Characterization of extended-spectrum cephalosporin-resistant *Enterobacterales* recovered from dairy manure in Southern Ontario

by

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ABSTRACT

CHARACTERIZATION OF EXTENDED-SPECTRUM CEPHALOSPORIN-RESISTANT *ENTEROBACTERALES* RECOVERED FROM DAIRY MANURE IN SOUTHERN ONTARIO

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Few studies have investigated and characterized extended-spectrum cephalosporin (ESC) resistance among *Enterobacterales* recovered from dairy manure and even less research has been conducted on ESC-resistance plasmids among *Escherichia coli* and *Klebsiella pneumoniae* from livestock in Canada. The objectives of this research focused on the distribution and persistence of ESC-resistant *E. coli* and *K. pneumoniae* strains and their ESC-resistance determinants through manure treatment on dairy farms in Southern Ontario. Isolates were recovered from manure samples spanning treatment pipelines on six dairy farms. *E. coli* and *K. pneumoniae* carrying $bla_{\text{CTX-M}}$ and a subset of *E. coli* carrying bla_{CMY} underwent whole genome sequencing (WGS) using Illumina short reads, with a subset of these isolates undergoing long read sequencing using Oxford Nanopore Technologies. Results suggest that *E. coli* is a driving force in the dissemination of ESC-resistance in *Enterobacterales* in dairy manure and *K. pneumoniae* also seems important but appears to be less prevalent.

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DECLARATION OF WORK PERFORMED

The work presented in this thesis was performed by Rebecca Elsie Vivian Anderson, with the following exceptions:

1. All manure samples were obtained by Roger Murray.
2. Bacterial species identifications using MALDI-TOF were coordinated by Sarah Lippert and processed by the Animal Health Lab (AHL), University of Guelph.
3. Illumina MiSeq whole genome sequencing were performed by the Advanced Analysis Centre (AAC), University of Guelph.
4. Illumina NextSeq whole genome sequencing were performed by the National Microbiology Lab (NML), Winnipeg, Manitoba.
5. A few rounds of DNA barcoding and loading of the MinION flow cell were performed by Gabhan Chalmers.
6. Basecalling and demultiplexing of long read sequences were performed by Gabhan Chalmers.
7. Multivariable logistic regression analysis and overview of remaining statistical analyses were guided by Dr. David Pearl.

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LIST OF ABBREVIATIONS

AD	Anaerobic digestion
AHL	Animal Health Laboratory
AMC	Amoxicillin-clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial resistance
ARG-ANNOT	Antimicrobial resistance gene annotation
ARGDIT	Antimicrobial resistance gene data integration toolkit
ARIBA	Antimicrobial resistance identification by assembly
ATCC	American type culture collection
BEACON	Bacterial genome annotation comparison
BLAST	Basic local alignment search tool
BSAC	British Society for Antimicrobial Chemotherapy
BWA	Burrow-Wheeler alignment
CARD	The Comprehensive Antibiotic Resistance Database
CAZ	Ceftazidime
CC	Clonal complex
CFU	Colony forming unit
cgMLST	Core genome multi locus sequence typing
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical Laboratory and Standards Institute
CTX	Cefotaxime
DEW	Dewatered
DIAMOND	Double index alignment of next-generation sequencing data
DNA	Deoxyribonucleic acid
DWOS	Digestate without solids
DWS	Digestate with solids
ESBL	Extended-spectrum ß-lactamase

ESC	Extended-spectrum cephalosporin
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp*
ETP	Ertapenem
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FOX	Cefoxitin
GC	Government of Canada
GEN	Gentamicin
HGT	Horizontal gene transfer
HTC	Heat-treated compost
ID	Identification
Inc	Incompatibility
IS	Insertion sequence
KAN	Kanamycin
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
MDR	Multi-drug resistant
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MOB	Relaxase mobility
MPF	Mating pair formation
MST	Minimum spanning tree
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NML	National Microbiology Laboratory
OIE	World Organization for Animal Health (Office International des Epizooties)
ONT	Oxford nanopore technology
PacBio SMRT-Seq	Pacific BioSciences single-molecule real time sequencing
PATRIC	Pathosystems resource integration center
PBP	Penicillin binding protein

PBRT	PCR-based replicon typing
PCR	Polymerase chain reaction
PGAP	Prokaryotic genome annotation pipeline
PHAC	Public Health Agency of Canada
RAST	Rapid annotation using system technology
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
ST	Sequence type
STEC	Shiga-toxin producing *Escherichia coli*
STR	Streptomycin
SUL	Sulfonamide
SXT	Sulfamethoxazole-trimethoprim
T4SS	Type four secretory system
TET	Tetracycline
UTI	Urinary tract infection
VBNC	Viable but non-culturable
VFDB	Virulence finder database
wgMLST	Whole genome multi locus sequence typing
WGS	Whole genome sequencing

CHAPTER ONE: Literature Review

1.0 INTRODUCTION

Antimicrobial resistance (AMR) is a concern for both human and animal health due to the medical importance of antimicrobials. Antimicrobials have been widely used in the farming industry, which has led to the increase in prevalence of resistant bacteria in animals and fecal excrement, including manure. Extended-spectrum cephalosporins (ESCs) are third and fourth generation cephalosporins that have an affinity for an extended spectrum of Gram-negative bacteria, including *Enterobacterales*. These drugs are of critical importance for human and veterinary medicine, but resistance to ESCs is unfortunately widespread in bacteria from humans and animals. Despite the use of ESCs in the dairy industry, there is a paucity of literature on ESC resistance in dairy cattle.

Since manure is spread on fields for fertilization, the resistant bacteria it contains may end up on crops, in soil and ground water and thus eventually in animal feed, human food and drinking water (Wang et al., 2019; Marti et al., 2013; Coleman et al., 2013). These mostly non-pathogenic but resistant bacteria represent a reservoir of AMR genes and provide an opportunity for their transmission to other bacteria through horizontal gene transfer (HGT), including to pathogens. Transmission of AMR genes through HGT, mainly through plasmid transfer, can occur in a variety of niches including not only the gastrointestinal tracts of humans and animals (Wallace et al., 2020), but also in manure (Lima et al., 2020).

Enterobacterales are commonly found in manure. The main species among them, *Escherichia coli*, carries a diversity of genes conferring resistance to ESCs on a variety of plasmids, both in animals and humans (Rozwandowicz et al., 2018; Ewers et al., 2012). Therefore, *Enterobacterales* will form the focus of the investigations conducted for the present thesis. This review will focus on the environmental, biological and molecular characteristics that form the basis for the transmission and persistence of AMR genes and ESC-resistance genes and plasmids through dairy manure treatment processes.

1.1 MANURE TREATMENTS AND DOWNSTREAM IMPLICATIONS

Farming practices such as antimicrobial use and manure treatment procedures depend heavily on geographical location, livestock size and commodity. Farm Environmental Management in Canada (Statistics Canada, 2019) provides guidelines for proper manure management including manure storage, land application, water management, chemical input regulation and appropriate land management (Statistics Canada, 2003; 2004a; 2004b; 2005; 2007). Despite these guidelines, there are no mandatory manure treatment protocols in place and as a result, a broad variety of treatment types and combinations thereof are used in the field. Treated manure is typically applied to farmland as fertilizer because of its rich nutrient content, which benefits crop development and reduces the need for chemical fertilizers (Pandey et al., 2018). However, soil amendment with treated manure has the potential to distribute any hazardous microbes it harbours, subsequently contaminating crops.

1.1.1 Manure treatment processes

Methods used to break down manure for crop fertilization include mainly anaerobic digestion (AD) at mesophilic (30 - 40˚C) or thermophilic (50 - 65˚C) temperatures, and composting (Maeda et al., 2010; Shepherd Jr et al., 2009; Lv et al., 2013). Manure microbial content is abundant with a diversity of pathogenic and commensal bacteria. For instance, *E. coli* and *Enterococci* are abundant in cattle manure (Bonetta et al., 2011) and are ubiquitous carriers of AMR genes. These bacteria can enter the environment, contributing to transmission and dissemination of AMR. Treatments that reduce bacteria to below detectable limits in manure are preferable over others to reduce the risk of transmission to humans and animals when applied to the environment.

Research has shown mesophilic digestion to be less effective at reducing the numbers of AMR genes and resistant bacteria, compared to thermophilic AD (Pandey et al., 2014; Watcharasukarn et al., 2009). Although resistance determinants such as mobile genetic elements (MGEs) and AMR genes have been shown to decrease in diversity and concentration with thermophilic AD, resistant bacteria remain evident (Zou et al., 2019; Sun et al., 2016). The microflora does shift after AD compared to raw manure, as shown in some cases that Proteobacteria dominate after mesophilic AD and Firmicutes dominant after thermophilic AD

(Zou et al., 2019; Sun et al., 2016). This may have implications regarding the spread of AMR since shifts in the dominant bacterial phyla in the manure may influence AMR prevalence and transfer dynamics.

Composting is a common and traditional way to manage waste (Inbar et al., 1993) and can effectively reduce the prevalence of pathogens such as *E. coli* O157:H7 (Shepherd Jr. et al., 2009). Compost takes around 90 days to mature and reach optimal pH, nutrient and water composition levels (Inbar et al., 1993), which is achieved by the microflora breaking down organic compounds and producing heat as a by-product. Variations in dominant bacterial species have been found depending on where the sample is taken within the compost pile (Maeda et al., 2010). This microbial diversity can be attributed to varying oxygen levels, temperature gradients and nutrient concentrations and may again influence distribution of AMR genes and their transmission within the environment.

1.1.2 Health concerns for humans and animals

ß-lactam antibiotics, including cephalosporins are considered critically important in both human and veterinary medicine (WHO, 2019; OIE, 2018), therefore the persistence of ESC-resistant *Enterobacterales* and transmissible plasmids carrying ESC resistance genes through the manure treatment process is extremely relevant for public health. Antimicrobial resistance genes can be transferred to other bacteria in the same environment through HGT, potentiating the risk of genetic transfer to opportunistic pathogens in a healthy human's microflora or during infection (Overdevest et al., 2011; Ben Sallem et al., 2012; Rebbah et al., 2018). Therefore, understanding the persistence of ESC-resistant bacteria in manure and its derivatives is important in a One Health perspective since these bacteria can eventually impede the success of treatment. The detailed risk of ESC-resistant *Enterobacterales* being transmitted to humans from contaminated manure has yet to be fully elucidated but goes beyond the scope of this review and research.

1.2 *ENTEROBACTERALES*

In 2016, the order *Enterobacteriales* was re-classified to *Enterobacterales* in attempts to group genera into more phylogenetically related families (Adeolu et al., 2016). *Enterobacterales* is comprised of seven families, including *Enterobacteriaceae*, *Erwiniaceae*, *Pectobacteriaceae*,

Yersiniaceae, *Hafniaceae*, *Morganellaceae* and *Budviciaeae* (Adeolu et al., 2016). Some of the former *Enterobacteriaceae* have been redistributed into new families of the *Enterobacterales* order. *Enterobacterales* are ubiquitous and, like other bacterial orders, have the ability to receive and donate AMR genes and other genetic material. They can harbour various plasmid types and carry a diversity of AMR genes. However, what is true for some species of *Enterobacterales*, such as *E. coli*, may not be true for all. On a larger scale, what is true for *Enterobacterales* may not be true for all Gram-negatives and especially for Gram-positive bacteria. Thus, although plasmid mobility and ubiquity of *Enterobacterales* provide an excellent model for studying the complexity of AMR transmission, one has to remain aware that they represent only one of many pathways of AMR transmission in the environment.

Enterobacterales which commonly cause infections in dairy cattle are *E. coli*, *Klebsiella pneumoniae* and *Salmonella enterica* (Geser et al., 2012a; Mollenkopf et al., 2012; Dahmen et al., 2013; Abdi et al., 2018). Frequent infections caused by these organisms include but are not limited to mastitis, metritis, diarrheal diseases and respiratory infections (Pol and Ruegg, 2007; Saini et al., 2012; Catry et al., 2016; Abdi et al., 2018). The diversity of infections caused by these bacteria reiterate the importance of ESC-resistance among *Enterobacterales* strains.

1.2.1 Sequence typing

Multi-locus sequence typing (MLST) is a method often used by epidemiologists for surveillance of pathogenic bacterial strains, or by researchers to understand the diversity and dynamics of strains within a population. Bacterial lineages within a species can be identified using MLST, which usually assesses allelic variation within the genome at seven different loci, typically housekeeping genes, to assign a sequence type (ST) to a bacterial isolate (Maiden et al., 1998). Two more recent variations of MLST include core-genome MLST (cgMLST) and whole-genome MLST (wgMLST). Both use hundreds to thousands of loci that span across either the core genome or whole genome, respectively. These methods are therefore much more discriminatory than the original MLST method. MLST can help track the spread of bacterial clones, which can help trace persistent strains of bacteria, such as *E. coli,* through sequential manure samples or to assess the diversity of strains carrying similar resistance plasmids.

1.2.2 Strains of clinical importance found in dairy manure

Depending on their virulence genes and overall genetic makeup, some strains of *E. coli* are more pathogenic than others and more commonly found in diseased animals. Some STs commonly found among *E. coli* from diagnostic samples of dairy cattle include ST10 and ST23 which can carry ESC-resistance genes such as $bla_{\text{CTX-M}}$ and are major causes of mastitis in dairy cattle (Table 1.1) (Afema et al., 2018; Dahmen et al., 2013; Freitag et al., 2017; Taponen et al., 2017; Gröhn et al., 2005). *Escherichia coli* ST69 found in dairy manure can carry $bla_{\text{CTX-M}}$ and is considered a human pandemic clone (Afema et al., 2018). In Eastern Canada, *Escherichia coli* ST88 predominantly carrying $bla_{\text{CMY-2}}$ has been isolated from dairy manure (Awosile et al., 2020). A summary of other STs found to carry $bla_{\text{CTX-M}}$ or bla_{CMY} in *Enterobacterales* from dairy cattle can be found in Table 1.1, and a more in-depth explanation of ESC-resistance determinants is described below.

1.3 ANTIMICROBIAL USE AND ESCs

Antimicrobials have been used in the farming industry for growth promotion, disease prevention and as treatment of bacterial infections (GC, 2019; PHAC, 2014). Due to the rise in AMR in both human and animal medicine, Canada has modified laws and regulations aimed at restricting antimicrobial use. The Canadian Food Inspection Agency and Health Canada have been working together to ensure the compliance and enforcement of new prescription requirements for medicated feed, as well as updating the Compendium of Medicating Ingredient Brochures to remove growth promotion claims of medically important antimicrobials (GC, 2019). Additionally, antimicrobials previously available over the counter now require a veterinarian's prescription. Despite these changes, antimicrobial-resistant bacteria and multi-drug resistant (MDR) bacteria may persist due to selective pressure and endemicity within the environments (Poole et al., 2011; Enne et al., 2001).

1.3.1 Extended-spectrum cephalosporins

The ß-lactam class of antibiotics includes penicillins, monobactams, carbapenems and cephalosporins. The family of cephalosporins is comprised of five generations, with each generation targeting a different array of bacterial species. The third and fourth generations are

classified as ESCs because of their affinity for a broader diversity of bacterial species, including additional Gram-negative bacteria. They are also more stable against ß-lactamases compared to previous generations. Due to the affinity of ESCs to a broad spectrum of bacteria, their functionality needs to be protected and preserved through responsible use.

1.3.2 Mechanism of action of ESCs

ß-lactam antibiotics target the bacterial cell wall of Gram-negative and Gram-positive bacteria, preventing proper peptidoglycan synthesis. ß-lactams bind to transpeptidases called penicillin-binding proteins (PBPs), preventing cross-linking of peptidoglycan and proper cell wall formation. This leads to lack of cellular integrity and ultimately, cell death. ß-lactams have a bactericidal effect, but lysis only occurs in growing cells since they are targeted during cell wall synthesis (Prescott, 2013).

1.3.3 Cephalosporin use on Canadian dairy farms

Cephalosporins were used on Canadian dairy farms more than other antimicrobial classes. In a previous study, third generation cephalosporins were shown to be among the top three antimicrobials used in Alberta, Ontario, Québec and the Maritimes, next to tetracyclines and penicillins in 2007-2008 (Saini et al., 2012). Specifically, ceftiofur, which is a third-generation cephalosporin, has been used more frequently in Ontario compared to Québec (Saini et al., 2012) to treat foot infections, respiratory infections, metritis and mastitis (Pol and Ruegg, 2007). ESCs are frequently used in the dairy industry, which contributes to the persistence of ESC-resistance determinants and resistant *Enterobacterales* in the gastrointestinal tract and feces of dairy cattle (Call et al., 2008; Mirzaagha et al., 2011).

