and farming practice, but the status of STEC carriage and ARB in farm dwelling rodent is unknown. To our knowledge, this study represents the first use of next-generation sequencing and bioinformatics techniques in combination with phenotypic susceptibility testing to assess the extent of antibiotic resistance in farm dwelling rodent *E. coli* isolates from the Midwest, USA. Additionally, we isolated and characterized STEC obtained from rodents inhabiting farms in two broad locations (i.e., Minnesota, Wisconsin). Different serotypes, sequence types were identified, and we observed diverse combinations of virulence factors among the isolates; a few isolates exhibiting high-risk virulence gene combinations. Our study, although based on a convenience sampling of a limited fraction of rodent pest populations, suggests the AMR reservoir status of farm-dwelling rodents and reveals the zoonotic potential of rodent-borne.

Escherichia coli strains are considered indicators of antimicrobial resistance and have been used for monitoring and surveillance of AMR from various sources. Even though antimicrobial resistance among STEC is not very common, an increase in resistance has been recently described (Freedman et al., 2016). Antimicrobial resistance profiling showed that all the presumptive STEC isolates (100%) from rodents were

phenotypically susceptible to all the 3 tested antimicrobials (i.e., Tetracycline, Ampicillin, Streptomycin). *In silico* analysis confirmed the antibiotic susceptibility status with no antibiotic resistance genes for all the 18 presumptive STEC isolates from rodents. We found only one presumptive STEC isolate from shrew (*B. brevicauda*) feces that displayed AMR, which was resistant to tetracycline. This antimicrobial is used in animal and human health; studies have found that up to 100% of STEC isolated from humans and cattle was resistant to this drug (Lekshmi et al., 2017). Antimicrobial resistance genes *tet*(A), *tet*(B) and *tet*(D) found in this study have been frequently reported in *E. coli*, STEC and also in other *Enterobacteriaceae* (Sow et al., 2007; Cergole-Novella et al., 2011; Begum et al., 2018). Additionally, AMR genotyping detected the mdf(A) gene in all STEC isolates which is associated with resistance against macrolides according to the information provided by the ResFinder database (Zankari et al., 2012; Kleinheinz et al., 2014). However, *mdf(*A) has been reported as an *E. coli* multidrug transporter (i.e., efflux pump) with a broad spectrum of drug resistance (Edgar et al., 1997). Since resistance against macrolides was not phenotypically tested in our study, correspondence with the phenotypic resistance could not be evaluated for this antibiotic group.

Four ExPEC related ST type (i.e., ST10, ST69, ST117, ST58) isolates were antimicrobial resistant phenotypically and carried matching resistance conferring genes. The three resistant isolates belonged to ST117 O85:H18 (tetracycline) with *tet*(B); ST69 O17:H18 (ampicillin) with *strA, strB*; and ST58 O9:H12 (tetracycline, ampicillin) with *strA, strB, tet*(A). However, all five of the ST10 isolates with different serotypes were tetracycline resistant with *tet(B)* gene only. Similar findings were made by Dong et al., who also reported resistance to ampicillin and tetracycline among isolates from a cattle

farm in South Korea (Dong et al., 2017). However, higher antimicrobial resistance levels to tetracycline, ampicillin, cephalothin and amoxicillin-clavulanic acid have been previously reported in a number of studies in STEC and ExPEC isolates (Karama et al., 2008; Amézquita-López et al., 2016; Iweriebor et al., 2015). Three isolates with 12 ARGs related to high risk clone ST744 and serotype O32:H9 were collected from house mouse in both Farm A (2) and Farm B (1). Isolation of multidrug resistant ST744 isolates from cattle was reported previously (Haenni et al., 2017)) and determined pathogenic in another study with a different serotype O89: H10 (Tacão et al., 2017). Another isolate from house mouse (Farm A) also exhibited high antibiotic resistance (n = 13) including mcr-9 and *blaSHV*-12 genes from ST388 and pathogenic serotype O45:H2. This ST type was reported in another study with MDR pattern isolated from wild bird (Skarżyńska et al., 2021). Collectively, the overall antibiotic resistance observed in our study was moderately high (56.3%) and multidrug pattern was also observed for 46.3% of the isolates. The very low antimicrobial resistance rates observed in potential STEC isolates suggests low risk, but overall AMR pattern is concerning. Results of this study suggest that the selection pressure exerted on rodents inhabiting in dairy cattle farms from which the isolates were recovered is not negligible. Since, cattle on dairy cattle operations in the USA are not supplemented with feed containing antimicrobial promoters that usually exert selective pressure on intestinal flora, presence of such antimicrobial resistant strains is unforeseen.

An interesting finding in this study was the occurrence of the plasmid-borne *mcr*-9 gene in an isolate from *Mus musculus* feces captured on a conventional farm with Streptomycin resistance (Figure 11). The *E. coli* isolate also carried ESBL, quinolone,

sulphonamide, trimethoprim, and tetracycline resistance genes. However, phenotypic resistance to colistin was not determined for the isolate as it was out of the scope for the current study. The occurrence of *mcr-9* gene was first reported in the USA in May 2019 from a clinical S*almonella Typhimurium* isolate (Carroll et al., 2019). Furthermore, isolates harboring *mcr*-9 were subsequently identified in six continents, including Europe, Asia, America, Oceania, South America, and Africa, which covered 21 countries (Li et al., 2020). In a USA study, phenotypic susceptibility to colistin in 100 *Salmonella enterica* and *E. coli* isolates from retail meat carrying the *mcr*-9 gene was reported. All the isolates were collected from the National Antimicrobial Resistance Monitoring System (NARMS) and were susceptible to colistin. The study suggests that the *mcr*-9 gene may not be associated with colistin resistance (Tyson et al., 2020). Furthermore, this particular isolate from our study also carried *blaSHV*-12 gene, which is an important ESBL producing pathogen causing nosocomial and community-acquired infections and have also been reported in *E. coli i*solated from livestock, poultry and wild birds (Yagi et al., 2000; Perilli et al., 2002; Díaz et al., 2010; Pouget et al., 2013; Alcalá et al., 2016).

Additionally, we identified 15 antibiotic resistant *Citrobacter* spp. (i.e., *Citrobacter freundii*, *Citrobacter braakii*) containing resistance genes to beta-lactam, quinolone, and tetracycline drug class. However, *Citrobacter* isolates phenotypically resistant to streptomycin lacked any conferring resistance genes (e.g., *strA, strB*) for that drug class in our genotypic analysis. *Citrobacter* is genetically related to *E. coli* being an *Enterobactericeae* and share virulence genes that are found on mobile genetic elements (Mundy et al., 2004). Thus, both have very similar morphological characteristics, which lead to phenotypic misidentification as *E. coli* in selective culture media (i.e.,

MacConkey agar) in our study. Furthermore, *Citrobacter freundii* is a highly infectious pathogen that causes colitis and transmissible colonic hyperplasia in mice, thus renamed as *Citrobacter rodentium* (Petty et al., 2010). Similar to enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *E. coli* virulence strategy, *C. rodentium* also uses a type III secretion system (T3SS) to induce attaching and effacing host gut (Mundy et al., 2004). However, *Citrobacter* rarely causes human infection, except for some rare opportunistic infections of the urinary tract, respiratory tract, wounds, and bloodstream infection (Anderson et al., 2018). Hence, we did not conduct any further analysis with the *Citrobacter* spp. as it was out of the scope of the current study.

Serotype and sequence type (ST) are important property when assessing the virulence potential of *E. coli*, particularly STEC, as some specific ones are highly associated with foodborne outbreaks. This is not only related with specific virulence gene presence (i.e., *stx, eae*), but the genetic pool of other virulence genes, ARG and sequence types associated with those particular strains (Karama et al., 2019). Moreover, *E. coli* genome presents high plasticity; with 3000 core genome and about 2000 accessory genome that makes the huge heterogeneity and genetic diversity among *E. coli* strains (Dobrindt et al., 2003; Land et al., 2015). Because of this, serotype and ST remains as a valuable tool for epidemiologic studies, not only for establishing the potential source, but also to evaluate the clonal and pathogenic potential of isolates. Although STEC O157:H7 is the most well known serotype due to its association with foodborne outbreaks, yet there is a worldwide increase in cases of human disease related to non-O157 STEC (Mathusa et al., 2010).

Rodent fecal samples may contain mixed populations of *E. coli* and STEC strains, many of which could share O-type antigens with a variety of H-antigens. It has been suggested that identifying *fli*C gene type may be helpful for detection of pathogenic STEC strains. Several H-types including H-, H2, H7, H8, H11, H12, H16, H19, H21, H25 and H28 are commonly associated with STEC strains and all have been found in our study. However, O serogroups when combined with different H antigens may contribute to different pathogenicity of the strains. For example, Serogroup O104 with the H7 or H21 can be more pathogenic than other combinations (Yan et al., 2015). Serotype O45 combined with H2, O26 combined with H2 and O121 with H8 appears to be a more pathogenic strain as it harbors Shiga toxin 2 called *Stx2f, Stx2a, Stx2c* (DebRoy et al., 2005; Karmali et al., 2003). Our isolates, on the other hand, were identified as O45:H8, and O121:H14, an O:H serotype combination that may not appear to be pathogenic. The WGS analysis and PCR results also revealed that all the O45:H8 isolates did not possess any of the *stx* and *eae* genes. This result also suggested that O:H serotype may reflect pathogenicity of *E. coli* strains.

We identified 34 ST and 35 serotypes in the 65 sequenced *E. coli* genomes, demonstrating a wide diversity among isolates. We also reported three strains of the Big Six groups: STEC O26, O45, and O121. The “Big Six” group (serogroups O26, O45, O103, O111, O121, and O145) has epidemiological importance in the US and around the world since they have caused multiple foodborne outbreaks (Hines et al., 2017; Scavia et al., 2018). On the basis of WGS characterization, ten of the isolates were identified as serotype O45:H8 and ST 75, which is a serotype and sequence type combination that is commonly associated with foodborne illnesses in humans (U.S. Department of

Agriculture, Food Safety and Inspection Service, 2012; U.S. Department of Health, and Human Services, Centers for Disease Control and Prevention, 2006). However, these ten isolates do not possess any *stx* genes (*stx1, stx2*), but it is possible for them to receive the gene from *stx*-converting phages disseminating throughout distinct *E. coli* lineages (Hooton et al., 2019). Additionally, O26:H2 represents a pathogenic serotype causing mild or uncomplicated diarrhea (Karmali et al., 2003). Furthermore, sequence type ST10, ST58, and ST117 identified in our rodent fecal isolates have been widely implicated with ExPEC infections in both human and animals (Roer et al., 2017; Mamani et al., 2019; Manges et al., 2019). Additionally, ST131 and ST69 are most common sequence types of *E. coli* causing urinary tract infections (UTI) worldwide (Riley 2014; Forde et al., 2019). Altogether, this information shows that some of the rodent isolates obtained in this study might represent a risk for public health.