1.4 RESISTANCE TO CEPHALOSPORINS

Gram-negative bacteria have four main mechanisms of resistance to cephalosporins. These include PBP modification, decreased permeability, increased efflux rate and the production of ß-lactamases (Prescott, 2013). PBP modification prevents the ß-lactams from inhibiting proper cell wall synthesis. Changes to porin structure may reduce or inhibit the influx of ß-lactams and limit access to PBPs, mutations may also increase efflux rates and result in expulsion of ß-lactams

before they can properly bind to the PBPs. The production of modifying enzymes and especially extended-spectrum ß-lactamases (ESBLs) and plasmid-encoded AmpC ß-lactamases capable of hydrolyzing ESCs, render them ineffective. AmpC ß-lactamase is produced by an intrinsic, constitutively expressed chromosomal *ampC* gene in some *Enterobacterales* species, including *E. coli.* The associated low-level production does not result in clinically relevant resistance levels. However, point mutations in the promoter region upstream of this gene can up-regulate the production of cephalosporinase (Caroff et al., 1999) and result in a fully resistant phenotype. Bacteria can have one or more of the previously mentioned resistance mechanisms, influencing the extent of their ESC-resistance.

1.4.1 Extended-spectrum ß-lactamases

Extended-spectrum ß-lactamases can be categorized using two major classification schemes. The Bush and Jacoby method focuses on the functional characteristics of the enzymes whereas the Ambler method focuses on the molecular structure of their amino acid sequence (Bush and Jacoby, 2009; Ambler, 1980). Widespread groups of ESBLs include CTX-M, SHV and TEM, which are all categorized under molecular class A (Ambler, 1980) and functional group 2 (Bush and Jacoby, 2009). Molecular Class A ESBLs provide resistance to monobactams and oxyimino-cephalosporins such as cefotaxime, but not carbapenems nor cephamycins (Cantón et al., 2012). Their activity can be blocked by inhibitors such as clavulanic acid (Jarlier et al., 1988). This contrasts with AmpC activity, discussed below, which are Molecular Class C ESBLs and can be blocked by boronic acid but not clavulanic acid (Coudron, 2005; Shoorashetty et al, 2011). The inhibition with clavulanate can be used as a diagnostic tool to differentiate between ESBLs and AmpC ß-lactamase production in clinical isolates (Jarlier et al., 1988; Coudron, 2005; Shoorashetty et al., 2011).

Since their emergence in 2011, CTX-M have become the predominant ESBLs found in pathogens from farm animals in Canada. There are five main groups of CTX-Ms based on amino acid sequence similarity, named after the first described variant of each respective group (Bonnet, 2004). As of 2020, more than 238 variants have been identified (Naas et al., 2020; http://bldb.eu/BLDB.php?prot=A#CTX-M) and found in various Gram-negative species including *Enterobacterales* such as *K. pneumoniae*., *E. coli, Proteus mirabilis* and *S. enterica*.

(Nijssen, 2004; Kim et al., 2005; Tian et al., 2016). The expression of *bla*$_{\text{CTX-M}}$ may be altered if mobile genetic elements (MGEs) are inserted or excised between its promoter and the gene (Bonnet, 2004; Ma et al., 2011). In addition, the hydrolytic activity of ESBLs for different ESC molecules depends on the variant (Bush and Jacoby, 2009). Variant CTX-M-15 has been found in both humans and animals worldwide and is the most prevalent CTX-M variant in human pathogens in Canada (Denisuik et al., 2013; Bevan et al., 2017). In contrast, CTX-M-1 is rarely found in *Enterobacterales* from humans in Canada, but is frequent in Western European countries, such as France, Germany and the Netherlands (Robin et al, 2016; Bevan et al, 2017). In addition, CTX-M-1 is more frequently found in animal isolates worldwide (Bevan et al., 2017; Zhang et al., 2018; Cormier et al., 2019a). Overall, variants CTX-M-1, 2, 14, 15, 27, 55 and -65 have been detected in *Enterobacterales* from dairy cattle worldwide (Tables 1.1 and 1.2).

Currently, there are over 244 variants of SHV (Naas et al., 2020; http://bldb.eu/BLDB.php?prot=A#SHV) and just like CTX-Ms, each variant can hydrolyze specific cephalosporins at different rates (Heritage et al., 1999). The first discovered variant, SHV-1, which is believed to have originated in *K. pneumoniae,* is not an ESBL (Matthew et al., 1979; Bush et al., 1995). SHV-1 is a narrow spectrum ß-lactamase conferring resistance to penicillins (Matthew et al., 1979) whereas other variants such as SHV-2 and SHV-3 are ESBLs (Jarlier et al., 1988). Some variants such as SHV-2 are slow at hydrolyzing cephalosporins and may be undetected during screening due to the resulting low minimal inhibitory concentrations (MICs) for ESCs (Heritage et al., 1999).

To date, there are more than 249 variants of TEM (Naas et al., 2020; http://bldb.eu/BLDB.php?prot=A#TEM). The overwhelmingly most common variant in *Enterobacterales* isolated from humans and animals globally is TEM-1. This variant is not an ESBL, and therefore, TEM is not discussed in greater detail in this review.

1.4.2 AmpC ß-lactamases

AmpC ß-lactamases are cephamycinases and therefore not formally considered ESBLs. As mentioned above, and contrary to the latter, AmpC ß-lactamases cannot be blocked by inhibitors such as clavulanic acid (Jacoby, 2009). These enzymes belong to the Ambler Class C and Bush and Jacoby Class 1 ß-lactamases (Bush and Jacoby, 2009; Ambler, 1980). The main

enzymes of this group are the CMYs, with CMY-2 being the predominant variant found in bacteria from dairy cattle (Table 1.1 and 1.2; Mollenkopf et al., 2012; Awosile et al., 2018; Rehman et al., 2017). However, more than 170 CMY variants have been identified to date (Naas et al., 2020; http://bldb.eu/BLDB.php?prot=C#CMY). *Escherichia coli* naturally carry *ampC* in its chromosome which is constitutively expressed at low levels and can yield full resistance to ESCs if a mutation has occurred in its promoter region at position -10, -42, -32, +24 or -35 (Corvec et al., 2007; Caroff et al., 1999). AmpC ß-lactamases are found regularly in bacteria isolated from cattle fecal samples (Mollenkopf et al., 2012; Haenni et al., 2014; Awosile et al., 2018; Awosile et al., 2020; Cormier et al., 2019a; Cormier et al., 2019b).

1.4.3 Transmission and acquisition of antimicrobial resistance

Horizontal gene transfer is the transmission of genetic material between cells through conjugation, transformation or transduction. Conjugation, otherwise known as bacterial mating, is the transfer of genetic material from a donor cell to a recipient cell through direct contact mediated by a pilus (Madigan et al., 2015). Conjugation is the main mechanism responsible for transfer of antimicrobial resistance and is pivotal for the dissemination of AMR within a bacterial population. Transformation is the uptake of extracellular free DNA, which is then incorporated into the recipient cell genome, but is less frequently involved in transmission of AMR genes (Madigan et al., 2015) and will not be discussed further. Transduction involves a viral vector such as a bacteriophage, transferring DNA from one cell to another through generalized or specialized transduction (Madigan et al., 2015). Recent studies have shown the role of generalized transduction in disseminating AMR genes in a bacterial population with regards to bacteriophages acting as vectors in environmental and poultry meat samples (Raj et al., 2018; Shousha et al., 2015). Phages carrying resistance genes for kanamycin, chloramphenicol, ampicillin and tetracycline were isolated from chicken meat and these AMR genes were successfully transduced to *E. coli in vitro* (Shousha et al., 2015). Phages carrying $bla_{\text{CTX-M}}$ isolated from water and soil samples were also successfully transduced into *E. coli in vitro* (Raj et al., 2018). These results demonstrate the presence and transmissibility of AMR genes, including ESC-resistance genes, in phage DNA to *Enterobacterales.* Although transduction is not central for the purpose of this study on ESC resistance, this mechanism of

genetic mobility is part of the complex transmission pathways of AMR in the environment, including dairy manure.

1.4.4 Mobile genetic elements

Antimicrobial resistance genes can move within a cell (e.g. between plasmids and the chromosome) through the action of MGEs. Important MGEs for the mobilization of ESBLS and AmpC genes include insertion sequences (ISs) and transposons. Although less involved in the transmission of ESC-resistance genes, integrons will also be discussed briefly, since they are associated with the mobilization of other AMR genes.

Insertion sequences are simple transposable elements comprised of short, inverted repeat sequences flanking a transposase gene. Most ISs need two flanking copies to mobilize genes (composite transposons, discussed below) and other need only a single copy to mobilize adjacent genes, such is the case with IS26, ISEcp1 and ISCR. However, IS26 can use both ends to flank bla_{SHV} from the chromosome of *K. pneumoniae* and mobilize it to plasmids and can increase expression of downstream genes (Ford and Avison, 2004). Two methods in which IS26 use both ends include the generation of a target duplication via the formation of a cointegrate and IS26 (Harmer et al., 2014). Secondly, the incorporation of translocatable unit at an already existing IS26, which is the most frequent method of mobilization (Harmer et al., 2014). Other important ISs for the spread of AMR include ISEcp1 and ISCR, which both use only one-ended transposition (Poirel et al., 2003; Toleman et al., 2006). The former, ISEcp1, has been found to mobilize $bla_{\text{CTX-M-15}}$ (Karim et al., 2001) and is likely to mobilize other $bla_{\text{CTX-M}}$ variants in a similar way.

Transposons can carry accessory genes, such as AMR genes. Composite transposons are formed when two ISs flank and mobilize the DNA between them, which can include accessory genes. Depending on where the transposon is inserted, alterations to gene function and expression may also occur. Similarly to ISs, transposon insertions can have structural side effects including, for instance, porin loss or damage to the mis-match repair system (Rodríguez-Martínez et al., 2009; LeClerc and Cebula, 2000). Reported transposons carrying ESC-resistance determinants include, among others, Tn3-like or Tn2 carrying $bla_{\text{CTX-M-15}}$ (Smet et al., 2010;

Zong et al., 2015), various composite transposons carrying *bla*$_{SHV-5}$ (Preston et al., 2004; Garza-Ramos et al., 2009) and transposon-like elements carrying *bla*$_{CMY-2}$ (Su et al., 2006).

Although integrons are usually not directly involved in the mobilization of ESBLs, they may be associated with ESBL genes through recombinational events. Thus, linkages between ESBLs and resistance to other antimicrobials encoded by integrons such as sulfonamides and aminoglycosides are important in the context of multidrug resistance and their spread through HGT in *Enterobacterales* (Stokes and Hall, 1989; Schmidt and Klopfer-Kaul, 1984). Integrons are comprised of three main regions and mobilize various AMR genes (Yamashita et al., 2014). These regions include the 5' and 3' conserved regions and a variable region in between. Specifically in class one integrons, the 5' conserved region carries the *intI* gene which encodes a recombinase, *attI* which is the recombination site, and the promoter site which induces expression of the gene cassettes once they are properly incorporated (Messier and Roy, 2001; Partridge et al., 2000; Collis and Hall, 1995; Lévesque et al., 1994). The 3' region carries resistance genes for quaternary ammonium disinfectants and sulphonamides (Paulsen et al., 1993; Sundström et al., 1988; Stokes and Hall, 1989). Class one integrons are the most common in *Enterobacterales* and often carry resistance cassettes for streptomycin-spectinomycin and for trimethoprim in their variable region (Kilani et al., 2015; Yamashita et al., 2014).

1.5 PLASMIDS

Plasmids are extrachromosomal DNA which replicate independently of the chromosome and are often non-essential for cell survival. Bacteria may not have any plasmids or may have one or more which can carry genes for virulence, AMR and genes that offer beneficial metabolic functions. If multiple AMR genes are located on the same plasmid, antimicrobial use may co-select for mobile multidrug resistant elements. Plasmids can be categorized based on their mobilization abilities, their incompatibility (Inc) group or their plasmid MLST (pMLST).

1.5.1 Mobility

Plasmids can be conjugative, mobilizable or non-mobilizable. Some genes that determine plasmid mobility can be identified through genomic analysis such as for conjugative plasmids, which carry all the genes necessary for proper mating pair formation (MPF), relaxosome

complex components and a type four secretory system (T4SS). Mobilizable plasmids carry the relaxosome components but use all or parts of the MPF and T4SS from co-resident conjugative plasmids. Non-mobilizable plasmids do not have the *oriT* locus necessary for autonomous mobility and may only get mobilized by co-integration into conjugative or mobilizable plasmids. Mobilizable and conjugative plasmids can be categorized based on their relaxase mobility (MOB) genes into nine main MOB families: MOB_B, $MOB_{C,}$ MOB_F, MOB_H, $MOB_{M,}$ $MOB_{P,}$ MOB_Q, MOB_T and MOB_V and (Garcillán-Barcia et al., 2009; Garcillán-Barcia et al., 2020).

Regardless of their conjugation abilities, plasmids can also be transferred to other bacteria through natural transformation and transduction, albeit less frequently. Plasmids host ranges are variable and plasmids with higher copy numbers have a higher likelihood of transfer and may be more stably inherited than others (Alonso et al., 2016; Schink et al., 2013).

1.5.2 Incompatibility groups

Incompatibility groups are defined based on a plasmid's ability to co-exist within a bacterial cell. If multiple plasmids are present in the same cell, they must be of different Inc groups. These groups are determined by their machinery for replication and partitioning (Novick, 1987; Novick et al., 1976). Each Inc group has distinct characteristics regarding copy number, host range, size range and mobility profile. For example, IncH plasmids have temperature-dependent conjugative abilities which are optimal at 22-30℃. They could therefore transfer at higher rates in cooler pockets of AD or composting. In North America, previously described plasmids carrying ESC-resistance genes in *Enterobacterales* isolated from cattle feces commonly include IncI and IncA/C for bla_{CMY-2} (Mollenkopf et al., 2012; Mulvey et al., 2009) and IncF, IncN and IncI1 for bla_{CTX-M} (Mollenkopf et al., 2012; Afema et al., 2018).

1.6 CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE

The AMR profile of *Enterobacterales* can be characterized using both phenotypic and genotypic methods. If the objectives are for therapeutic purposes, phenotypic methods may suffice, whereas if the data are used for epidemiologic surveillance or evolutionary and phylogenetic purposes, genome sequencing may be required. Phenotypic screening tests can confirm genomic expression of AMR genes and help identify the presence of novel AMR genes,

but they cannot determine the location of the gene, nor identify the variant of determinant responsible for the observed phenotype. In order to assess the type and location of genetic determinants involved, the genome needs to be sequenced. Typing of bacterial strains is also necessary to identify clonal lineages of bacteria and to assess strain relatedness in the frame of epidemiological investigations, which can be done most efficiently through genome sequencing.

1.6.1 Phenotypic methods

Disk diffusion and minimum inhibitory concentration (MIC) determination can identify whether a strain is resistant, intermediate or susceptible to antimicrobials (CLSI, 2018). Both are quantitative methods, with disk diffusion also being qualitative, that can be utilized to produce phenetic trees based on antibiograms, visualizing the similarities of AMR phenotypes. Although phenotypic methods provide useful information regarding MICs, they do not identify the gene variants responsible for their resistances. As previously mentioned, disk diffusion can identify the production of ESBLs and AmpC enzymes and differentiate the two groups based on inhibition with clavulanic acid or boronic acid used together with a cephalosporin. Expression of AMR genes may not always be evident using phenotypic tests. Alterations in protein structure due to amino acid changes may increase or reduce the MIC. If the alteration results in a decreased MIC, this resistance may remain below the clinical breakpoint and go unnoticed, such is often the case for SHV-2 (Heritage et al., 1999). Low levels of expression due to weak promotors or low plasmid copy numbers can also result in AMR gene inconspicuousness.

Standardized procedures and interpretation schemes allow for reliable assessment and classification of MICs. Different organizations provide documents describing them, including among others the Clinical Laboratory and Standards Institute (CLSI) (https://clsi.org), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (https://eucast.org) and the British Society for Antimicrobial Chemotherapy (BSAC) (http://www.bsac.org.uk). In North America, the CLSI guidelines are predominantly followed and used for interpreting antimicrobial susceptibility tests. There are minor discrepancies between these sets of protocols and interpretation schemes, and discretion is advised when comparing and interpreting results. In addition, there are often different criteria for human and veterinary medicine.

1.6.2 PCR-based methods

Polymerase chain reaction (PCR) is an easy, quick and reliable method to detect AMR genes. Most research laboratories routinely use PCR and are readily able to use it for AMR genes in a sample or isolate. Purified PCR products can be sequenced using Sanger sequencing to identify gene variants. Since ESC-resistance genes have many variants, it is imperative that the genes are properly identified when assessing the epidemiology of ESC resistance.

PCR-based replicon typing (PBRT) is a method used to identify major plasmid families in *Enterobacteriaceae* using series of single and multiplex PCRs (Carattoli et al., 2005). This method is based on genetic regions specific to each incompatibility group which can include *rep*, iterons or RNAI. It has been continually adapted to include a wider range of plasmid families and distinction between IncF plasmids among various bacterial species (García-Fernandez et al., 2008; Villa et al., 2010). PBRT can determine the replicon types of the plasmids residing in the cell. Although more than 25 major incompatibility groups are now identifiable using this technique, rare or novel Inc types may not be included and would be unidentifiable with this approach (Carattoli et al., 2005; García-Fernandez et al., 2008; Villa et al., 2010; García-Fernandez and Carattoli, 2010; Villa et al., 2012, Dolejska et al., 2013; Johnson et al., 2012).