A huge diversity of virulence genes (e.g., *espP, usp, katP, terC, iss, lpfA)* often associated with pathogenic *E. coli* was found in the genomes of rodent isolates in this study (Sarowska et al., 2019) (Table 5). There are some STs that have been described as pathogenic (e.g., UPEC, ETEC), but the strains with those STs did not carry any virulence genes for those pathotypes in our dataset. These results emphasized the need to analyze the data as a whole including virulence genes, ARGs, serotypes; ST or serotype alone should not be considered as an accurate predictor of pathotype or virulence. Some strains have the same ST, yet they show differences in their virulence profile as well as their pathogenic gene content. For example, there were 5 strains that were ST10 and from these none were classified as STECs. All were negative for any of the Shiga toxin or attaching genes (*stx, eae*, or *saa* genes), and thus considered low risk for causing

infection in human or animal. However, there might be other unknown genes that are associated with the STEC phenotype in our rodent isolates. Another possibility is the STEC associated gene loss for rodent host adaptation, as rodents do not acquire STEC infections similar to humans or are less susceptible to Shiga toxin (Russo et al., 2014). This demonstrates that a single characteristic (e.g. ST or serotype) is not enough to make an inference of the potential pathogenic trait of any STECs. Ideally, information such as ST, *stx* type, virulence genes, and serotype would be considered to make a more informed prediction of the pathogenic potential of any *E. coli* or STEC in conjunction with historical available data on clinical cases. One example is of a O113:H21 strain containing *stx2a, saa* (a gene that encodes an auto-agglutinating adhesion), but lacking *eae* gene and reported to cause HUS cases (Shen et al., 2015). Similarly, in the case of any STEC that has all those pathogenic attributes but has not been linked to any human cases may be actually harmful to humans. While we cannot predict the actual disease outcome of a strain with certain genetic attributes, it still warrants a warning about its presence in foods or environment and potential threat to public health.

Single-nucleotide polymorphisms (SNPs) based phylogenetic analyses can help in determining the potential source, phylogenetic nature, lineage, and timeline of transmission of each group. For example, Martinez-Urtaza et al. (2017), have showed SNP analysis very useful for the ST36 lineage of *Vibrio parahaemolytic*a. In the current study, phylogenetic reconstruction revealed many different clades among these *E. coli*, STECs, and ExPECs isolated from rodents (Figure 12). As expected, most of these isolates clustered based on ST and serotype, and it appears that the geographic origin and source type of an isolate had only a marginal influence on clustering. However, for a few

ST types (i.e., ST2485, ST10, ST58) it was observed that mammalian source (e.g., swine, bovine) was clustered more closely together than avian source (e.g., turkey, chicken), indicating host specificity for *E. coli* strains (Figure 13). While we expected that isolates within the same ST and from the same geographical area would cluster together, we did identify a case where one ST75 swine isolate from Germany clustered with ten other isolates from USA and with different sources including rodents, poultry, cattle (Figure 4). Another interesting observation was that the rodent-borne ST131 clustered more closely with the cattle source ST131 from Iowa, where the human source ST131 formed a separate clade. This was expected as the rodents from our study were collected from cattle farm and for the rodent obtaining the ST131 clone from cattle or farm environment is more likely the case than from human. Although this connection is intriguing, we did not have background information about the specific animals surveyed in the Iowan study which might have helped to explain the phenomenon. Other studies are required to discover the extent of this finding, perhaps by investigating cattle transport between areas or other factors that influence the survival of particular strains in specific settings.

The dramatic plasticity of the *E. coli* genome accelerates the adaptation of this species into various environments and host species adaptation, which provides numerous opportunities for new variants to emerge via the gains and losses of genes (Nogueira, T. et al., 2009). From our analysis we did not find any unique genetic signature associated with rodent-borne *E. coli* isolates. The presence of mobile genetic elements, such as plasmids and pathogenicity islands, could play a role in phylogenetic reconstructions; however, these elements are not considered in phylogeny when using a reference genome as in the current study, since they are not part of the core genome. To understand if the

rodent-borne *E. coli* isolates within our cohort have an identifiable population structure, future study with more rodent isolates and pan genome analysis considering both the core and accessory genomes of *E. coli* is necessary. In addition, future analyses using only the genomes of strains that are located within each individual or among selected clades can produce a more detailed evolutionary history.

This study focused on a small subset of rodent populations collected from three food animal farms, concentrating only on the target (i.e., tetracycline, ampicillin, streptomycin) antimicrobial resistant *E. coli* and STEC. Thus, its findings may not be generalized for all food animal farms, given that the diversity and number of resident rodents in a particular farm may vary largely due to the biosecurity and environmental conditions of the farm. Similar studies employing a larger sample size and covering greater geographical area and diversity of *E. coli* and other foodborne pathogens should be conducted. Additionally, phenotypic antibiotic susceptibility testing with large panels of therapeutic antibiotics (e.g., NARMS panel) would reveal the true scenario of AMR in the rodentborne *E. coli* isolates. However, this study adds to the knowledge that rodent pests carry potential pathogenic *E. coli* and AMR, which is a great public health concern and should not be neglected while taking control measures.

This is the first study that presents a genomic survey of antibiotic resistant *E. coli* and STEC isolated from farm dwelling rodent pests in the Midwest USA. Our results provide insights into the large diversity of *E. coli* and STEC isolated from rodents inhabiting dairy cattle farms in Minnesota and Wisconsin. We found that *E. coli* isolates from rodents lacked common key virulence-associated traits (i.e., *stx, eae, saa*) of STEC, however there might be other unknown genes that are associated with the STEC

phenotype in our rodent isolates. Additionally, ExPEC related ST, virulence were observed and most *E. coli* isolates contained high numbers of antimicrobial resistance genes, including the plasmid mediated colistin resistance *mcr-9* gene and ESBL producing *blaSHV*-12 gene. Hence, prudent use of antimicrobials in livestock farming is important to reduce the burden of antimicrobial resistant organisms entering the food chain. In addition, considering that some of the serotypes, sequence types were found in combination with virulence factors in the isolates, rodents may be considered a source of potential pathogenic AMR *E. coli* and STEC in the food animal farms. This also indicates the presence and abundance of those pathogenic bacteria in the food animals and farm environment as well. Therefore, some of the food products derived from these farms (such as milk, cheese, and meat) could become contaminated with potentially pathogenic STEC, risking public health. Farmworkers and their families may also be at risk due to close contact with the rodent pests, farm animals and animal feces. Therefore, appropriate control measures must be enforced at different food production levels to avoid pathogenic AMR *E. coli* and STEC spread to humans, foods, and animals. Specifically, improving biosecurity and good manufacturing practices would help avoid the contact of different food products with animal feces that can carry these human pathogens and become a vehicle of foodborne diseases.

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Table 4: List of primers used for the detection of *E. coli* and STEC

Primer	Organism	Sequence
lacZ F	*E. coli*	5′ TTGAAAATGGTCTGCTGCTG 3′
lacZ R	*E. coli*	5′ TATTGGCTTCATCCACCACA 3′
yaiO F	*E. coli*	5′ TGATTTCCGTGCGTCTGAATG 3′
yaiO R	*E. coli*	5′ ATGCTGCCGTAGCGTGTTTC 3′
uidA F	*E. coli*	5′ TGGTAATTACCGACGAAAACGGC 3′
uidA R	*E. coli*	5′ ACGCGTGGTTACAGTCTTGCG 3′
uspA F	*E. coli*	5’-CCGATACGCTGCCAATCAGT-3’
uspa R	*E. coli*	5’-ACGCAGACCGTAAGGGCCAGAT-3’
stx1 F	*STEC*	5’-TCTCAGTGGGCGTTCTTATG -3’
stx1 R	STEC	5’-TACCCCCTCAACTGCTAATA -3’
stx2 F	STEC	5’-GCGGTTTTATTTGCATTAGC -3’
stx2 R	STEC	5’-TCCCGTCAACCTTCACTGTA -3’
Eae F	STEC	5’-ATGCTTAGTGCTGGTTTAGG -3’
Eae R	STEC	5’-GCCTTCATCATTTCGCTTTC -3’

Table 5: Description of the virulence-associated genes among 65 *E. coli* isolates

Gene	Description of genes	Isolate no.	Percent Present
chuA	Outer membrane hemin receptor	65	98.5%
cia	Colicin Ia	61	92.4%
etsC	Putative type I secretion outer membrane protein	47	71.2%
fyuA	Siderophore receptor	39	59.1%
gad	Glutamate decarboxylase	34	51.5%
hlyF	Hemolysin F	30	45.5%
ibeA	Invasin of brain endothelial cells	20	30.3%
iroN	Enterobactin siderophore receptor protein	16	24.2%
irp2	High molecular weight protein 2 non-ribosomal peptide synthetase	14	21.2%
iss	Increased serum survival	14	21.2%
kpsE	Capsule polysaccharide export inner-membrane protein	12	18.2%
kpsM	capsular antigen / Polysialic acid transport protein	11	16.7%
ompT	Outer membrane protease (protein protease 7)	10	15.2%
papC	Outer membrane usher P fimbriae	8	12.1%
sitA	Iron transport protein	8	12.1%
terC	Tellurium ion resistance protein	8	12.1%
traT	Outer membrane protein complement resistance	7	10.6%
usp	Uropathogenic specific protein	7	10.6%
yfcV	Fimbrial protein	6	9.1%
neuC	Polysialic acid transport protein	6	9.1%
lpfA	Long polar fimbriae adhesin	6	9.1%
astA	Aggregative heat stable toxin	6	9.1%
hra	Heat-resistant agglutinin	5	7.6%
f17G	F17G adhesin subunit	5	7.6%
f17A	F17 fimbrial protein	5	7.6%
espP	Serine protease EspP	4	6.1%
tsh	Temperature sensitive hemagglutinin	4	6.1%

cvaC	Colicin V	4	6.1%
mchF	ABC transporter protein MchF	4	6.1%
iucC	Aerobactin siderophore	4	6.1%
iutA	Iron transport	4	6.1%
cnf1	Cytotoxic necrotizing factor 1	4	6.1%
cdtB	Cytolethal distending toxin subunit B	3	4.5%
cib	Colicin Ib	3	4.5%
pic	Serine protease autotransporter	3	4.5%
celb	Endonuclease colicin E2	3	4.5%
eilA	Efflux pump	2	3.0%
air	Aerotaxis receptor	2	3.0%
mcmA	Microcin M	2	3.0%
papA	Pap fimbrial major pilin protein precursor	2	3.0%
tcpC	Tir domain-containing protein	2	3.0%
vat	Vacuolating autotransporter toxin	2	3.0%
cma	Colicin M toxin	2	3.0%
cba	Colicin B	2	3.0%
afaE8	afimbrial adhesin	2	3.0%
afaD	Afimbrial adhesion	1	1.5%
afaC	Outer membrane usher protein	1	1.5%
afaB	Periplasmic chaperone	1	1.5%
afaA	Transcriptional regulator	1	1.5%
cea	Colicin E1	1	1.5%
clbB	Hybrid nonribosomal peptide/polyketide megasynthase	1	1.5%
mchC	MchC protein	1	1.5%
mchB	Microcin H47 part of colicin H	1	1.5%
ireA	Iron-regulated siderophore receptor	1	1.5%
katP	Catalase-peroxidase	1	1.5%