A multitude of studies have used PBRT to identify plasmids that carry ESC-resistance determinants. For instance, a surveillance study in Brazil sampled feces from healthy cattle to assess whether ESBL-producing *Enterobacterales* were present in animal sources for a global exporter of beef (Palmeira et al., 2020). Using PBRT, these authors showed that $bla_{\text{CTX-M-8}}$ was frequently associated with an IncI1 plasmid (Palmeira et al., 2020). In Uganda, a pilot study looking at the epidemiology of ESC-resistance determinants in *S. enterica* and *E. coli* from dairy cattle manure was conducted using these same techniques (Ball et al., 2019). These researchers found $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM} among the *E. coli* isolates and identified the Inc types of the plasmids residing in the isolates. However, as in many other studies, a main shortcoming of the study was that it did not explore which plasmids carried which genes (Ball et al., 2019). In another study, in the United States, the transferability of ESC-resistance plasmids from bacterial species isolated from dairy cattle was the primary interest (Poole et al., 2017). Both PCR and PBRT techniques were used as preliminary approaches for selecting a sub-set of isolates for

whole genome sequencing (WGS) (Poole et al., 2017). These three studies show the versatility of PBRT including usefulness for surveillance and epidemiological purposes. However, they also illustrate that it can often represent only preliminary steps to initiate further, more focused and costly analyses on a subset of isolates and plasmids.

1.7 WHOLE GENOME SEQUENCING METHODS AND ANALYSIS

Whole genome sequencing can accurately identify AMR genes and their variants. In addition to AMR characterization, genes encoding for virulence factors, toxin-antitoxin addiction (TA) systems, metal resistance genes, advantageous metabolic genes, insertion sequences, transposons and integrons can be also identified (Smith et al., 2015; Abraham et al., 2018). The cost of WGS has steadily decreased while sequence assembly and processing has improved, making it a quintessential tool for research purposes. Whole genome sequencing is ideal for studying plasmids, but it can be difficult to assemble entire plasmids due to repeated sequences and dynamic regions such as the shufflon on *pilV* in IncI plasmids, as well as ISs.

1.7.1 Sequencing methods

Next generation sequencing (NGS) is commonly used to obtain short read sequences. The technologies used for this are mainly Illumina platforms which provide short but accurate reads between 100-300bp in length (Luo et al., 2012; Quail et al., 2012). Two popular methods used for obtaining long read sequences include Oxford Nanopore Technology (ONT) (Jain et al., 2018) and Pacific Biosciences single-molecule real time sequencing (PacBio SMRT-seq) (Wenger et al., 2019). The length of reads produced ranges from 10kb to more than 100kb and is necessary to successfully close chromosomes and plasmids. The main drawback to long read sequencing is a higher price tag than short read sequencing and a reduced per base accuracy.

If a study is primarily aimed at identifying gene variants, such as ESC-resistance and other AMR gene variants, short read sequencing will suffice. Although not focused on dairy cattle, a Canadian study comparing CTX-M variants in *E. coli* isolated from beef cattle and urban wastewater from the same geographical locations in Alberta, used WGS via Illumina MiSeq and HiSeq technologies. The results of that study showed that CTX-M-14, 15, 27 and 55 were recovered from both sources and MLST sequence types were also identified with these

short-read sequences, providing insight on the epidemiology of CTX-M within two distinct niches (Cormier et al., 2019b). Another Canadian study used Illumina HiSeq 2500 technology to sequence the whole genomes of both *Streptococcus uberis* and *Streptococcus dysgalactiae* isolated from clinical mastitis in dairy cattle from the Maritime Provinces (Vélez et al., 2017). The goal was to identify AMR genes in these isolates and determine the correlation between their AMR phenotypes and genotypes (Vélez et al., 2017).

With the advancements in long-read sequencing over recent years, there has been an increase in sequencing AMR bacterial isolates from animal sources. However, few studies focus on ESC-resistant *Enterobacterales* from dairy cattle using hybrid sequencing and assembly techniques. Therefore, the following studies include results from both dairy cattle sources and broiler chickens to stress the importance of ESC-resistance investigation within livestock and food producing animals.

To investigate whether the emergence of $bla_{\text{CTX-M-1}}$ in broiler chickens was due to clonal expansion or horizontal gene transfer of plasmids harbouring this gene, a group in Norway used Illumina short reads on 31 *E. coli* isolates carrying $bla_{\text{CTX-M-1}}$. Among these isolates, 28 carried the $bla_{\text{CTX-M-1}}$ gene on a conjugative plasmid (Mo et al., 2020). Based off their MLST and cgMLST profiles, nine of these were further sequenced with PacBio technologies and they all harboured $bla_{\text{CTX-M-1}}$ on IncI1 plasmids, with most being the epidemic IncI1-pMLST3 (Mo et al., 2020; Carattoli et al., 2018). They concluded both clonal expansion and HGT were major contributors to the dissemination of $bla_{\text{CTX-M-1}}$ in Norway (Mo et al., 2020). In England, ESC-resistant *E. coli* recovered from dairy feces were compared to ESC-resistant *E. coli* recovered from healthy humans living in the same region to identify zoonotic transmission through close interactions (Findlay et al., 2020). Isolates were first sequenced using Illumina technologies, with one isolate additionally sequenced with ONT (Findlay et al., 2020). They found evidence of transmission of plasmids carrying $bla_{\text{CTX-M}}$ between farms, but concluded these plasmids were not contributing to the spread in human populations. Similar to the previous study, they found $bla_{\text{CTX-M-1}}$ on the epidemic IncI1-ST3 plasmid (Findlay et al., 2020; Carattoli et al., 2018). Both studies had very different objectives but took similar approaches with sequencing a larger data set using short reads first, then a smaller sample set for hybrid assembly with either PacBio or

ONT. These studies stress the importance of using both long and short reads to assemble the genome of AMR bacteria, to identify the location of these genes and understand their mobility.

1.7.2 Assembly-based methods

Assembly-based methods using short or long read DNA sequences first organize reads into contiguous fragments called contigs and then compare these sequences to a reference database, for the subsequent purpose of gene identification. Benefits to this method include the identification of both novel and known genes as well as MGEs. However, this method is time consuming and computationally expensive, especially when working with large data sets and genomes. There is not a single assembly method that is consistently superior to all others and the quality of assemblies depends largely on DNA source, quality of library, reads quality and depth of sequencing. Thus, it is recommended to use multiple tools to identify the optimal pipeline for a desired outcome and given data set.

The main assembly software programs such as SPAdes, Velvet and ABySS use a de Bruijn graph-based approach (Bankevich et al., 2012; Zerbino et al., 2008; Simpson et al., 2009), which forms a network using overlapping, pre-divided short reads, called k-mers to perform a *de novo* assembly of the whole genome. Using the de Bruijn graph, the genome is assembled via the most optimal route, called a Eulers path (Compeau et al., 2011). Benefits of this assembly method include efficiency and ability to compute large amounts of data, but sequencing inaccuracies may make this method prone to errors. However, the latter can be mitigated with high sequence coverage in combination with known error types that are typically built into assembly algorithms.

To mitigate the issue of assembling plasmids and obtaining reliable and accurate assemblies, hybrid assembly using both long and short reads or hierarchical strategies, are recommended. Pipelines commonly used for these purposes include Unicycler, Canu and most recently, Flye (Wick et al., 2017; Koren et al., 2017; Kolmogorov et al., 2019).

Unicycler assembles short reads first using SPAdes to create a de Bruijn graph and then generates short read bridges necessary to link contigs based on k-mer multiplicity. Long reads are then mapped to the graph to create longer bridges by overpassing the short-read bridges,

simplifying the graph and identifying the most likely Eulers path (Wick et al., 2017). Once all contigs are merged, the final step of polishing can be performed by both Bowtie2 (Langmead and Salzberg, 2012) and Pilon (Walker et al., 2014) to correct for any mismatches or insertions and deletions (indels) (Wick et al., 2017). Although this is an accurate and efficient pipeline for hybrid assemblies, challenges arise if short reads are not of high quality or sequencing depth is insufficient.

In contrast to Unicycler's hybrid assembly, Canu uses a hierarchical method which focuses on assembling the genome using long reads only and polishes with short reads after assembly is complete (Koren et al., 2017). Canu is comprised of three main steps: correction, trimming and assembly which can be used individually or in succession. The first step takes the raw long reads and uses the overlaps and estimated read lengths to correct and comprise a consensus read. This results in a smaller dataset, cleaner assembly and a less expensive first round before entering the more computationally expensive second round of trimming. The consensus read then gets trimmed, scanned for errors and adjusted accordingly before contigs and assembly graphs are formulated. A unique feature of Canu is k-mer weighting using MinHash, which proportionately weighs a k-mer based on its frequency, with a more frequent k-mer "weighing less" than a rare k-mer. Benefits to this assembler include a faster runtime and better assembly with lower read coverage compared to hybrid assembly (Koren et al., 2017). A challenge with this assembler revolves around the polishing step, which needs to be highly accurate to prevent any consequential errors.

Flye is a newer, slightly different tool than both Unicycler and Canu, which utilizes long reads only to create disjointigs, rather than a de Bruijn graph to assemble genomes (Kolmogorov et al., 2019). Disjointigs are concatenated segments of DNA which are then compiled into a single genomic segment. This single segment is used to create an assembly graph which is untangled using reads, by bridging any repeat segments. The final assembly needs to be polished using Pilon, however the end product is an accurately assembled genome. A drawback to this assembler is that it struggles with assembling repeats that are less than 1% divergent, however, Flye is less computationally expensive, quicker, and shows a comparable output to Canu (Kolmogorov et al., 2019).

1.7.3 Read-based methods

In contrast to assembly-based methods, read-based methods directly map reads to a reference database for gene identification, therefore no pre-assembly is required. This method recognizes the presence or absence of genes and can identify single nucleotide variations (SNVs) and gene variants but lacks discovery potential of novel genes and cannot resolve recombination events. Commonly used and publicly available tools for this approach include Bowtie2 (Langmead and Salzberg, 2012) and Burrow-Wheeler Aligner (BWA) (Li and Durbin, 2009). Read-based assembly is less time intensive and computationally demanding since *de novo* assembly is not performed, nor is the prediction of protein coding genes. Pitfalls include non-identifiable single-nucleotide polymorphisms (SNPs) which may result in novel AMR gene variants going undetected.

1.7.4 Downstream sequence analysis

Once a genome is fully assembled and polished, genes of interest can be identified. There are a variety of tools that take advantage of genomic repositories, each with a specific purpose but fluctuating with the frequency of their up-dates. Assembly-based annotation databases such as ResFinder (Zankari et al., 2012), PlasmidFinder (Carattoli et al., 2014), VirluenceFinder (Joensen et al., 2014), ISFinder (Siguier et al., 2006), CARD (Jia et al., 2017), INTEGRALL (Moura et al., 2009) and Mimeo (Taranto, 2020), are publicly accessible. Search algorithms which use the previously mentioned public databases include basic local alignment search tool (BLAST) (Altschul et al., 1990), double index alignment of next-generation sequencing data (DIAMOND) (Buchfink et al., 2014) and BASys (Van Domselaar et al., 2005).

Since WGS is a popular tool in research settings, but qualified personnel needed to perform sequence and assembly analysis and interpret results are scarce, automated pipelines have been developed to perform these tasks. Popular and publicly available automated pipelines for assembly and sequence annotation include MEGAnnotator (Lugli et al., 2016), MyPro (Liao et al., 2015), Prokka (Seemann, 2014), NCBI prokaryotic genome annotation pipeline (PGAP), Rapid Annotation using System Technology (RAST) (Aziz et al., 2008) and Pathosystems Resource Integration Center (PATRIC) (Wattam et al., 2016) which perform the aforementioned tasks, in addition to quality control. An automated tool for Bacterial gEnome Annotation

ComparisoON (BEACON) is a publicly available pipeline that compares the results of multiple annotation software's, in addition to performing its own annotation (Kalkatawi et al., 2015). The tools mentioned here provide insight on the multitude of options available for prokaryotic gene annotation, with each tool having benefits and pitfalls and should be carefully chosen according to the research objectives.

An effective and commonly used annotation database called Antimicrobial Resistance Identification by Assembly (ARIBA) uses both assembly and read based methods, with paired-end reads as the input, to recognize antimicrobial resistance determinants (Hunt et al., 2017). Both private or public databases for AMR genes are used by ARIBA and include but are not limited to NCBI, ResFinder (Zankari et al., 2012), CARD (Jia et al., 2017), Antimicrobial Resistance Gene ANNOTation (ARG-ANNOT) (Gupta et al., 2013) and MEGARes (Lakin et al., 2017). ARIBA is advantageous at providing information on sequence functionality such as nonsense, missense or indels (Hunt et al., 2017). In addition, ABRicate is a conglomerate of repositories for AMR genes, plasmid types and virulence factors used for genomic annotation including NCBI AMRFinderPlus, CARD, ResFinder, PlasmidFinder, ARG-ANNOT, VFDB, EcOH and MEGARES which is capable of screening large datasets of contigs (https://github.com/tseemann/abricate).

Like for most bioinformatic tools, the quality of results relies heavily on the quality of the input reads. A challenge for databases such as those accessible through ARIBA, is that any improperly assembled or missed genes could dramatically influence search and annotation results, and if databases are not updated frequently, vital details could be missed. A newly developed tool called Antimicrobial Resistance Gene Data Integration Toolkit (ARGDIT) has been developed to help overcome these issues. ARGDIT is comprised of two main functions with the first being validating databases followed by integrating them (Chiu and Ong., 2018). This tool mitigates obsoleteness in the aforementioned databases and provides a comprehensive and reliable index for gene annotation. Among the databases available to date, CARD remains the most comprehensive and reliable.

1.8 THESIS PROPOSAL

1.8.1 Rationale

The motivation behind this research stems from the lack of information surrounding ESC-resistance in dairy manure, especially in Canada. Dairy manure is an omnipresent transmission route of AMR genes into environmental and anthropological niches, which has downstream public health implications. A better understanding on the epidemiology of ESC-resistance, factors that aid in persistence through manure treatments and conjugative ability between and within bacterial species will better equip researchers and epidemiologists to combat and trace the spread of AMR originating from agricultural sources.

This research is part of a larger, collaborative project, including work from laboratories in Romania, Germany, the Netherlands and London, ON., to provide a more comprehensive understanding of the epidemiology of ESC-resistant *Enterobacterales* throughout various manure treatments. The results from the proposed research will be specific to Canada, but will complement the results found in Europe, to gain a better understanding of the global epidemiology of ESC-resistance in manure.

1.8.2 Hypothesis and Objectives

We hypothesize that transmission of plasmids carrying $bla_{\text{CTX-M}}$ or bla_{CMY} occurs between strains of *Enterobacterales* such as *E. coli* and *K. pneumoniae* in manure and likely in the animal itself, and some strains and plasmids may persist or spread better than others throughout treatment processes. Thus, to test these hypotheses, we proceeded to assess the persistence of ESC-resistant *E. coli* strains through manure treatment processes using phenotypic analysis, resistance gene identification and WGS. To further address these hypotheses, statistical analyses assessed the reduction of ESC-resistant *Enterobacterales* downstream manure treatment pipelines using multivariable logistic regression models which incorporated farm source, sampling date, and sample as random intercepts, with manure process as a fixed effect. Secondly, we assessed the diversity of plasmids carrying ESC-resistance determinants and their potential persistence through manure treatment processes using whole genome sequencing and identification of plasmid genetic relatedness. Finally, we sought out to characterize ESC-resistant

K. pneumoniae carrying *bla*$_{\text{CTX-M}}$ to better understand their role in the dissemination and persistence of ESC-resistance in dairy manure.

1.8.3 Methodology

To thoroughly address the stated objectives, both phenotypic and genotypic approaches were used. Manure samples were obtained via convenience sampling from six dairy farms in Southern Ontario over a twelve-month period, starting in November 2018 through October 2019.

Since the prevalence of ESC-resistant *Enterobacterales* in these samples were unknown, but assumed to be low, the samples were enriched using two different ESCs to optimize their recovery for this study. The recovered bacterial isolates were screened for common ESC-resistance genes using traditional PCR, prior to taxonomic identification using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). To identify phenotypic similarities between bacterial isolates and in support of strategic selection of isolates for whole genome sequencing, *Enterobacterales* that harboured the genes of interest underwent antimicrobial susceptibility testing. These antibiograms helped identify potentially similar strains of *E. coli* amongst and between the farms, partially addressing objective one.

Multivariable logistic regression models were used to assess significant changes through manure treatment processes comparing the raw input to anaerobically digested manure as well as recycled bedding and the final environmentally applied output, with regards to recovery of ESC-resistant *Enterobacterales*. To account for differences in prevalence between sources and sampling periods, farms and sampling dates were included as fixed effects with manure treatments and sample or replicate as random effects. Changes after anaerobic digestion were assessed for all six farms, whereas changes in the environmentally applied output and recycled bedding were only assessed on the four farms which provided these samples. In addition to these models, exact logistic regression models were used to determine any relationship between the presence of *bla*$_{\text{CMY}}$ or *bla*$_{\text{CTX-M}}$ and resistant phenotypes to each of the antimicrobials tested. Together, these results further supported objective one.