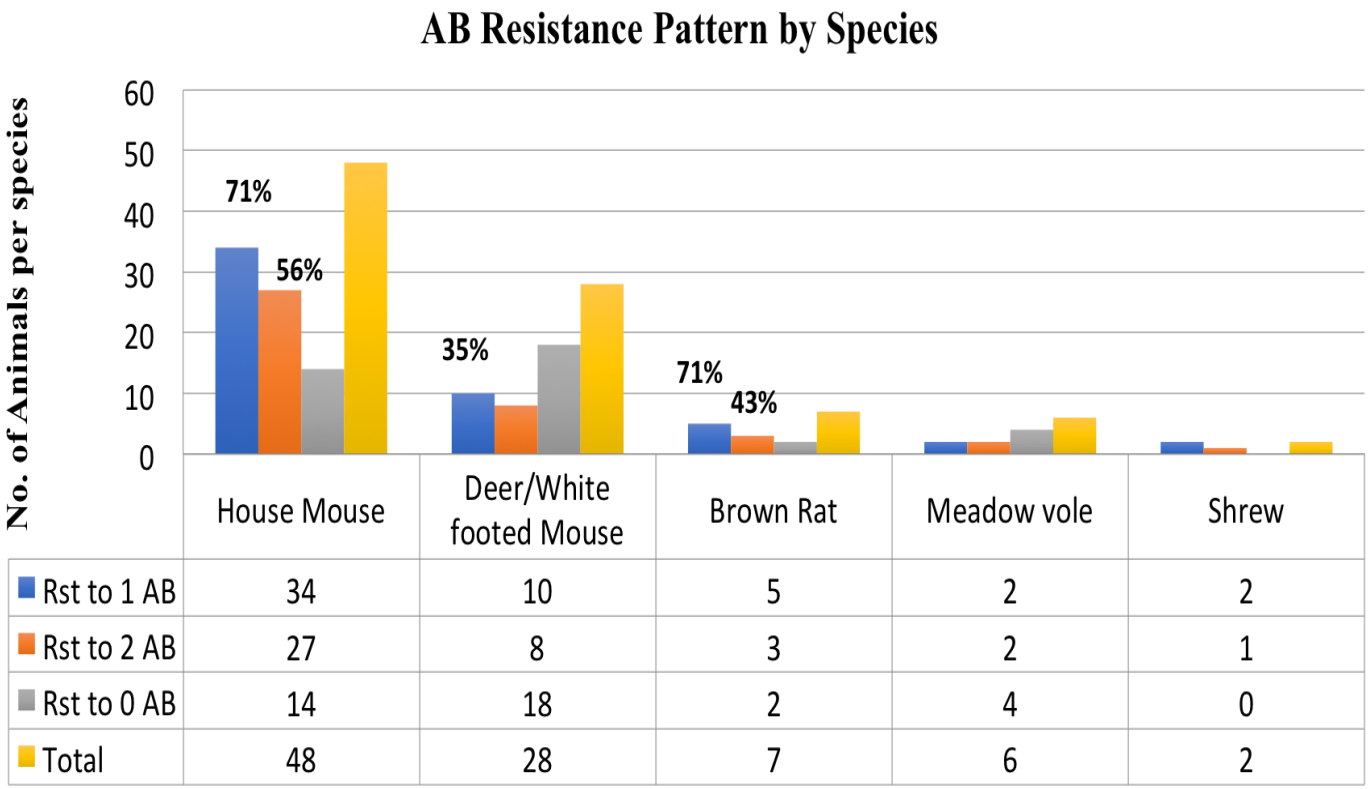

Figure 10: Phenotypic antibiotic resistance percentages (%) of *E. coli* exhibited by different rodent (House mouse = *Mus musculus*, Meadow vole = *Microtus pennsylvanicus*, White footed mouse = *Peromyscus leucopus*, Deer mouse = *P. maniculatus*, Brown rat = *Rattus norvegicus)* and shrew (*Blarina brevicauda*) species (n = 92) in our study.

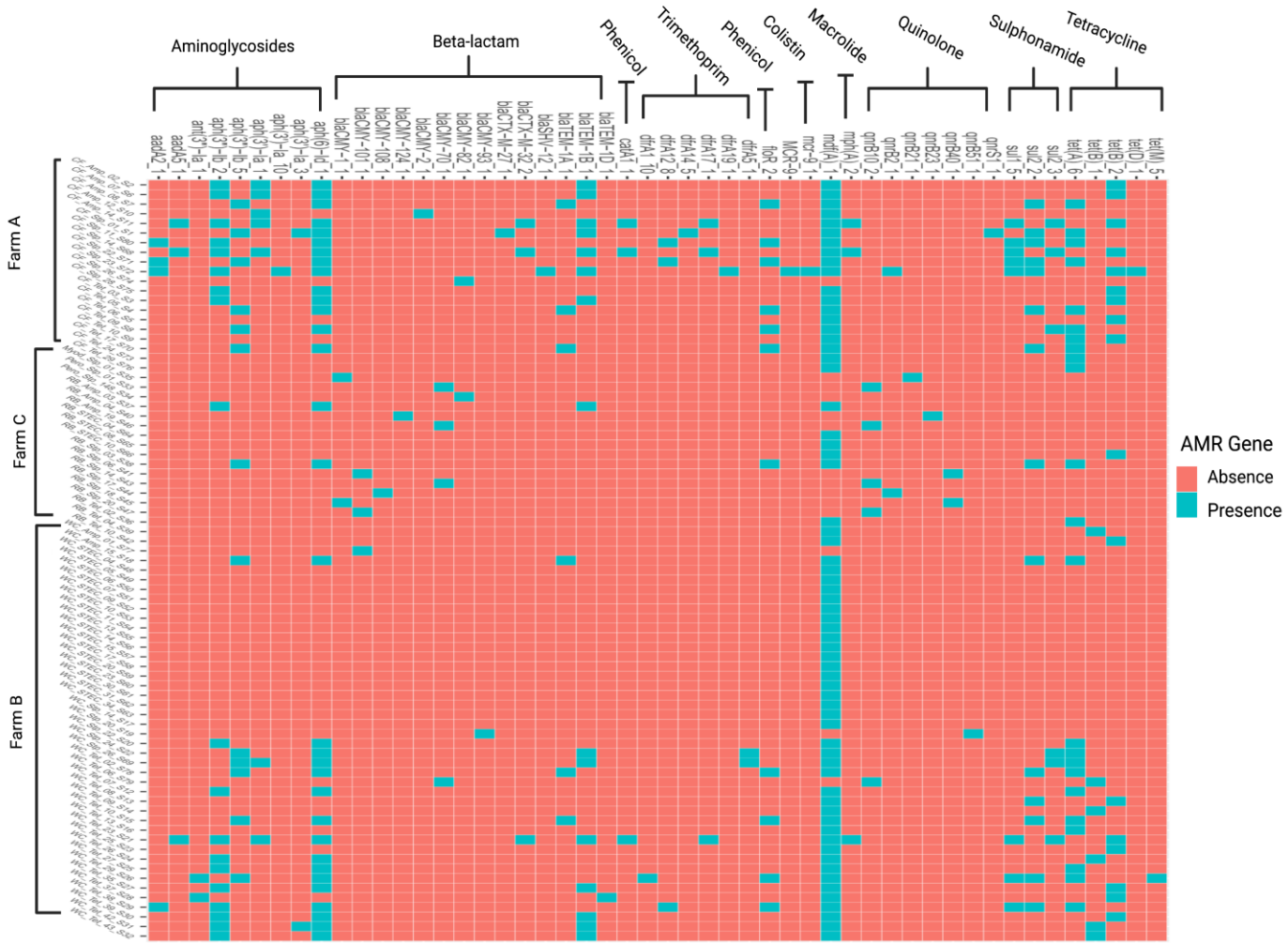

Figure 11: Matrix of the presence/absence of antibiotic resistance genes detected by WGS in *E. coli* genomes isolated from farm dwelling rodents in Minnesota (Farm A & B) and Wisconsin (Farm C) (n = 80). Genetic determinants of resistance are grouped according to their corresponding antimicrobial classes. Blue filling indicates that the gene is present.

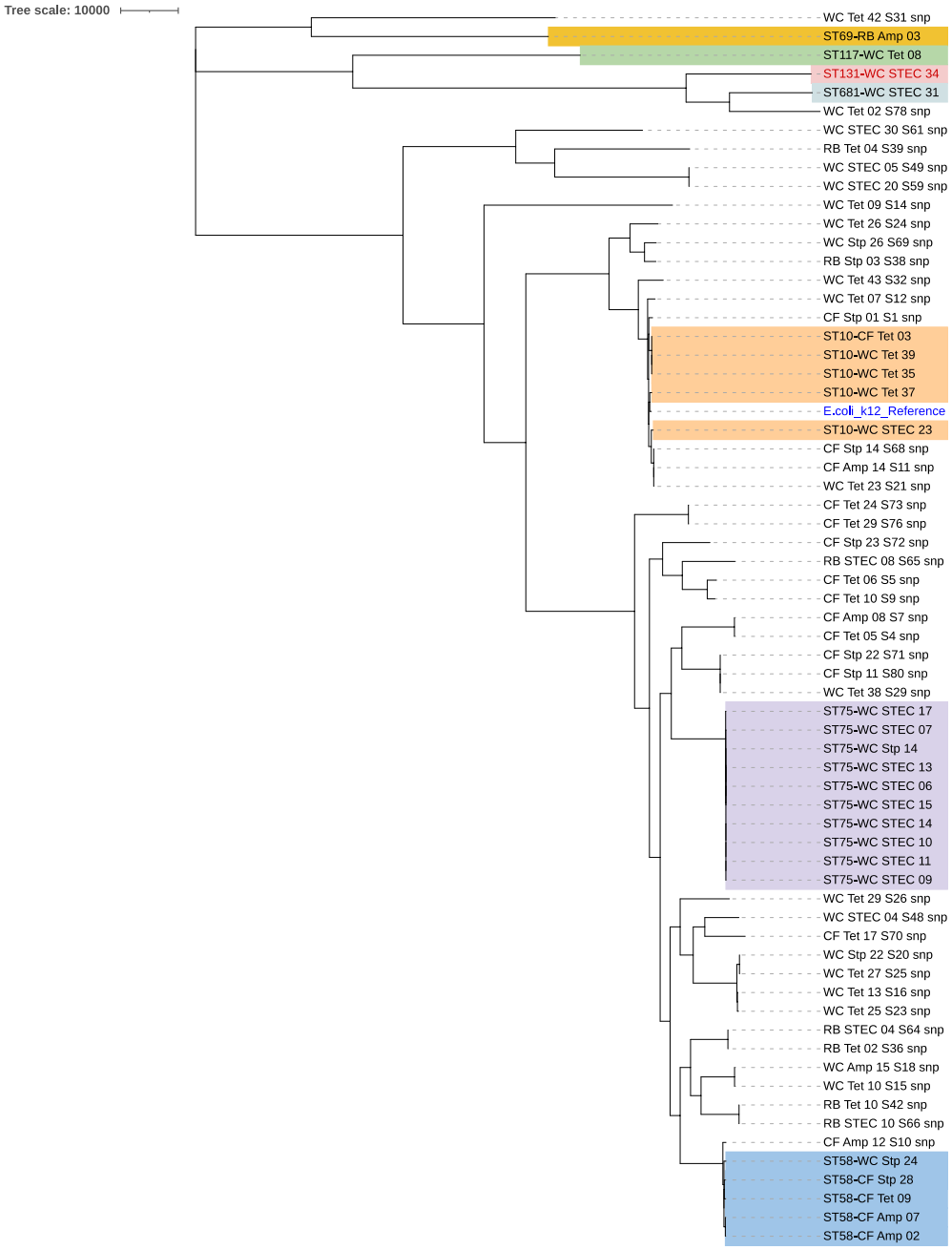

Figure 12: Phylogenetic relationships among the 65 genomes of *E. coli* sequenced in this study by SNP analysis based on 3,829 core SNPs. *E. coli* k12 was used as Reference strain. The evolutionary history was inferred by using the Maximum-likelihood (ML) tree built using the genetic distance and showing the existence of many diverse clades with clustering based on STs. Clades are colored based on different STs as labeled.