Although a main goal was to assemble the plasmids in ESC-resistant *Enterobacterales*, it was also of interest to gain an understanding of the ESC-resistance genes in our bacterial library.

To accomplish this, Illumina MiSeq and NextSeq were used on all *E. coli* and *K. pneumoniae* carrying *bla*$_{\text{CTX-M}}$, and a subset of *E. coli* carrying *bla*$_{\text{CMY}}$. These sequenced isolates provided an in depth understanding of the epidemiology of ESC-resistance among dairy farms in Southern Ontario, completing objective one and partially completing objective three. ONT long read sequencing was performed on a subset of *E. coli* and *K. pneumoniae* isolates previously sequenced with Illumina technologies, to assemble their ESC-resistance plasmids, completing objectives two and three.

1.9 TABLES

Table 1.1. Sequence types of *Enterobacterales* isolated from dairy cattle globally and their associated ESC-resistance genes.

ST	ESC-Resistance Gene Variant	Geographical Location	Year Isolated	Sample Type	Bacterial Species	Reference
10	CTX-M-1	Germany	2009-2013	Milk	*E. coli*	Freitag et al., 2017
	CTX-M-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
	CTX-M-14	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
	CTX-M-14	France	2009-2011	Milk	*E. coli*	Dahmen et al., 2013
	CTX-M-15	Germany	2009-2013	Milk	*E. coli*	Freitag et al., 2017
	CTX-M-15	Germany	2009-2013	Milk	*E. coli*	Freitag et al., 2017
	CTX-M-27	United States	2012	Feces	*E. coli*	Afema et al., 2018
23	CTX-M-1	France	2009-2011	Milk	*E. coli*	Dahmen et al., 2013
	CTX-M-14	France	2009-2011	Milk	*E. coli*	Dahmen et al., 2013
	CTX-M-27	United States	2012	Feces	*E. coli*	Afema et al., 2018
32	CTX-M-27	United States	2012	Feces	*E. coli*	Afema et al., 2018
44	CTX-M-2, CMY-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
45	CTX-M-14	France	2011	Milk	*K. pneumoniae*	Dahmen et al., 2013
46	CTX-M-2, CMY-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
48	CTX-M-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
	CTX-M-14	United States	2012	Feces	*E. coli*	Afema et al., 2018
	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
	CTX-M-27	United States	2012	Feces	*E. coli*	Afema et al., 2018
57	CTX-M-14,	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
58	CTX-M-1	France	2009-2011	Milk	*E. coli*	Dahmen et al., 2013
	CTX-M-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
	CTX-M-14	France	2009-2011	Milk	*E. coli*	Dahmen et al., 2013
	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
	CTX-M-27	United States	2012	Feces	*E. coli*	Afema et al., 2018
69	CTX-M-14	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
	CTX-M-55	United States	2012	Feces	*E. coli*	Afema et al., 2018
86	CTX-M-14	United States	2012	Feces	*E. coli*	Afema et al., 2018
	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
88	CTX-M-14, CMY-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
	CTX-M-15	France	2007-2009	Feces	*E. coli*	Madec et al., 2012
	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
	CMY-2	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2020
	CMY-2	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2020
90	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
117	CTX-M-1	Germany	2009-2013	Milk	*E. coli*	Freitag et al., 2017
154	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
155	CTX-M-14	United States	2012	Feces	*E. coli*	Afema et al., 2018
162	CTX-M-14	United States	2012	Feces	*E. coli*	Afema et al., 2018
163	CTX-M-14	United States	2012	Feces	*E. coli*	Afema et al., 2018
219	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018

227	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
221	CTX-M-8	Brazil	2009-2011	Milk	*K. pneumoniae*	Nobrega et al., 2021
	CTX-M-8	Brazil	2009-2011	Feces	*K. pneumoniae*	Nobrega et al., 2021
345	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
349	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
361	CTX-M-15	Germany	2009-2013	Milk	*E. coli*	Freitag et al., 2017
	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
362	CTX-M-14	Germany	2009-2013	Milk	*E. coli*	Freitag et al., 2017
	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
394	CTX-M-15	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
448	CTX-M-14	United States	2012	Feces	*E. coli*	Afema et al., 2018
540	CTX-M-1	Germany	2009-2013	Milk	*E. coli*	Freitag et al., 2017
	CTX-M-14	United States	2012	Feces	*E. coli*	Afema et al., 2018
	CTX-M-15	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
	CTX-M-65	United States	2012	Feces	*E. coli*	Afema et al., 2018
685	CTX-M-65	United States	2012	Feces	*E. coli*	Afema et al., 2018
744	CTX-M-14	United States	2012	Feces	*E. coli*	Afema et al., 2018
	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
	CTX-M-15, CMY-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
	CTX-M-27	United States	2012	Feces	*E. coli*	Afema et al., 2018
746	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
761	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
	CTX-M-24	United States	2012	Feces	*E. coli*	Afema et al., 2018
	CTX-M-27	United States	2012	Feces	*E. coli*	Afema et al., 2018
1163	CTX-M-55	United States	2012	Feces	*E. coli*	Afema et al., 2018
1163	CTX-M-27	United States	2012	Feces	*E. coli*	Afema et al., 2018
1167	CTX-M-15	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
1266	CTX-M-15	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
1284	CMY-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
1431	CTX-M-2	Germany	2009-2013	Milk	*E. coli*	Freitag et al., 2017
1508	CTX-M-15	Germany	2009-2013	Milk	*E. coli*	Freitag et al., 2017
1629	CTX-M-27	United States	2012	Feces	*E. coli*	Afema et al., 2018
2210	CTX-M-15	France	2007-2009	Feces	*E. coli*	Madec et al., 2012
2211	CTX-M-15	France	2007-2009	Feces	*E. coli*	Madec et al., 2012
2212	CTX-M-15	France	2007-2009	Feces	*E. coli*	Madec et al., 2012
2213	CTX-M-15	France	2007-2009	Feces	*E. coli*	Madec et al., 2012
2214	CTX-M-15	France	2007-2009	Feces	*E. coli*	Madec et al., 2012
2215	CTX-M-15	France	2007-2009	Feces	*E. coli*	Madec et al., 2012
2324	CTX-M-2, CMY-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
2325	CTX-M-15,	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
2437	CTX-M-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
2438	CMY-2	Japan	2007-2009	Feces	*E. coli*	Ohnishi et al., 2013
3018	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
3042	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
4096	CTX-M-27	United States	2012	Feces	*E. coli*	Afema et al., 2018
4249	CTX-M-15	United States	2012	Feces	*E. coli*	Afema et al., 2018
5447	CTX-M-1	Germany	2009-2013	Milk	*E. coli*	Freitag et al., 2017

Table 1.2. ESC-resistance determinants found in *Enterobacterales* isolated from dairy cattle globally without known sequence types.

ESC-Resistance Gene Variant	Geographical Location	Year Isolated	Sample Type	Bacterial Species	Reference
CTX-M-1	United States	Unknown	Feces	*E. coli*	Mollenkopf et al., 2012
	United States	Unknown	Feces	*E. coli*	Mollenkopf et al., 2012
	Spain	2014-2016	Rectal swab	*E. coli*	Tello et al., 2020
	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2018
	Switzerland	2011	Feces	*E. coli*	Geser et al., 2012
	Czech Republic	2008	Rectal swab	*E. coli*	Dolejska et al., 2011
	Czech Republic	2008	Rectal swab	*E. coli*	Dolejska et al., 2011
	Czech Republic	2008	Milk filters	*E. coli*	Dolejska et al., 2011
	Czech Republic	2008	Milk filters	*E. coli*	Dolejska et al., 2011
	France	2000-2004	F	*E. coli*	Meunier et al., 2006
	France	2000-2004	Urine	*E. coli*	Meunier et al., 2006
CTX-M-2	Japan	Unknown	Feces	*E. coli*	Sato et al., 2014
	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2018
	Japan	2007-2009	Feces	*K. pneumoniae*	Ohnishi et al., 2013
	Japan	2007-2009	Feces	*K. pneumoniae*	Ohnishi et al., 2013
	Japan	2007-2009	Feces	*C. freundii*	Ohnishi et al., 2013
	Japan	2007-2009	Feces	*E. cloacae*	Ohnishi et al., 2013
CTX-M-9	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2018
CTX-M-14	Japan	Unknown	Feces	*E. coli*	Sato et al., 2014
	United States	Unknown	Feces	*E. coli*	Mollenkopf et al., 2012
	Spain	2014-2016	Rectal swab	*E. coli*	Tello et al., 2020
	Switzerland	2009-2011	Milk sample	*E. coli*	Geser et al., 2012
CTX-M-15	United States	Unknown	Feces	*E. coli*	Mollenkopf et al., 2012
	United States	Unknown	Feces	*E. coli*	Mollenkopf et al., 2012
	Spain	2014-2016	Rectal swab	*E. coli*	Tello et al., 2020
	France	2000-2004	Urine	*E. coli*	Meunier et al., 2006
CTX-M-32	Spain	2014-2016	Rectal swab	*E. coli*	Tello et al., 2020
CMY-2	Japan	Unknown	Feces	*E. coli*	Sato et al., 2014
	Spain	2014-2016	Rectal swab	*E. coli*	Tello et al., 2020
	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2020
	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2018
	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2018
	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2018
CMY-4	Spain	2014-2016	Rectal swab	*E. coli*	Tello et al., 2020
CTX-M-1, CTX-M-2	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2018
CTX-M-1, CMY-2	United States	Unknown	Feces	*E. coli*	Mollenkopf et al., 2012
	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2018
CTX-M-2, CMY-2	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2018
CTX-M-9, CMY-2	Canada	2014-2015	Rectal swab	*E. coli*	Awosile et al., 2018

CHAPTER TWO: Whole genome sequencing of *Escherichia coli* and characterization of other *Enterobacterales* isolated from dairy manure in Ontario, Canada

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ABSTRACT:

Extended-spectrum cephalosporins (ESCs) are important antimicrobials in human and veterinary medicine. The ESC-resistance genes *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY}}$ and *bla*$_{\text{SHV}}$ have been found in bacteria from livestock, including beef cattle. However, genome sequences of ESC-resistant *Enterobacterales* from dairy cattle in Canada, are sparse. In this study, the persistence of ESC-resistant *Escherichia coli*, through manure treatment processes were assessed and ESC-resistance plasmids were characterized. Manure samples were obtained from six farms in Southern Ontario over a one-year period. Samples were enriched using ESCs and recovered isolates were screened with PCR for *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY}}$ and *bla*$_{\text{SHV}}$. *E. coli* (n = 248) carrying *bla*$_{\text{CTX-M}}$ or *bla*$_{\text{CMY}}$ underwent whole genome sequencing using Illumina MiSeq/NextSeq. These isolates were typed using multi-locus sequence typing (MLST) and their resistance gene profiles. No *E. coli* carrying *bla*$_{\text{SHV}}$ were detected. A subset of *E. coli* (n = 28) were sequenced using Oxford Nanopore Technologies. Plasmids were assembled using Unicycler and subsequently characterized via their resistance genes, replicon type, plasmid MLST, phylogenetic analysis and mauve alignments. Recovery of ESC-resistant *Enterobacterales* were significantly reduced in manure outputs. Fifty *E. coli* STs were identified, in addition to 13 novel STs, with ST10, ST46, ST58, ST155, ST190, ST398, ST685 and ST8761 being identified throughout the treatment pipeline. ESC-resistance gene variants included CTX-M-1, -14, -15, -17, -24, -32, -55 and CMY-2. Plasmids carrying *bla*$_{\text{CTX-M}}$ are more diverse than plasmids carrying *bla*$_{\text{CMY}}$. Although reduced, the recovery of ESC-resistant *Enterobacterales* in environmentally applied manure is still evident and contains diverse ESC-resistant strains and determinants, potentiating horizontal gene transfer within downstream niches.

2.0 INTRODUCTION

The end product of dairy manure treatment, including any residual antimicrobial-resistant bacteria, is applied to the environment for crop fertilization. This application could potentiate the risk of antimicrobial resistance (AMR) transmission to humans and animals through water run-off and contaminated crop consumption (Zekar et al., 2017; Hölzel et al., 2018). Characterizing the diversity of resistant bacteria and associated plasmids as well as identifying which strains survive manure treatment processes will provide a better understanding of AMR plasmid transmission pathways and population dynamics in manure, which is important to mitigate transmission to humans and other animals.

Manure is treated to optimize the nutrient composition for fertilizing crops, reduce net volume, reduce green-house gas emissions and provide recycled bedding for livestock. This treatment modifies its microflora and incidentally reduces the prevalence of antimicrobial resistant bacteria and AMR genes. For instance, composting is an effective method to reduce the prevalence of *Escherichia coli* and proteobacteria in general (Shepherd Jr et al., 2009; Qian et al., 2016). Composting at thermophilic temperatures can reduce absolute abundance of AMR genes measured in copies per gram of dry compost, such as *sul2* and *tet,* if done at a consistently high temperature (Qian et al., 2016). Anaerobic digestion at thermophilic temperatures reduces the amount of sulphonamide- and tetracycline-resistant bacteria in manure (Zou et al., 2019) and the prevalence of extended-spectrum ß-lactamase (ESBL)-producing *Enterobacterales* (Iwasaki et al., 2019).

Critically important antimicrobials such as extended-spectrum cephalosporins (ESCs) are widely used in human and veterinary medicine, including for the treatment of severe and life-threatening bacterial infections. Resistance to ESCs can prevent resolution of infections and increase mortality. The production of extended-spectrum β-lactamases (ESBLs) is one of the main resistance mechanisms to ESCs and the most common enzymes among them include the CTX-Ms, SHVs, and TEMs (Sepp et al., 2019; Bevan et al., 2017; Liakopoulos et al., 2016; Bradford, 2001). Common variants of CTX-M detected in bacteria from dairy cattle include CTX-M-1, 2, 9, 14, 15 and 27 (Afema et al., 2018; Awosile et al., 2018). Few studies have described SHV variants in *Enterobacterales* from dairy cattle in North America (Awosile et al., 2018). Due to the human health implications associated with ESBLs, further investigation is

warranted. Although TEMs have not been frequently investigated in dairy cattle, the most commonly identified variant is the non-ESBL TEM-1. In addition to ESBLs, cephamycinases are frequently responsible for resistance to ESCs, in particular the CMY enzymes. In North America, the most common CMY variant in *Enterobacterales* from dairy cattle is CMY-2 (Mollenkopf et al., 2012; Awosile et al., 2018; Awosile et al., 2020; Rehman et al., 2017; Nilsson, 2011), which is consistent in other geographical regions such a Czech Republic and Japan (Manga et al., 2019; Onishi et al., 2013; Sato et al., 2014).

The few genome-based studies of ESC-resistant *Enterobacterales* from cattle in Canada show that $bla_{\text{CTX-M-15}}$ is commonly found in *Enterobacterales* from dairy cattle (Rehman et al., 2017). The same variant as well as $bla_{\text{CTX-M-1, 14, 27, 32, 55}}$ and $_{\text{-65}}$ have been found in beef cattle (Cormier et al., 2019b; Adator et al., 2020). In *Enterobacterales* from Canadian beef cattle, bla_{CMY} is more frequent than $bla_{\text{CTX-M}}$ (Cormier et al., 2019b), but the situation is not clear in dairy cattle.

The reduction of resistant bacteria and resistomes from raw to anaerobic digestion at both mesophilic and thermophilic temperatures in dairy manure has been assessed *in vitro* (Flores-Orozco et al., 2020; Iwasaki et al., 2019) but few studies have been performed under realistic field conditions *in situ*. ESC-resistant strains and plasmids carrying $bla_{\text{CTX-M}}$ are of higher priority than those carrying bla_{CMY}, due to recent emergence in farm animals in Canada and their frequent location on multi-drug resistant (MDR) plasmids, in addition to their clinical importance in humans. In general, there is a lack of genomic research on ESC-resistant *Enterobacterales* and their ESC-resistance plasmids from dairy cattle and its manure in Canada whereas research on beef cattle is more predominant. Therefore, to aid in the understanding of how various manure treatments affect ESC-resistant *Enterobacterales* in dairy manure, manure samples from a variety of treatment stages have been obtained for this study.

Our objectives were twofold. The first was to investigate the diversity and persistence of ESC-resistant *E. coli* strains through manure treatment processes using phenotypic analysis, resistance gene identification, and whole genome sequencing. The second was to assess the diversity of plasmids carrying ESC-resistance determinants and their potential persistence

through manure treatment processes using whole genome sequencing and assessment of plasmid genetic relatedness.