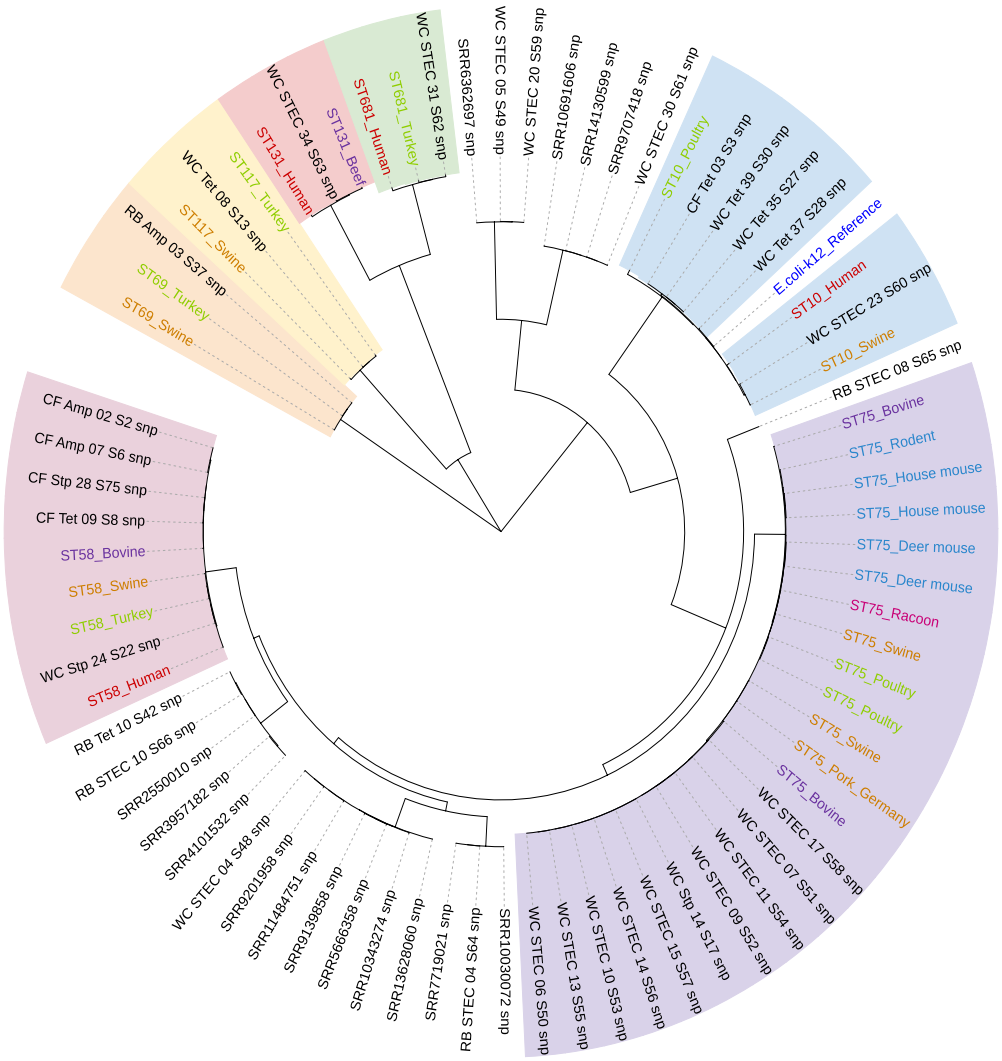

Figure 13: Circular phylogenomic tree with color annotations depicting the relationship between rodent *E. coli* isolates of pathogenic ST's from this study (n=32) and 44 reference strains of matching STs of human and animal clinical origin. The evolutionary history was inferred by using the Maximum-likelihood (ML) tree. Clades are colored based on different STs as labeled. Isolates of the same host (i.e., human, animal, or bird) were highlighted with the same color.

GENERAL CONCLUSIONS

This dissertation examined the metagenetic fecal community of farm dwelling rodent pests as well as the molecular epidemiology and genomic characterization of *E. coli* and STEC isolated from rodents inhabiting dairy cattle farms in Minnesota and Wisconsin. The research herein represents a novel multidisciplinary approach that blends classical mammalogy, genomics, and traditional culture techniques to characterize rodent-borne bacteria. This approach was leveraged to specifically address the lack of research focused on rodent pests of food animal farms and the potential foodborne zoonoses and antimicrobial resistant bacterial strains carried by them in the United States.

In Chapter 2, we focused on reviewing and collecting data from published literatures (n =149) across the world on the rodent-agriculture interface, especially rodent-borne zoonoses, presence and/or abundance of foodborne pathogens, and documented cases of antimicrobial resistant bacteria in rodent hosts. We confirmed a lack of concurrent studies and existing knowledge gaps regarding rodent foodborne zoonoses and AMR status in United States agricultural systems. Additionally, a focus of Chapter 2 was to highlight common species of both native (i.e., *Peromyscus leucopus, Peromyscus maniculatus, Microtus pennsylvanicus, Ictidomys tridecemlineatus, Tamias striatu, Sciurus carolinensis*) and invasive (i.e., *Mus musculus, Rattus norvegicus*) rodents with natural history traits contributing to their common appearance on farms and within food-production systems. Finally, we proposed a One Health metagenomic research approach and further comparative genomic studies concentrating on specific foodborne pathogens circulating in rodent hosts.

Chapter 3 aimed to characterize the fecal metagenomic community of farm dwelling rodent pests using Nanopore single-molecule sequencing technology and full-length 16S amplicon sequencing as a surveillance tool. The overall objectives were to: determine if the feces collected from farm-dwelling peridomestic rodents contained potential foodborne bacterial pathogens; identify what specific rodent species (i.e., native vs. invasive) carry these pathogens, and investigate if rodent species-specific patterns for pathogen carriage exist. Rodents were captured from three different food animal farms, mainly dairy cattle farm as part of convenience sampling. We live trapped rodents (n = 90) following approved protocols from three different farms including two dairy cattle farms in Minnesota and one mixed species farm in Wisconsin. We captured five different species of rodents including both native (*Microtus pennsylvanicus, Peromyscus leucopus, P. maniculatus*) and invasive (*Mus musculus, Rattus norvegicus*) species from our study sites. Additionally, we captured two shrews (*B. brevicauda*) and included the shrew fecal microbiota as an outgroup sample. A majority of the rodents captured in our study were house mice (*Mus musculus*, n= 48) and White-footed mice (*Peromyscus leucopus*, n= 24). However, this observation does not necessarily reflect farm-level dominant species, as we observed hundreds of Norway rats (*Rattus norvegicus*) on a particular farm during our field work. This species of rat is notoriously difficult to collect using live Sherman-trap techniques, thus necessitating a targeted pre-baiting and trapping strategy. Our analyses of the full length 16S gene indicated the presence of multiple putative foodborne pathogens (i.e., *Salmonella* spp., *Campylobacter* spp., *Staphylococcus aureus,* and *Clostridium* spp.) and many important mastitis pathogens (i.e., *Streptococcus* spp., *Staphylococcus* spp.). Collectively, the sampled rodent species displayed similar fecal