2.1 MATERIALS AND METHODS

2.1.1 Sample processing and enrichment

One-hundred sixty-four manure samples were obtained from six farms located in Southern Ontario from November 2018 to October 2019 (Table S2.1). Originally, seven farms were sampled, however due to low turnover rate and lack of anaerobic digestion, farm six was excluded from this study. Farms one through four had two mesophilic anaerobic digesters. They were sampled before digestion and at the secondary digester during digestion, with the exception of farm one being sampled in the holding pit immediately following digestion (Fig. 2.1). On farms one and three, manure from the secondary digester is separated into solids and liquids, which were both sampled (Fig. 2.1). Farm five utilizes a three step digestion processes involving two mesophilic anaerobic digesters, followed by a third thermophilic anaerobic digester. Raw manure, digesters one and two, and output from digester three, which was separated into a solids and liquids tank, were sampled (Fig. 2.1). Farm seven is the most complex and utilizes a primary mesophilic anaerobic digester, followed by two screw presses and a rotating kiln that performs at thermophilic temperatures to produce compost. Samples were taken before digestion, at the digestate pit, after the first screw press, after the second screw press and following heat treatment (Fig. 2.1). Further details regarding sampling procedures used for each farm were previously described (Tran et al., 2021). Manure samples were immediately refrigerated and processed within twenty-four hours. Samples were enriched by adapting a previously described protocol which selects for ESC-resistant *Enterobacterales* (Cormier et al., 2019a). Each sample was tested in triplicate by inoculating 10 mL (or 10 g equivalent) of manure into 90 mL of *Escherichia coli* (EC) broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 2 mg/L of cefotaxime and incubated overnight with constant shaking at 37˚C. After incubation, 10 uL were plated onto MacConkey agar (Becton, Dickinson and Company) containing 1 mg/L ceftriaxone, incubated overnight at 37˚C and examined for growth.

2.1.2 Selection of ESC-resistant bacteria

Three or more colonies were selected per plate based on lactose fermentation and morphology: 1) lactose fermenter with non-mucoid morphology; 2) lactose fermenter with mucoid morphology; 3) lactose fermenters with a different morphology or non-lactose fermenters with varying morphologies were selected until each unique morphology was sub-cultured. Selected colonies were plated onto MacConkey agar (Becton, Dickinson and Company) (1 mg/L ceftriaxone) and incubated overnight at 37˚C. Subcultures were then made on Luria-Bertani (LB) agar (Becton, Dickinson and Company) (1 mg/L ceftriaxone), incubated overnight at 37˚C and frozen in brain-heart infusion (BHI) broth (Becton, Dickinson and Company) containing 20% glycerol and stored at -70˚C.

2.1.3 Detection of ARGs and bacterial species identification

Polymerase chain reaction (PCR) was performed for *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY}}$ and *bla*$_{\text{SHV}}$ with reagents and thermocycler conditions as previously described (Table 2.1). The *bla*$_{\text{TEM}}$ gene was not screened for systematically since the most frequent variant in *Enterobacterales* from animals in Canada is *bla*$_{\text{TEM-1}}$, which does not confer resistance to ESCs. Bacterial lysates were obtained by the boiling method. Bacterial isolates carrying *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY}}$ or *bla*$_{\text{SHV}}$ were identified using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF; Bruker Daltonik GmbH, Bremen, Germany) at the Animal Health Laboratory, University of Guelph, Guelph, ON. Forty-two ESC-resistant bacteria which tested negative for *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY}}$ or *bla*$_{\text{SHV}}$, originating from all six farms and covering all manure processing stages and various time periods, were identified using MALDI-TOF and tested for *bla*$_{\text{TEM}}$ using primers in Table 2.1. Among the latter, *E. coli* isolates which were negative for *bla*$_{\text{TEM}}$ were further investigated by sequencing PCR products of their chromosomal AmpC promoter region using primers in Table 2.1 and thermocycler conditions as previously described (Caroff et al., 1999).

2.1.4 Antimicrobial susceptibility testing

Enterobacterales carrying *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY}}$ or *bla*$_{\text{SHV}}$, underwent antimicrobial susceptibility testing using the disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2018; CLSI, 2019). Antimicrobials used for susceptibility

testing included ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefotaxime (CTX), cefoxitin (FOX), sulfonamide (SUL), sulfamethoxazole-trimethoprim (SXT), tetracycline (TET), streptomycin (STR), kanamycin (KAN), gentamicin (GEN), ciprofloxacin (CIP) and chloramphenicol (CHL). Inhibition zone diameters data from each isolate were used to create phenetic trees in BioNumerics v7.6, using the Pearson correlation coefficient and the unweighted pair group method with arithmetic mean. Clusters from these phenetic trees were determined using a 95% similarity cut-off to select a subset of *E. coli* carrying bla_{CMY} for sequencing.

2.1.5 Illumina sequencing

Since CTX-M diversity was our main interest, *E. coli* carrying $bla_{\text{CTX-M}}$ from all six farms were sequenced (n = 227). A subset of *E. coli* carrying bla_{CMY} from farms three and seven were selected for sequencing using stratified random sampling, with one or two isolates selected per phenetic cluster (n = 28). Seven of these isolates carried both bla_{CMY} and $bla_{\text{CTX-M}}$. DNA extractions were completed using the Epicentre MasterPure$^{\text{TM}}$ DNA Purification kit (Epicentre, Madison, WI, USA), following the manufacturer's instructions. DNA sequencing was performed on 188 isolates using Illumina NextSeq (Nextera XT library prep, PE150; Illumina, San Diego, CA, USA) at the National Microbiology Laboratory in Winnipeg, MB, and the remaining isolates (n = 60) using Illumina MiSeq (PE300) at the Advanced Analysis Centre, University of Guelph, Guelph, ON, CA.

2.1.6 MinION sequencing

A subset of *E. coli* carrying $bla_{\text{CTX-M}}$ (n = 24) and bla_{CMY} (n = 9) were sequenced using a MinION MIN-101B device (Oxford Nanopore Technologies, Oxford, UK). These isolates were selected based on Illumina MiSeq data in addition to phenetic trees, to cover the highest diversity of $bla_{\text{CTX-M}}$ variants over as many manure treatment stages and time periods as possible, to assess potential plasmid distribution. These isolates were from farms three and seven only, since these two farms had the highest recovery of *E. coli* carrying $bla_{\text{CTX-M}}$ and a large diversity of phenetic clades to select from, which encompassed all manure treatment stages and time periods being assessed. DNA extractions were the same as described as above. Sequencing libraries and barcoding preparation was done using the SQK-LSK109 and EXP-NBD104/114 ligation and native barcoding kits (Oxford Nanopore Technologies) according to the manufacturer's

instructions. Three flow cells (version FLO-MIN106 R9.4) were used and run for 48h each. Basecalling of fast5 files and demultiplexing was performed using Guppy Basecaller v3.3 (Oxford Nanopore Technologies) with barcode trimming enabled.

2.1.7 Genomic assembly and analysis

Whole genome sequences using short reads only were assembled using BioNumerics v7.6 (Applied Maths, Saint-Martens-Latem, Belgium) using the SPAdes *de novo* assembler, and assembly-free and assembly-based allele calling. Resistance genes, plasmids and virulence genes were identified using the *E. coli* functional genotype scheme in BioNumerics at a 90% similarity threshold which utilizes PlasmidFinder, ResFinder and VirulenceFinder databases from the Center for Genomic Epidemiology, Technical University of Denmark, DTU. Multi-locus sequence types (MLSTs) were assigned using cgMLST application for BioNumerics and the BioNumerics *E. coli*/*Shigella* Enterobase scheme with Achtmann Sequence Types (https://www.applied-maths.com/applications/wgmlst). Any non-identifiable sequence types (STs) using this approach were assessed via an MLST online database (Centre for Genomic Epidemiology; Cosentino et al., 2012) and short reads were mapped to novel alleles. If no errors were identified in the assessment, the isolate was designated with a novel ST with a prefix STN (Table S2.2). Minimum spanning trees (MSTs) were generated using the wgMLST (core enterobase) function for cgMLST trees, and the wgMLST (all loci) function for wgMLST trees in BioNumerics with 10x bootstrapping and no multithreading, using 2,513 and 17,380 loci, respectively.

Long and short reads were assembled using hybrid assembler Unicycler v0.4.8 (Wick et al., 2017), in parallel with Flye v2.6 (Lin et al., 2016) and polished with short reads using Racon v1.4.0 (Vaser et al., 2017) and Pilon v1.23 (Walker et al., 2014). Assemblies were visualized using Bandage v0.8.1 (Wick et al., 2015) and annotated with Abricate (https://github.com/tseemann/abricate). Sequenced genomes were analyzed using Geneious v9.1.8 (Biomatters, Auckland, New Zealand) and plasmids were aligned and visualized using Mauve plug-in v2.3.1 (Darling et al., 2004). Plasmids assembled and closed with Unicycler which had consistent long read coverage were used for analysis. If Unicycler was unsuccessful at

assembling complete plasmids, polished Flye assemblies were used for downstream analysis (Spreadsheet S1).

2.1.8 Phylogenetic analysis

Core phylogenetic SNP analyses were performed using paired end llumina shorts reads. These analyses were completed using Snippy v4.4.5 according to developer's guidelines, under Core SNP Phylogeny (https://github.com/tseemann/snippy) with *E. coli* K12 substrain MG1655 (GenBank Accession #U00096) as a reference sequence. The clean.full.aln files were analyzed with Gubbins v2.4.0 (Croucher et al., 2014) and the output clean.core.aln file was analyzed with SNP-sites v3.0 (Page et al., 2016). FastTree v2.2.11 (Price et al., 2010) was used to create maximum likelihood phylogenetic trees which were analyzed in Geneious. Strains with >25% missing data were excluded from analysis and are summarized in Spreadsheet S1.

Phylogenetic SNP analyses were performed on the hybrid assembled, ESC-resistance plasmids using a pre-determined protocol (Cormier et al., *manuscript in preparation*). A gene presence/absence analysis was first conducted on all ESC-resistance plasmids independently of replicon types. The ESC-plasmids (n = 30) were analyzed with Prokka v1.14.6 (https://github.com/tseemann/prokka) and the output .gff files were analyzed with Roary v3.13.0 (Page et al., 2015). A SNP analysis was then performed on plasmids of the same replicon type. The same steps previously mentioned with Prokka and Roary were used, however SNP-sites were run on the core.gene.alignment output file from Roary. These SNP sites (14601, 13750, 14124 and 12621), along with the core.gene.alignment file, were used to create a maximum likelihood phylogenetic tree with IQtree v2.0.3 (Minh et al., 2020). All trees were visualized in Geneious.

2.1.9 Statistical analysis

Exact logistic regression models were constructed to assess associations with phenotypic resistance to each of the twelve antimicrobials with predictor variables $bla_{\text{CTX-M}}$ or bla_{CMY}. For the purpose of exact logistic regression models, isolates with intermediate susceptibility profiles were included in the resistant category. However, for the consideration of multi-drug resistance, only resistant phenotypes were included. Multi-drug resistance was defined as being resistant to

at least one antimicrobial in three or more classes, as determined using CLSI guidelines. Classes were categorized as follows: aminoglycosides (KAN, GEN and STR), tetracyclines (TET), quinolones (CIP), folate pathway inhibitors (SUL and SXT), phenicols (CHL), cephems (CTX and FOX) and other ß-lactams (AMP). For the purpose of MDR classification, AMC was excluded from the analysis since resistance to this β-lactam + inhibitor combination was mainly due to the presence of *bla*$_{CMY}$ and correlated strongly with FOX resistance. Exact logistic regression models were constructed in Stata v16.1 (StataCorp LP, College Station, TX).

Multi-level logistic regression models were fitted to identify any significant associations between the stage of manure processing (i.e., raw vs. processed) and the recovery of four categories of *Enterobacterales* (i.e., present vs. absent): 1) ESC-resistant *Enterobacterales* carrying *bla*$_{CTX-M}$, *bla*$_{CMY}$ or *bla*$_{SHV}$, 2) ESC-resistant *E. coli* carrying *bla*$_{CTX-M}$ and/or *bla*$_{CMY}$, 3) *E. coli* carrying *bla*$_{CTX-M}$ and 4) *E. coli* carrying *bla*$_{CMY}$. Models were fitted at the sample and replicate levels. Changes from raw to anaerobically digested manure were assessed for all six farms, which was digestate with solids on all farms except for farm five, in which digestate one with solids was used. Due to the sampling structure (Fig. 2.1), changes in environmentally applied manure and recycled bedding were assessed for farms one, three, five and seven only. Environmentally applied manure samples were digestate without solids for all four farms and recycled bedding including dewatered manure on farms one, three and five, and heat-treated compost on farm seven. All samples were processed in triplicate.

Individual models fitted at the replicate level included each manure process (treated, environmentally applied manure or bedding) in reference to the raw input manure, and the recovery group of interest as fixed effects. The random intercepts included in the models accounted for clustering/autocorrelation among samples from the same farm and the same farm on the date of sampling with an additional random intercept to account for clustering at the sample level. All variables were forced into each model. Multi-level models were fitted using adaptive quadrature with the “melogit” or “meqrlogit” commands in Stata v16.1 (StataCorp LP, College Station, TX). Descriptive statistics alone were reported for outcomes with a prevalence of 100% or 0% since these data are not suitable for multi-level modeling. A significance level of $p < 0.05$ was used for all statistical analyses.

2.2 RESULTS

2.2.1 Recovery of ESC-resistant bacteria at different stages of manure processing

Each of the six farms have individualized treatment pipelines with varying level of ESC-resistant *Enterobacterales* reduction. Despite differences in manure processing, they all have three main components: 1) raw input manure; 2) anaerobically digested manure; 3) environmentally applied output manure (Fig. 2.1). All farms were included in mixed effect logistic regression analysis to identify changes in recovery of ESC-resistant *Enterobacterales* from raw to anaerobically digested manure, using digestate with solids (Tables 2.2; S2.3 and 2.3) at the replicate level. Farms one, three, five and seven have dewatered manure used for recycled bedding, in addition to the environmentally applied digestate for fertilization. These four farms were used for mixed effect logistic regression models to assess changes in recovery of ESC-resistant *Enterobacterales* in their environmentally applied manure and recycled bedding, at the replicate level (Table 2.3).

Recovery of ESC-resistant *Enterobacterales* in environmentally applied manure and recycled bedding decreased significantly (Table 2.3). Similarly, recovery of ESC-resistant *E. coli* decreased in recycled bedding. However, the decrease in recovery of ESC-resistant *E. coli* in anaerobically digested manure and environmentally applied manure were not significant (Table 2.3). Recovery of *E. coli* carrying $bla_{\text{CTX-M}}$ or bla_{CMY} decreased significantly in recycled bedding only (Table 2.3). Based on the odds ratios, the decrease was more drastic and consistent across categories in recycled bedding than in environmentally applied manure (Table 2.3).

2.2.2 Bacterial species and antimicrobial resistance profiles

The main species of interest for this study was *E. coli,* however, seven other genera of *Enterobacterales* were recovered (Fig. 2.2). *Escherichia coli* comprises 69.3% of the 707 recovered *Enterobacterales* carrying $bla_{\text{CTX-M}}$, bla_{CMY} or $bla_{\text{SHV.}}$ More *E. coli* isolates carry bla_{CMY} (n = 261) than $bla_{\text{CTX-M}}$ (n = 222) and seven carry both genes. The next most frequent species were *Klebsiella pneumoniae* (11.8%) and *Proteus mirabilis* (10.3%) (Fig. 2.2).

Seventy-nine percent of ESC-resistant *E. coli* isolates were MDR (Table 2.4). Aside from the ß-lactams, the antimicrobials to which *E. coli* were most frequently resistant included tetracycline (71.6%), sulphonamides (68.6%), sulfamethoxazole-trimethoprim (58.0%) and streptomycin (57.1%) (Table 2.4). The same trend was seen among the non-*E. coli* *Enterobacterales* isolates in general, with resistance to tetracycline being the most common (87.6%), followed by sulphonamides (72.8%), sulfamethoxazole-trimethoprim (70.5%) and streptomycin (65.0%) (Table S2.4). In contrast, resistance to gentamicin and ciprofloxacin were the least frequent amongst *E. coli* with 8.6% and 3.4%, respectively, and amongst all other ESC-resistant *Enterobacterales* with 4.1% resistance to gentamicin and no resistance to ciprofloxacin (Tables 2.4 and S2.4).

Antimicrobial resistance phenotypes associated with the presence of $bla_{\text{CTX-M}}$, compared to resistance phenotypes in the presence of bla_{CMY}, are reported in Table 2.5. The odds of $bla_{\text{CTX-M}}$-positive isolates showing a resistant phenotype to CIP (OR 42.46), SXT (OR 4.83), SUL (OR 3.66), KAN (OR 2.15), TET (OR 1.93) and STR (OR 1.83) were positive when compared to isolates carrying bla_{CMY}. In contrast, in the presence of $bla_{\text{CTX-M,}}$ the odds of an isolate showing a resistant phenotype to AMC (OR 0.0024) and FOX (OR 0.0007) were negative when compared to isolates with $bla_{\text{CMY,}}$ which was expected. These results indicate that *E. coli* carrying $bla_{\text{CTX-M}}$ are significantly more likely to be resistant to non-ß-lactams than *E. coli* carrying $bla_{\text{CMY.}}$

The distribution of the number of antimicrobial classes to which *E. coli* isolates are resistant was mono-modal for those carrying $bla_{\text{CTX-M}}$, with a mode of five (Fig. 2.3). Resistance to all seven classes of antimicrobials tested were found only in *E. coli* carrying $bla_{\text{CTX-M}}$. In contrast, *E. coli* carrying bla_{CMY} show a bi-modal distribution with modes at two (cephems and ampicillin) and six classes of antimicrobials (Fig. 2.3).

2.2.3 ESC-resistance gene variants

Seven CTX-M variants were identified among the 227 *E. coli* carrying the $bla_{\text{CTX-M}}$ gene: CTX-M-1 (n = 25), CTX-M-14 (n = 28), CTX-M-15 (n = 125), CTX-M-17 (n = 1), CTX-M-24 (n = 11), CTX-M-32 (n = 4) and CTX-M-55 (n = 34). One isolate carried both $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-32}}$. Only the CMY-2 variant was identified among the 28 sequenced *E. coli* carrying the bla_{CMY} gene. One hundred and thirty-eight of the sequenced *E. coli* carried bla_{TEM}, with eight

variants identified: TEM-1 (*n* = 124), TEM-34 (*n* = 4), TEM-104 (*n* = 3), TEM-135 (*n* = 1), TEM-198 (*n* = 1), TEM-207 (*n* = 1), TEM-216 (*n* = 2) and TEM-230 (*n* = 2). The TEM-207 variant is the only ESBL and was found in one ST58 *E. coli* carrying $bla_{\text{CTX-M-15}}$. Two isolates also carried $bla_{\text{OXA-10}}$, and three isolates carried $bla_{\text{CARB-2}}$, both of these genes are not ESBLs. No bla_{SHV} genes were detected in these *E. coli*.

ESC-resistant isolates which tested negative for $bla_{\text{CTX-M}}$, bla_{CMY} or bla_{SHV} were tested for bla_{TEM} and identified at the species level. *E. coli* with mutations in the chromosomal *ampC* promoter region (*n* = 4) and *E. coli* carrying bla_{TEM} (*n* = 2) were the most frequent. Additional resistance genes were identified among the sequenced ESC-resistant *E. coli* (*n* = 248) that were otherwise not screened for using phenotypic approaches (Spreadsheet S1). Resistance genes for macrolides were found in most strains, including *mdf(A)* (*n* = 244), *mph(A)* (*n* = 52), *mph(E)* (*n* = 5), *msr(E)* (*n* = 5) and *erm(B)* (*n* = 1). Isolates which carried *mph(E)* also carried *msr(E).* Genes associated with resistance to fosfomycin, rifampin and lincosamides included *fosA3* (*n* = 5), *arr-2* (*n* = 17), *lnu(F)* (*n* = 18), respectively, were less frequent. Details regarding other identified AMR genes are summarized in Spreadsheet S1.

2.2.4 *Escherichia coli* strain diversity

Fifty *E. coli* STs were identified and are listed in Tables 2.6 and S2.5. In addition to these known STs, twenty-one isolates have novel STs which were given temporary MLST numbers for this study and begin with the prefix STN (Table S2.2). The most frequent STs include ST58 (*n* = 30), ST46 (*n* = 22), ST398 (*n* = 16), ST10 (*n* = 14), ST190 (*n* = 13), ST744 (*n* = 12), ST685 (*n* = 12) and ST155 (*n* = 12). On farms one and three, the diversity of STs for isolates carrying $bla_{\text{CTX-M}}$ was higher in raw manure compared to environmentally applied manure (Table 2.6). Conversely, on the remaining four farms, the diversity of STs for isolates carrying $bla_{\text{CTX-M}}$ was higher in environmentally applied manure (Table S2.5). All farms that dewater their manure, showed fewer STs in recycled bedding (Table 2.6).

Each farm presents unique epidemiological characteristics with regards to strains carrying $bla_{\text{CTX-M}}$ (*n* = 227) with some STs being unique to each farm (Table 2.6 and Table S2.5). However, some STs are found in more than one farm, including ST10 (*n* = 3), ST38 (*n* = 2), ST46 (*n* = 2), ST58 (*n* = 4), ST69 (*n* = 2), ST88 (*n* = 2), ST155 (*n* = 4), ST162 (*n* = 2), ST165 (*n*

= 2), ST398 (*n* = 2), ST540 (*n* = 2), ST685 (*n* = 2), ST744 (*n* = 2), ST746 (*n* = 2), ST1722 (*n* = 2) and ST8761 (*n* = 2) with the number of farms in parenthesis (Table 2.6 and S2.5). STs which were found in some farms through the entirety of the treatment process including raw, anaerobically digested and environmentally applied manure, include ST10, ST46, ST58, ST155, ST190, ST398, ST685 and ST8761. Fewer isolates carrying *bla*$_{\text{CMY}}$ only belonged to the same STs than those carrying *bla*$_{\text{CTX-M}}$ only. These included ST10, ST155, ST162, ST165, ST398 and ST746 (Table S2.5). STs found in raw manure only included STN1 (*n* = 2), STN3 (*n* = 2), STN9 (*n* = 1), STN10 (*n* = 2), STN12 (*n* = 2), ST57 (*n* = 4), ST69 (*n* = 2), ST219 (*n* = 2), ST224 (*n* = 1), ST609 (*n* = 1), ST1201 (*n* = 3), ST1140 (*n* = 3), ST3298 (*n* = 6), ST3714 (*n* = 1), ST4429 (*n* = 2) and ST5314 (*n* = 2), with the number of isolates in parenthesis. Few STs were found only in environmentally applied manure which included STN6 (*n* = 1), ST316 (*n* = 1), ST657 (*n* = 3), ST939 (*n* = 1) and ST1152 (*n* = 1), with the number of isolates in parenthesis.

To further assess strain relatedness cgMLST and wgMLST MSTs were created (Fig. 2.4 and S2.1). These trees demonstrate that most *bla*$_{\text{CTX-M}}$ variants including *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-55}}$, and *bla*$_{\text{CMY-2}}$ are not restricted to single clonal lineages but have spread horizontally and reside in a variety of unrelated STs (Fig. 2.4 and Fig. S2.1). Groups of multiple genetically related isolates were found at different processing stages in farms one, three, and seven (annotated with red arrows in Fig. 2.4), thus suggesting spread of these clonal lineages through the entire manure processing. However, except for the ST46 isolates from farm three, which are tightly related and carry the same *bla*$_{\text{CTX-M-15,}}$ the looser relatedness of the ST58 isolates from farm one (all carrying *bla*$_{\text{CTX-M-15}}$) and the diversity of *bla*$_{\text{CTX-M}}$ variants found in the ST190 isolates from farm seven suggest a longer evolutionary history, either on the farm or, less likely through repeated penetrations into these farm environments. In contrast, two other clusters of related isolates (annotated with a black arrow in Fig. 2.4) were found in more than one farm. Thus, suggesting the widespread dissemination of these clonal lineages in dairy cattle manure in Ontario. The relatedness of the ST744 isolates from four different farms is further supported by the presence of the *bla*$_{\text{CTX-M-24}}$ gene in all of them and in none of the other isolates of this study. The group of ST155 also found on four farms, were genetically slightly more diverse and carried not exclusively but mainly the *bla*$_{\text{CTX-M-15}}$ gene, similar trends were observed with ST10. Nevertheless, it includes a pair of tightly related isolates from two different farms.

Although wgMLST trees split the main clusters shown in Fig. 2.4, the relatedness between strains within those clusters remained (Fig. S2.1). Core phylogenetic trees using SNP analysis with WGS short reads, were created for isolates from farms three and seven (Fig. S2.2 and S2.3). Branch lengths are derived from the proportion of nucleotide pairwise differences with *E. coli* K12 as a reference. The cgMLST MSTs show relationships between isolates across all six farms, whereas the SNP trees show each farm separately (Fig. S2.2 and S2.3). Clusters shown in cgMLST MSTs (Fig. 2.4) were generally conserved in the phylogenetic SNP trees, and isolates which were not clustered in cgMLST MSTs, remained so in the phylogenetic SNP trees (Fig. S2.2 and S2.3).

2.2.5 Diversity of ESC-resistance plasmids

A sub-sample of *E. coli* isolates from farms three and seven were chosen for long read sequencing and hybrid assembly. Twenty-four isolates carrying $bla_{\text{CTX-M}}$ and nine isolates with bla_{CMY} were sequenced. Of these, 32 ESC-resistance genes were carried on plasmids and in two instances $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-55}}$ were found on the chromosome, with the latter isolate carrying an additional $bla_{\text{CTX-M-55}}$ on a plasmid (Spreadsheet S1). Most of the plasmids were fully assembled using Unicycler (n = 29). However, two were assembled using polished Flye assemblies and one could not be closed (Spreadsheet S1). Plasmids (n = 30) were compared using a presence and absence of genes, with expected clustering of plasmids by incompatibility group, except for replicons belonging to the IncF family, which were dispersed (Fig. 2.5). Two plasmids were excluded from phylogenetic analysis due to discrepancies between Unicycler and Flye assemblies

Hybrid assembly of ESC-resistant *E. coli* showed a wide diversity of plasmids carrying $bla_{\text{CTX-M}}$ variants and $bla_{\text{CMY-2}}$. Of the four $bla_{\text{CTX-M-1}}$-positive isolates, two were from different farms and from isolates of different STs but carried $bla_{\text{CTX-M-1}}$ on an IncI1/ST3 plasmid (Table 2.7). The other two isolates were from the same sample. They presented different antimicrobial susceptibility profiles, different AMR gene profiles, and were from different STs, but both carried the $bla_{\text{CTX-M-1}}$ gene on a IncN/ST1 plasmid (Table 2.7 and Spreadsheet S1). One of these isolates carrying an IncN/ST1 also carried an IncC/ST3 plasmid harbouring $bla_{\text{CMY-2}}$. The $bla_{\text{CTX-M-14}}$ gene was found in farm three only and was located on IncI1 plasmids in all five investigated

isolates. Four of these plasmids were ST166 despite originating from isolates of three different STs (Table 2.7).

The *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-55}}$ variants were carried on more diverse plasmids. Plasmids carrying *bla*$_{\text{CTX-M-15}}$ included a co-integrated IncF and IncI1 plasmid, various IncF replicon types and one IncY. They were all carried in isolates with different STs (Table 2.7). The plasmids carrying *bla*$_{\text{CTX-M-55}}$ were even more diverse and included IncF (n = 3), co-integrated IncHI2 and IncN (n = 2), IncHI2 (n = 1), IncY (n = 1) and plasmid p0111 without an Inc type (n = 1). They were also all carried in isolate with different STs. The two IncFIB/IncFIC plasmids carrying *bla*$_{\text{CTX-M-55}}$ have the same pMLST and are from isolates recovered from different farms, with different MLST profiles. The three plasmids which were either entirely IncHI2 or co-integrated IncHI2/IncN had the same pMLST profile for IncHI2/ST2 (Table 2.7). In addition, one isolate (279-2a) carried two separate *bla*$_{\text{CTX-M-55}}$ copies, one on the chromosome and the other on an IncHI2/IncN plasmid. This isolate had an intermediate susceptibility for cefoxitin, despite lack of *bla*$_{\text{CMY}}$ gene, with no mutations in the chromosomal *ampC* promoter region.

Plasmids of three incompatibility groups carried *bla*$_{\text{CMY-2}}$ (Table 2.7). Plasmids of the IncC group with pMLST profile ST3 (n = 4) from different *E. coli* STs, farms, and time points were found to carry consistently *bla*$_{\text{CMY-2}}$ and the same additional resistance genes *floR, tet(A), aph(6)-Id, aph(3'')-Ib, sul1, sul2, dfrA12, aadA2,* (Table 2.7). IncI1 plasmids (n = 4) did not carry any resistance genes beyond *bla*$_{\text{CMY-2}}$. Three of these are IncI1/ST20, are closely related, and structurally similar (Fig. 2.6). Two isolates carrying IncI1/ST20 were both ST162 recovered from different farms, with the third plasmid being harbored in ST609. Only one IncY plasmid carrying *bla*$_{\text{CMY-2}}$ was identified and carried fewer, but similar resistance genes as those found on the IncC/ST3 plasmids (Table 2.7) and was found in an ST155 isolate. SNP analysis shows that IncI1 plasmids carrying *bla*$_{\text{CTX-M}}$ or *bla*$_{\text{CMY}}$ cluster in accordance to their pMLST and in general also to their ESC-resistance genes (Fig. 2.6).

2.3 DISCUSSION

Broader research investigating the epidemiology of ESC-resistance in livestock, particularly in dairy cattle, and their surrounding environments is needed. We hypothesized that both persistence of some ESC-resistant strains through manure treatment pipelines and HGT of

ESC-resistance plasmids between strains in the treatment facility occur simultaneously. Persistence of ESC-resistant strains and plasmids could contribute to the transmission of ESC-resistance within agricultural environments and consequentially into the food chain (Marti et al., 2013). This is an increasingly pressing issue due to the rise in prevalence of AMR and MDR *Enterobacterales* in agriculture and the importance of fresh produce and plant-based foods in a healthy diet.

2.3.1 Recovery of ESC-resistant *Enterobacterales* decreases downstream the treatment pipeline

Recovery of ESC-resistant *Enterobacterales* carrying *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY}}$ or *bla*$_{\text{SHV}}$ and *E. coli* carrying *bla*$_{\text{CTX-M}}$ or *bla*$_{\text{CMY}}$ all decreased downstream the treatment pipeline. Despite the persistence of some ESC-resistance in the environmentally applied manure, the decrease is indicative of the effectiveness of manure treatments at reducing the prevalence of ESC-resistant *Enterobacterales*. To re-iterate the differences in manure outputs, all farms produce environmentally applied manure, four of the farms also produce recycled bedding, also known as the solid composite, removed from the environmentally applied manure sample (digestate without solids). The reduction in prevalence of ESC-resistant *Enterobacterales* was the most drastic in recycled bedding, next to environmentally applied manure. This could have beneficial implications for livestock and husbandry purposes. Contrary to the general trend and the case with ESC-resistant *Enterobacterales*, we did not observe a significant reduction in recovery of *E. coli* carrying *bla*$_{\text{CTX-M}}$ or *E. coli* carrying *bla*$_{\text{CMY}}$ during manure processing. Since fewer isolates with *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY}}$ were recovered in the samples in general, this could be an issue of statistical power associated with small sample size. Additionally, since *bla*$_{\text{CTX-M}}$-positive strains are very diverse, one could also suspect that a higher transmission rate due to conjugation ability of *bla*$_{\text{CTX-M}}$ plasmids could also be a potential explanation for such an observation. In addition, *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-55}}$ were found on at least two chromosomes, which could be another reason for its stability within these niches.

2.3.2 Recovery of ESC-resistant non-*E.coli Enterobacterales* and their potential role in transmission of ESC-resistance

The *Enterobacterales* other than *E. coli* recovered from our manure samples include a number of bacterial species which have not been thoroughly characterized with regards to ESC-resistance in the literature (Fig. 2.2). For instance, to our knowledge this is the first time that *Rahnella aquatilis* carrying *bla*$_{\text{CTX-M}}$ has been identified and the first time *R. aquatilis* and *Raoultella ornithinolytica* carrying *bla*$_{\text{CTX-M}}$ have been isolated from animal-associated sources. These species do not intrinsically carry *bla*$_{\text{CTX-M}}$ and are indicative of a broad transmission of ESC-resistance plasmids amongst *Enterobacterales* in general. Other than *E. coli*, the two most frequently recovered ESC-resistant species of *Enterobacterales* were *K. pneumoniae* and *P. mirabilis*. These species may play an important role in the epidemiology of ESC-resistance in dairy manure and the surrounding environments. They certainly warrant further investigations, including comparative studies on ESC resistance plasmids.

2.3.3 Additional AMR genes are frequently associated with plasmid carrying *bla*$_{\text{CTX-M}}$, but less with plasmids carrying *bla*$_{\text{CMY}}$

It is not surprising that ESC-resistant strains are non-susceptible to additional antimicrobials since plasmids carrying *bla*$_{\text{CTX-M}}$ or *bla*$_{\text{CMY}}$ often carry other AMR genes (Table 2.7) (Tran et al., 2021; Moffat et al., 2020; Smet et al., 2010). Out of all *Enterobacterales* screened, particularly in *E. coli*, the top three resistances found other than ß-lactams were tetracyclines, sulphonamides and potentiated sulphonamides. These are the most frequently used antimicrobials on dairy farms in Canada, other than ß-lactams (Saini et al., 2012) and these findings are consequently expected.

The increased likelihood of phenotypic resistance to six groups of antimicrobials in isolates carrying *bla*$_{\text{CTX-M}}$ compared to those carrying *bla*$_{\text{CMY}}$ (Table 2.5) suggests co-transmission of *bla*$_{\text{CTX-M}}$ and other AMR genes and potentially co-location on the same plasmids. Particularly striking is the strong association of ciprofloxacin resistance in isolates carrying *bla*$_{\text{CTX-M}}$ compared to those carrying *bla*$_{\text{CMY}}$. This suggests that isolates harbouring *bla*$_{\text{CTX-M}}$ are more likely to also carry resistance determinants to quinolones, than those harbouring *bla*$_{\text{CMY}}$. In contrast, the lack of positive associations between *bla*$_{\text{CMY}}$ and phenotypic resistance to non ß-

lactams suggests *bla*$_{CMY}$ is frequently the only AMR gene found on plasmids, as was observed with those of IncI1 group. The association with *bla*$_{CTX-M}$ and ciprofloxacin resistance is further supported by the presence of *qnr* genes on plasmids carrying *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-55}$ and lack thereof in plasmids carrying *bla*$_{CMY-2}$. This is an important observation with regards to both human and veterinary medicine since quinolones are critically important antimicrobials (Rodríguez-Martínez et al., 2016).