core microbiota with *Lactobacillus* spp. being the most abundant in all species. We did not observe rodent species-specific patterns regarding potential pathogen carriage in our sample. While comparing the fecal microbiota of rodents and shrews, we observed substantial differences suggesting the influence of different food habits (i.e., omnivore vs. insectivore). Interestingly, we found that the shrew fecal microbiota was represented with potential foodborne pathogens (i.e., *Salmonella enterica*) in high abundance. Although, standard culturing techniques and molecular methods are still required to confirm the presence of these putative pathogens, our surveillance goal was served by the rapid nanopore sequencing method. Our study provided baseline data for rodent and shrew fecal microbiota, and paved the way for future research focused on small mammals as potential reservoirs for foodborne zoonotic pathogens.

In Chapter 4, we focused on well-known foodborne pathogen STEC and antibiotic resistant *E. coli* to evaluate the carrier status and abundance of these potential pathogens in rodents inhabiting food animal farms. As two out of the three food animal farms were primarily dairy cattle farms, and cattle being a major reservoir for STEC, it was rational to determine the presence and abundance of this particular pathogen in the resident rodent pest community. Additionally, *E. coli* is ubiquitous being a major gut bacterium and antibiotic resistance in *E. coli* is very common with multidrug classes through the horizontal gene transfer (HGT) mechanism. Thus, it is important to evaluate the AMR status in rats and mice that are in close contact with food animals as they are often treated with antibiotics for treatment or preventive measures. Biological samples (feces, colon) collected from the work in Chapter 3 (n = 92) were used for isolating target organisms (i.e., STEC, *E. coli*) using appropriate microbiological methods and whole genome

sequencing was performed for genotyping. Phenotypic antibiotic screening obtained 46 drug resistant *E. coli* and 15 *Citrobacter* spp., where a majority of the isolates were resistant to tetracycline 49%, followed by streptomycin (42%), and ampicillin (30%). Multidrug (≥ 2) resistance was obtained for 45% isolates. We isolated 19 presumptive STEC isolates with conforming phenotypes from farm B and Farm C. Further genotyping revealed 34 different sequence types (STs) and a total of 35 different serotypes of *E. coli* from our rodent isolates. Pathogenic STs included ST131, ST69, ST58, ST75, ST117 and serogroup O45, O26, O121. However, presumptive STEC isolates from our study lacked the typical shiga-toxin producing genes (*stx, eae*) and contained other virulence associated genes such as *terC, lpfA, astA, fyuA, espP,* and *katP*. This result indicates that rodent STEC isolates from our study may not be pathogenic or may have other mechanisms of pathogenicity, which needs to be determined with animal challenge experiments. Phylogenetic analysis showed clustering of isolates according to STs, where distinct clades were formed close together by highly pathogenic STs with similar pathotypes (e.g., ST131, ST69, ST117). Comparative phylogenetic analysis with clinical isolates revealed that the geographic origin and source type of an isolate had only a marginal influence on clustering. In our study we did not observe any specific pattern for *E. coli* virulence or genotypic feature related to the farm's geographic location, or rodent species. However, a majority (69%) of the pathogenic STs and serotypes were observed in Farm B rodents, possibly due to the more diverse food animal population (e.g., dairy cattle, hogs) and farming practice (free-range) of that particular farm. Based on phylogenetic analyses, we did not identify unique genetic signatures associated with rodent-borne *E. coli* or STEC isolates in our study. However, more comprehensive

analyses focusing on pan genomes might reveal such rodent host specific features and accessory genetic elements. Major limitations of the study were small sample size, number of food animal farms and geographic region. However, this study provides basis for future research by providing exclusive genomic data on rodent-borne *E. coli* and STEC isolates.

Finally, future research directions include the development of *in vitro* and/or *in vivo* tests for confirming the virulence of suspected pathogenic strains of *E. coli* and STEC from rodents in our collection, as the presence of virulence-associated factors does not guarantee virulence. Additionally, larger sample sizes including more diverse rodent populations from different food animal farms (poultry, swine), broader geographical regions, and application of different statistical methods (e.g., regression modelling) are fundamental to assess the role of rodent reservoir with any species-specific pattern of pathogen carriage. Clearly, additional Norway rat (*Rattus norvegicus*) specimens are required to develop a better understanding of the zoonotic pathogen carriage status of this common farm-dwelling species, as per our observations. Furthermore, although we focused on *E. coli* and STEC, other foodborne pathogens such as *Salmonella*, *Campylobacter*, *Listeria* might be present in high abundance in rodent feces and were not the focus of our study. Another interesting finding from our metagenetic analysis was shrew feces (*B. brevicauda*) exhibited a high abundance of *Salmonella*, which might pose a public health risk and additional studies are required to confirm this finding.

Collectively, this dissertation has contributed to a better understanding of the rodent fecal core bacterial community and potential pathogenic carrier status in the midwestern United States. More specifically, we provided important data on rodent

diversity in food animal farms and the first insights of the shrew (*B. brevicauda*) fecal microbiota. We observed moderately high AMR and MDR traits in rodent isolates from our collection in all farms irrespective of geographical location or rodent species pattern, with low AMR in potential STEC strains. To conclude, this thesis provides a concrete example for the need to account for small mammal species (i.e., rats, mice, shrews) inhabiting food animal farms when generating surveillance and control strategies for zoonotic foodborne pathogens.

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