CMY-2 was the only variant identified among the sequenced *bla*$_{CMY}$-positive isolates. This differs from the *bla*$_{CTX-M}$-positive isolates where several CTX-M variants were identified, notably the most common variants found in human *E. coli* (Cantón et al., 2012; Bevan et al., 2017). The *bla*$_{TEM}$ gene was not initially screened for due to the most frequently identified variant in animal and environmental niches being the non-ESBL TEM-1 (Singh et al., 2018; Cormier et al., 2019b). However, our genome sequencing of 248 *E. coli* strains confirmed this hypothesis by showing that TEM-1 was overwhelmingly the most frequent TEM variant identified (89.9%). The other TEM variants were also generally non-ESBL β-lactamases and only one ESBL variant, TEM-207, was detected and it was infrequent (0.7%).

2.3.4 *E. coli* recovered through the treatment pipeline appear to be comprised of mostly multiple lineages with few closely related lineages.

In this study, eight of fifty STs were identified through the entirety of the pipelines from raw to environmentally applied manure. When looking at cgMLST and core SNP analysis, it is evident that many of these STs are likely comprised of multiple lineages (Fig. 2.4; S2.2 and S2.3). ST46 isolates remained tightly related through the different treatment stages, suggesting clonal spread, whereas ST58 and ST685 isolates were less tightly related, possibly indicating multiple lineages. In contrast, ST10 and ST398, were genetically very diverse and although they were found through the entire treatment process, it is unlikely that they belong to the same lineages. Three ST155 isolates from farm seven and three ST8761 isolates from farm three, each from different sampling stages did not cluster in cgMLST nor SNP analysis, thus strongly suggesting that these are isolates from different lineages. Similarly, ST190 which was found only on one farm includes both a tightly related cluster of isolates (likely the same lineage) and a less related cluster of isolates (likely of different lineages). In addition, STs which were found

through the entire pipeline are frequently found on more than one farm (Table 2.6 and S2.5). This widespread distribution suggests that isolates of these STs may not always be epidemiologically related and representative of persistence when found at different processing stages in a farm, particularly when detailed analysis such as cgMLST and SNP analysis demonstrates a significant diversity (Fig. 2.4; S2.2 and S2.3).

2.3.5 Few STs parallel other cattle studies and those found in human clinical studies

A few STs identified in environmentally applied manure such as ST10, ST398 and ST744 have been described in human clinical isolates or other livestock samples. *E. coli* ST10 is typically a commensal in the human gastrointestinal tracts and is ubiquitous in the environment (Day et al., 2019; Müller et al., 2016), it also carries thermotolerance genes (Kamal et al., 2020). Incidentally, two ESC-resistant *E. coli* ST10 were recovered in heat treated compost, and upstream of heat treatment in both anaerobically digested manure and the liquid composite after the dewatering process (Fig. 2.4 and Table 2.6 and 2.7). Recovering ST10 before and after thermophilic treatment suggests ST10 is stable through thermophilic manure treatments. However, as mentioned above, the ST10 isolates recovered are diverse and not closely related (Fig. 2.4 and Fig. S2.3). ST398 is less frequently associated with human infections but has been found previously in both pig manure and human bloodstream infections (Ludden et al., 2019). The ST398 isolates from farm seven were recovered over the full one-year time span from December 2018 to October 2019 and were recovered from every stage of the treatment process, including environmentally applied manure but excluding heat-treated compost. This suggests long term persistence through the whole treatment process which may have contributed to the SNP diversity visible in core genes trees (Fig. S2.3). Due to this ST being associated with livestock colonization (Ludden et al., 2019), the elimination of ST398 from recycled bedding is certainly beneficial. ST744 has been identified in dairy cattle manure (Afema et al., 2018). Although, these previous ST744 isolates from the USA carried $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-27}}$, contrary to the presence of this ST carrying $bla_{\text{CTX-M-24}}$ in farms one, three, five and seven in raw, environmentally applied manure and heat-treated compost, suggest a broad clonal spread and possible adaptation to the dairy manure environment. Finally, it is important to note that human associated pathogenic STs such as ST131 were not identified in this study. Taken together, the results of this study show that some ESBL-producing clonal lineages can survive

the whole manure treatment process, some can persist over long periods of time (e.g. ST398 in farm seven), and some such as ST744 are probably adapted to this specific environment.

2.3.6 Plasmids carrying *bla*$_{\text{CTX-M}}$ are diverse and include phage-like and epidemic plasmids

Out of the hybrid assembled isolates, *bla*$_{\text{CTX-M-1}}$ was carried on IncN/ST1 in two isolates, and on the IncI1/ST3 plasmid in the other two isolates. IncN/ST1 plasmids carrying *bla*$_{\text{CTX-M-1}}$ have been identified in *E. coli* from crow feces in Canada and in *Salmonella enterica* from poultry in the US, and widely in Europe, in both human and animal isolates (Jamborova et al., 2018; Denagamage et al., 2019; Jakobsen et al., 2015; Dolejska et al., 2012). However, these plasmids had not been described in dairy cattle from Canada. IncI/ST3 plasmids carrying *bla*$_{\text{CTX-M-1}}$ have also been found recently in crow feces (Jamborova et al., 2018) and in a number of domestic species in Canada (Cormier et al., *manuscript in preparation*), but to our knowledge they have not been described in Canadian dairy cattle. Both of these plasmids may represent epidemic plasmids (Carattoli et al., 2018) which have recently been introduced into Canadian environments, possibly from European sources. Monitoring of these plasmids is warranted in order to follow their distribution and spread.

Plasmids carrying *bla*$_{\text{CTX-M-14}}$ were found on one farm only and were consequently more conserved (Fig. 2.5 and 2.6). *bla*$_{\text{CTX-M-14}}$ was found repeatedly on IncI1 plasmids of the same pMLST ST166, but in three different strains and at different points in time and processing stages. It certainly represents a good example of a plasmid spreading by horizontal gene transfer through the manure processing process. IncI1/ST166 plasmids have been described in *Salmonella* from human clinical samples and animal sources in Asia but these plasmids have neither been described in human nor in animal sources in North America (Wong et al., 2015; Cha et al., 2020). Plasmids carrying *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-55}}$ were more diverse and found on both farms investigated for plasmid diversity. This illustrates once more the mobilization of these genes afforded by their adjacent mobile genetic elements onto diverse plasmids with varying host ranges, conjugation abilities and advantageous accessory genes (Rozwandowicz et al., 2018). The observed distribution of resistance to multiple classes of antimicrobials and associations between *bla*$_{\text{CTX-M}}$ and resistance to antimicrobials of classes other than β-lactams mentioned

earlier are further supported by the frequent presence of additional resistance genes on the plasmids carrying the most frequent *bla*$_{\text{CTX-M}}$ variants (i.e. *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-55}}$).

IncY plasmids carried *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CMY-2}}$ and are phage-like plasmids, which likely transfer through transduction (Schneider, 2017). Although other phage like plasmids carrying *bla*$_{\text{CTX-M-15}}$ have been described (Colavecchio et al., 2017), to our knowledge, IncY plasmids carrying *bla*$_{\text{CTX-M-15,}}$ *bla*$_{\text{CTX-M-55}}$ or *bla*$_{\text{CMY-2}}$ from dairy cattle have not been described. However, IncY plasmids are frequently identified in ESC-resistant strains (Mollenkopf et al., 2012). The frequent association of IncY plasmids and ESC-resistance strains may be due to their presence in strains carrying IncF or IncI1, which are known to carry ESC-resistance genes (Ben Sallem et al., 2014; Dierikx et al., 2010; Smet et al., 2010; Moffat et al., 2020). Our results show that these phage-like plasmids carry diverse ESC resistance genes and may represent an alternate route of ESC-resistance dissemination, worth more detailed studies.

Fewer replicon types carrying *bla*$_{\text{CMY}}$ were identified and in contrast to plasmids carrying *bla*$_{\text{CTX-M}}$ there were no identified IncF plasmids carrying *bla*$_{\text{CMY}}$. This lower diversity could be a result of smaller sample size for WGS of *E. coli* carrying *bla*$_{\text{CMY}}$. The sequenced IncC plasmids, which until recently have been classified as IncA/C (Ambrose et al., 2018), all carried the genes for resistance to phenicols, tetracyclines, aminoglycosides and folate pathway inhibitors typically associated with these plasmids (Ambrose et al., 2018). This fits the higher mode seen in the bi-modal resistance distribution seen among the larger sample of *bla*$_{\text{CMY}}$-positive isolates shown in Fig. 2.3. In contrast, IncI1 plasmids carrying *bla*$_{\text{CMY}}$ usually carry no additional resistance genes (Baudry et al., 2009; Pietsch et al., 2018) and fit the lower mode of this bi-modal distribution. The presence of these IncI1 plasmids as vectors of *bla*$_{\text{CMY}}$ explains the negative association between this gene and resistance to antimicrobials other than β-lactams, when compared to *bla*$_{\text{CTX-M}}$. In Canadian beef cattle, identified plasmids carrying *bla*$_{\text{CMY-2}}$ have always been IncA/C (Mataseje et al., 2010; Martin et al., 2011) and our results suggest that the situation may be different in dairy cattle. Further studies are needed to assess the exact proportions of IncI1 and IncC plasmids in feces and manure from dairy cattle. Although the plasmids sequenced in this study originated from bacteria isolated from raw and treated manure, the general associations between *bla*$_{\text{CTX-M}}$ variants, *bla*$_{\text{CMY-2}}$ and replicon types were in agreement with those from cattle

and described in the literature, (Mollenkopf et al., 2012; Madec et al., 2012; Sato et al., 2014; Rehman et al., 2017).

2.3.7 Similar CTX-M and CMY plasmids are found at different processing stages and over time

Similar plasmids carrying *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY}}$ were identified in more than one processing stage, suggesting persistence through treatments (Table 2.7). For the plasmids carrying *bla*$_{\text{CTX-M}}$, these include the IncI1/ST166 carrying *bla*$_{\text{CTX-M-14}}$ mentioned earlier and IncHI2/IncN plasmids carrying *bla*$_{\text{CTX-M -55}}$. These plasmids were found in different isolates at both extremities of the manure process, with the latter even surpassing thermophilic treatment. For plasmids carrying *bla*$_{\text{CMY-2}}$, IncI1/ST20 persisted through the entire treatment pipeline. IncI1/ST20 plasmids carrying *bla*$_{\text{CMY-2}}$ have not been described in animals from Canada but have occasionally been found in human clinical samples in Canada and the US (Mataseje et al., 2010; Folster et al., 2014). The relations between these plasmids in bacterial populations from animals and humans may therefore warrant further investigations.

2.3.8 Similar CTX-M and CMY plasmids are found in diverse STs within the same farm and across farm three and seven

Similar plasmids were identified in diverse STs. Some of them were found in diverse strains on the same farm and some in both farm three and seven. Recovery of similar plasmids in diverse isolates on the same farm, could indicate HGT during processing as indicated by IncI/ST166 plasmids carrying *bla*$_{\text{CTX-M-1}}$ found in two STs on farm three and IncHI2/IncN carrying *bla*$_{\text{CTX-M-55}}$ found in the ubiquitous ST10 and ST219, also from farm three. Recovery of the same plasmids in genetically unrelated isolates from different farms suggests that HGT of some plasmids may play a significant role in the spread of ESC resistance at a larger scale. For instance, this is illustrated in this study by the presence of the epidemic *bla*$_{\text{CTX-M-1}}$ /IncI1/ST3 plasmid (Carattoli et al., 2018; Smith et al., 2015; Dahmen et al., 2013; Valcek et al., 2019), and probably also by the *bla*$_{\text{CMY-2}}$/IncI1/ST20, *bla*$_{\text{CMY-2}}$/IncC/ST3 and *bla*$_{\text{CTX-M-55}}$/ IncFIB/FIC/F18:A-:B58 plasmids.

2.3.9 Final Conclusion

The finding of numerous *Enterobacterales* species other than *E. coli* carrying *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY}}$ in the same samples as *E. coli* warrant further comparative studies on plasmids from other *Enterobacterales*, such as *K. pneumoniae* or *P. mirabilis* to better understand the ESC-resistance plasmid epidemiology. Statistical analyses showed a significant reduction in ESC-resistant *Enterobacterales* in general and of *E. coli* in particular downstream treatment pipelines. However, ESC-resistant *Enterobacterales* and ESC-resistance plasmids were still recovered in all environmentally applied samples. Thus, risk assessments to address downstream implications to human health regarding integration into the human gut microbiome through consumption of contaminated crops are needed.

Although eight *E. coli* MLST profiles were identified across the different stages of the treatment pipeline, this may have been for some of them the consequence of the widespread occurrence of these strains in general. However, in a few cases, our data strongly suggest that some strains can persist through the whole manure processing and that some may even have adapted to the manure treatment environment. Further investigations are needed to confirm these observations on a broader scale. Our results have also shown the horizontal spread of some plasmids in bacterial populations across the manure treatment process. The characteristics of such plasmids will need to be investigated further in order to identify the factors allowing them to spread and their presence in bacteria from the end products of manure treatment. Finally, epidemic plasmids IncI1/ST3 and InsI1/ST20 were identified carrying *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CMY-2}}$, respectively. The role of dairy manure in their global spread will also need further investigations.

2.4 ACKNOWLEDGEMENTS

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2.5 TABLES

Table 2.1 Primers used for PCR screening to characterize ESC-resistant isolates recovered from dairy manure samples across six farms in Southern Ontario.

Target	Primer Sequence	Product size (bp)	Reference
bla$_{\text{CTX-M}}$	F: ATGTGCAGYACCAGTAARGTKATGGC R: TGGGTRAARTARGTSACCAGAAYCAGCGG	593	Mulvey et al., 2003
bla$_{\text{CMY}}$	F: GACAGCCTCTTTCTCCACA R: TGGACACGAAGGCTACGTA	1000	Kozak et al., 2009
bla$_{\text{SHV}}$	F: AGGATTGACTGCCTTTTTG R: ATTTGCTGATTTCGCTCG	393	Kozak et al., 2009
bla$_{\text{TEM}}$	F: TTCTTGAAGACGAAAGGGC R: ACGCTCAGTGGAACGAAAAC	1150	Briñas et al., 2002
AmpC	F: AATGGGTTTTCTACGGTCTG R: GGGCAGCAAATGTGGAGCAA	191	Caroff et al., 1999

Table 2.2 Comparison of the recovery of ESC-resistant *Enterobacterales* and *E. coli* isolates from raw input manure to manure post treatment with anaerobic digestion at mesophilic temperatures individually and both manure outputs on four farms, individually and pooled.

Farm	Process[a,]	ESC-Resistant *Enterobacterales* (CTX-M, CMY or SHV)			ESC-Resistant *E. coli* (CTX-M or CMY)		
		Sample	Replicate[b]	Isolate[c]	Sample	Replicate[b]	Isolate
1	Raw	100 (8/8)	87.5 (21/24)	45.2 (38/84)	87.5 (7/8)	79.2 (19/24)	32.1 (27/84)
	Treated	75.5 (6/8)	62.5 (15/24)	71.4 (25/35)	62.5 (5/8)	58.3 (14/24)	60.0 (21/35)
	Environmental	50.0 (2/4)	50.0 (6/12)	100 (11/11)	50.0 (2/4)	50.0 (6/12)	81.8 (9/11)
	Recycled bedding	57.1 (4/7)	38.1 (8/21)	80.0 (12/15)	42.9 (3/7)	33.3 (7/21)	73.3 (11/15)
2	Raw	83.3 (5/6)	55.5 (10/18)	79.0 (15/59)	83.3 (5/6)	50.0 (9/18)	20.3 (12/59)
	Treated	100 (6/6)	100 (18/18)	80.0 (36/45)	100 (6/6)	100 (18/18)	73.3 (33/45)
3	Raw	100 (8/8)	100 (24/24)	59.0 (49/83)	100 (8/8)	100 (24/24)	45.8 (38/83)
	Treated	100 (8/8)	83.3 (20/24)	60.1 (42/69)	100 (8/8)	83.3 (20/24)	46.4 (32/69)
	Environmental	100 (8/8)	87.5 (21/24)	77.4 (41/53)	100 (8/8)	87.5 (21/24)	67.9 (36/53)
	Recycled bedding	0 (0/7)	0 (0/21)	0 (0/0)	0 (0/7)	0 (0/21)	0 (0/0)
4	Raw	100 (8/8)	100 (24/24)	47.19 (42/89)	100 (8/8)	100 (24/24)	30.3 (27/89)
	Treated	87.5 (7/8)	79.2 (19/24)	59.3 (32/54)	87.5 (7/8)	70.8 (17/24)	48.1 (26/54)
5[d]	Raw	100 (8/8)	95.8 (23/24)	45.1 (37/82)	100 (8/8)	95.8 (23/24)	40.2 (33/82)
	Treated	87.5 (7/8)	75.0 (18/24)	35.3 (24/68)	87.5 (7/8)	75.0 (18/24)	32.4 (22/68)
	Environmental	50.0 (4/8)	41.7 (10/24)	66.7 (18/27)	50.0 (4/8)	41.2 (10/24)	66.7 (18/27)
	Recycled bedding	43.0 (3/7)	33.3 (7/21)	57.1 (8/14)	28.6 (2/7)	28.6 (6/21)	42.9 (6/14)
7	Raw	100 (8/8)	100 (24/24)	57.3 (74/129)	100 (8/8)	70.8 (17/24)	15.5 (20/129)
	Treated	100 (8/8)	95.8 (23/24)	62.2 (61/98)	100 (8/8)	91.7 (22/24)	29.59 (29/98)
	Environmental	100 (8/8)	91.7 (22/24)	66.7 (56/84)	100 (8/8)	91.2 (22/24)	33.3 (28/84)
	Recycled bedding[e]	37.5 (3/8)	25.0 (6/24)	46.7 (7/15)	37.5 (3/8)	20.8 (5/24)	33.3 (5/15)
Pooled	Raw	97.8 (45/46)	91.3 (126/138)	48.5 (255/526)	95.7 (44/46)	84.1 (116/138)	29.8 (157/526)
	Treated	91.3 (42/46)	81.9 (113/138)	59.6 (220/369)	89.1 (41/46)	78.9 (109/138)	44.2 (163/369)
	Environmental	78.6 (22/28)	70.2 (59/84)	72.0 (126/175)	78.6 (22/28)	70.2 (59/84)	52.0 (91/175)
	Recycled bedding	34.5 (10/29)	24.1 (21/87)	61.4 (27/44)	27.6 (8/29)	20.7 (18/87)	50.0 (22/44)

[a]All raw samples were the manure input; treated manure from all six farms were digestate with solids samples; environmental manure is the environmentally applied manure output, which on farms one, three, five and seven were digestate without solids; recycled bedding on farms one, three and five were dewatered manure whereas on farm seven this was heat-treated compost.

[b]Each sample was processed in triplicate to equate three biological replicates per sample.

[c]Isolates represent the selected colonies with unique morphologies from each plate, one plate per replicate, therefore three plates per sample (triplicate).

[d]Treated manure on farm five was sampled from the first digester, and environmental manure was digestate three without solids taken after the third digestion process on after the solids have been removed. This farm utilizes two mesophilic digesters with the third digester performing at thermophilic temperatures.

[e]Heat-treated compost is unique to farm seven and is dewatered material that has been treated at thermophilic temperatures and left to mature under composting conditions.

Numerical values within each cell represent the percentage, with the proportions in parenthesis.

Table 2.3 Multivariable logistic regression models comparing recovery of ESC-resistant *Enterobacterales* and *E. coli* in anaerobically digested, environmentally applied manure and recycled bedding to the referent raw manure at the replicate level.

Sampling Stage[a]	Recovery of Interest[b]	Odds Ratio	p-value	95% CI
Anaerobically digested manure	ESC-Resistant *Enterobacterales*	0.25	0.079	0.0553 – 1.1742
	ESC-Resistant *E. coli*	0.49	0.336	0.1181 – 2.0732
	E. coli CTX-M	1.22	0.696	0.4532 – 3.2704
	E. coli CMY	0.63	0.445	0.1915 – 2.0659
Environmentally applied manure	ESC-Resistant *Enterobacterales*	**3.0×10^{-3}**	**0.002**	**7.0×10^{-5} – 0.1258**
	ESC-Resistant *E. coli*	0.17	0.154	1.44×10^{-2} – 1.9571
	E. coli CTX-M	1.55	0.417	0.5383 – 4.4577
	E. coli CMY	0.23	0.103	0.0384 – 1.3464
Recycled bedding	ESC-Resistant *Enterobacterales*	**1.0×10^{-4}**	**0.000**	**1.53×10^{-6} – 0.0106**
	ESC-Resistant *E. coli*	**1.62×10^{-5}**	**0.010**	**8.81×10^{-9} – 0.0686**
	E. coli CTX-M	**4.65×10^{-2}**	**0.000**	**1.01×10^{-2} – 0.2139**
	E. coli CMY	**6.96×10^{-3}**	**0.001**	**3.49×10^{-4} – 0.1387**

Results in bold are considered statistically significant.

[a]Anaerobically digested manure includes digestate with solids manure samples from farms one, two, three, four and seven; digestate one with solids from farm five; Environmentally applied manure includes digestate without solids samples from farms one, three and seven; digestate three without solids from farm five; Recycled bedding includes dewatered samples from farms one, three and five, and heat-treated compost from farm seven.

[b]ESC-Resistant *Enterobacterales* carrying $bla_{\text{CTX-M}}$, bla_{CMY} or bla_{SHV} and ESC-Resistant *E. coli* carrying $bla_{\text{CTX-M}}$ or bla_{CMY}

Table 2.4 Frequency of resistance to twelve antimicrobials and of multi-drug resistance among *E. coli* isolates carrying *bla*$_{CTX-M}$ or *bla*$_{CMY}$.

Farm (# of isolates)	Percentage of *Escherichia coli* isolates conferring a resistant phenotype[a]												MDR[a] (%)
	CHL	CIP	SUL	SXT	KAN	GEN	STR	TET	AMP	AMC	CTX	FOX	
1 (*n* = 68)	33.8	7.3	66.1	55.8	29.4	17.6	58.8	58.8	100	47.0	100	45.5	72.1
2 (*n* = 45)	53.3	6.5	73.3	60.0	28.9	15.2	55.5	86.7	100	65.2	100	65.2	93.3
3 (*n* = 106)	37.7	1.8	71.6	65.0	22.6	17.9	68.8	80.1	100	54.7	98.1	50.9	85.0
4 (*n* = 53)	45.2	0	67.9	54.7	9.4	0	56.6	71.6	100	83.0	100	83.0	88.7
5 (*n* = 103)	17.4	4.8	46.6	34.9	5.8	2.9	37.8	46.6	100	84.4	100	80.5	55.3
7 (*n* = 115)	34.8	1.7	85.2	73.9	15.3	0.8	62.3	87.8	100	18.2	99.1	18.2	88.7
Overall (*n* = 490)	34.4	3.4	68.6	58.0	17.6	8.6	57.1	71.6	100	55.5	99.4	53.7	79.0

[a]MDR; multi-drug resistant is defined as being resistant to at least one antimicrobial in three or more antimicrobial classes, therefore isolates with an intermediate phenotype were not considered resistant. Resistance to AMC was not included in consideration for multi-drug resistance since isolates carrying *bla*$_{CMY}$ are naturally resistant to AMC and would by default be considered MDR.

Abbreviations: Ampicillin (AMP); amoxicillin-clavulanic acid (AMC); cefotaxime (CTX); cefoxitin (FOX); sulfonamide (SUL); sulfamethoxazole-trimethoprim (SXT); tetracycline (TET); streptomycin (STR); kanamycin (KAN); gentamicin (GEN); ciprofloxacin (CIP) and chloramphenicol (CHL).

Table 2.5 Associations between expression of phenotypic resistance to twelve antimicrobials in *E. coli* carrying *bla*$_{CMY}$ compared to *E. coli* isolates carrying *bla*$_{CTX-M}$ using exact logistic regression models.

Antimicrobials	CMY			CTX-M		
	Odds Ratio	p-value	95% CI	Odds Ratio	p-value	95% CI
AMC	1.0*	<0.0000	0 - + ∞	**0.0024**	**0.0000**	**0.0000 – 0.0142**
AMP[a]	N/A	N/A	N/A	N/A	N/A	N/A
CTX[a]	N/A	N/A	N/A	N/A	N/A	N/A
FOX[a]	N/A	N/A	N/A	**0.0007**	**<0.0000**	**0.0002 - 0.0024**
CHL	1.0349	0.9304	0.7030 – 1.5247	0.7932	0.2609	0.5357 - 1.1711
CIP	**0.0263***	**<0.0000**	**0.0 - 0.1517**	**42.4697***	**0.0000**	**7.3647 - + ∞**
GEN	0.9848	1.0000	0.5051 - 1.9294	0.9323	0.9547	0.4708 - 1.8196
KAN	**0.4327**	**0.0003**	**0.2673 - 0.6926**	**2.1497**	**0.0009**	**1.3503 - 3.4494**
STR	**0.5339**	**0.0012**	**0.3603 - 0.7875**	**1.8375**	**0.0019**	**1.2422 – 2.7323**
SXT	**0.2285**	**<0.0000**	**0.1510 - 0.3423**	**4.8334**	**0.0000**	**3.1919 - 7.4097**
SUL	**0.2342**	**<0.0000**	**0.1480 - 0.3646**	**3.6613**	**0.0000**	**2.3543 - 5.7878**
TET	**0.4694**	**0.0004**	**0.3011 - 0.7248**	**1.9341**	**0.0024**	**1.2506 - 3.0223**

[a]Due to the complete correlation between these genes and their phenotypic resistance, an exact logistic regression analysis could not be done.

*median unbiased estimate

Bolded results are considered significant at a <0.05 p-value. Isolates with an intermediate phenotype were classified as resistant for these analyses.

Abbreviations: Ampicillin (AMP); amoxicillin-clavulanic acid (AMC); cefotaxime (CTX); cefoxitin (FOX); sulfonamide (SUL); sulfamethoxazole-trimethoprim (SXT); tetracycline (TET); streptomycin (STR); kanamycin (KAN); gentamicin (GEN); ciprofloxacin (CIP) and chloramphenicol (CHL).

Table 2.6 Sources and MLST diversity of *E. coli* strains carrying *bla*$_{CTX-M}$ among the four farms which separate their manure outputs into recycled bedding and environmentally applied manure.

Farm	Raw	Other[a]	Output: EAM[b]	Output: Bedding[c]
1	**ST38 (*n* = 1)**			
	ST58 (*n* = 1) [15]	**ST58 (*n* = 1) [15]**	**ST58 (*n* = 1) [15]**	**ST58 (*n* = 1) [15]**
		ST88 (*n* = 1)		
	ST540 (*n* = 1)			
	ST685 (*n* = 1)			
	ST744 (*n* = 2)			
	ST3298 (*n* = 2)			
	STN3 (*n* = 1)			
3	**ST10 (*n* = 1) [1]**	**ST10 (*n* = 1) [15]**	**ST10 (*n* = 1) [15]**	N/A[d]
	ST46 (*n* = 2) [15]	**ST46 (*n* = 5) [15]**	**ST46 (*n* = 4) [15]**	
	ST58 (*n* = 1) [14]	**ST58 (*n* = 1) [14]**	**ST58 (*n* = 1) [14]**	
			ST155 (*n* = 1)	
		ST162 (*n* = 1)		
	ST224 (*n* = 1)			
	ST398 (*n* = 1)		**ST398 (*n* = 1)**	
		ST641 (*n* = 1)		
	ST744 (*n* = 2)			
			ST746 (*n* = 2)	
	ST4429 (*n* = 1)			
		ST7978 (*n* = 1)		
	ST8761 (*n* = 1) [55]	**ST8761 (*n* = 1) [55]**	**ST8761 (*n* = 1) [55]**	
	STN8 (*n* = 1)			
	STN7 (*n* = 1)			
5		ST23 (*n* = 2)		
		ST155 (*n* = 3) [15]	**ST155 (*n* = 1) [15]**	**ST155 (*n* = 1) [15]**
		ST718 (*n* = 1)		
			ST744 (*n* = 1)	
			ST939 (*n* = 1)	
			ST1152 (*n* = 1)	
		ST1722 (*n* = 1)	**ST1722 (*n* = 1)**	
	STN1 (*n* = 1)			
7		**ST10 (*n* = 2) [15]**	**ST10 (*n* = 4) [15]**	**ST10 (*n* = 2) [15;55]**
		ST38 (*n* = 2)		
		ST58 (*n* = 2)	**ST58 (*n* = 3)**	
	ST88 (*n* = 1)			
		ST100 (*n* = 1)		
	ST155 (*n* = 1) [15]	**ST155 (*n* = 1) [15]**	**ST155 (*n* = 2) [1;55]**	
		ST162 (*n* = 2)		
	ST190 (*n* = 1)	ST190 (*n* = 7)	ST190 (*n* = 2)	
	ST219 (*n* = 1)			
	ST398 (*n* = 2) [15;32]	**ST398 (*n* = 7) [1;14;15;32]**	**ST398 (*n* = 2) [1;32]**	

	ST540 (*n* = 3)	**ST540 (*n* = 1)**	
ST683 (*n* = 1)	ST683 (*n* = 2)		
ST685 (*n* = 3) [1;15]	**ST685 (*n* = 2) [1;15]**	**ST685 (*n* = 3) [15]**	
		ST744 (*n* = 1)	ST744 (*n* = 1)
ST1201 (*n* = 2)			
	ST1722 (*n* = 1)		
	ST3580 (*n* = 1)		
ST5314 (*n* = 2)			
	ST5597 (*n* = 2)		
	ST7982 (*n* = 1)		
			ST8761 (*n* = 1)
	ST9062 (*n* = 2)	ST9062 (*n* = 1)	
	ST11633 (*n* = 1)	ST11633 (*n* = 2)	
		STN6 (*n* = 1)	
	STN9 (*n* = 1)		
	STN11 (*n* = 2)		

[a]Other treatments include any other intermediate manure processing stages on that farm.

[b]EAM represents environmentally applied manure (digestate without solids) on each farm.

[c]Bedding represents dewatered manure from farms one, three and five and heat-treated compost from farm seven.

[d]ESC-resistant isolates were not recovered from farm three dewatered samples at any time.

Bolded MLST profiles are found in more than one farm, and any profile beginning with STN, has a novel MLST profile and was given a temporary name for this study. Number of samples in which each ST were identified are represented in parenthesis. For STs recovered at three or more stages, the CTX-M variants identified at that stages are in square brackets.

Table 2.7 Characteristics of *Escherichia coli* genomes assembled using both long and short read sequencing and their ESC-resistance plasmids.

Gene	ID	F	Sample	SM	Plasmid			Isolate	
					Replicon Type	pMLST	Plasmid size (bp)	MLST	Additional resistance genes on plasmid carrying *bla*CTX-M or *bla*CMY
CTX-M-1	280-3a	7	DWS	Apr	IncN	ST1	42, 484	ST190	None
CTX-M-1	280-2c	7	DWS	Apr	IncN	ST1	42, 481	ST398	None
CTX-M-1	302-1b	7	DWS	Jun	IncI1	ST3	105, 449	ST3580	*tet(A), sul2*
CTX-M-1	254-3a	3	DWS	Mar	IncI1	ST3	104, 156	ST162	*sul2*
CTX-M-14	200-2a	3	Raw	Jan	IncI1	ST166	95, 660	ST58	None
CTX-M-14	202-3b	3	DWOS	Jan	IncI1	ST166	93, 630	ST58	None
CTX-M-14	200-3b	3	Raw	Jan	IncI1	ST166	91, 896	ST4429	None
CTX-M-14	270-3a	3	DWOS	Apr	IncI1	ST166	90, 813	ST398	None
CTX-M-14	162-3b	3	DWOS	Nov	IncI1	Novel	90, 590	STN2	None
CTX-M-15	329-1a	3	Raw	Aug	IncI1/IncFIB /IncFIC	ST31/F46:A -:B20	208, 008	ST8761	*tet(A), tet(M), sul2, sul3, bla*TEM-1*, aadA17, cmlA1, dfrA12, floR, aph(3')-1a*
CTX-M-15	238-2b	7	DEW	Feb	IncFIB	Unknown	111, 929	ST683	None
CTX-M-15	331-3a	3	DWOS	Aug	IncFIB	Unknown	110, 943	ST155	None
CTX-M-15	190-2a	7	DEW	Dec	IncFIB, IncFIB(K)	F-:A-:B53	104, 690	ST162	*tet(A), bla*TEM-1*, dfrA14, qnrS1*
CTX-M-15	343-3b	7	DEW	Aug	IncY	N/A	98, 488	ST540	*tet(A), sul2, bla*TEM-1*, dfrA14, aph(3")-Ib, aph(6)-Id, qnrS1*
CTX-M-55	260-3b	7	DEW	Mar	IncY	N/A	130, 626	ST11633	*tet(A), sul2, aadA5, dfrA17 floR, aph(3")-Ib, aph(6)-Id, mdf(A)*
CTX-M-55	342-3a	7	DWOS	Aug	IncFIB/ IncFIC	F18:A-:B58	129, 958	ST744	*sul3, aadA1, floR, aph(3')-Ia, ant(3")-1a, lnu(F)*
CTX-M-55	254-2c	3	DWS	Mar	IncFIB/ IncFIC	F18:A-:B58	112, 048	ST7978	*tet(A), aph(3')-Ia*
CTX-M-55	259-1a	7	DWOS	Mar	IncFII	F33:A-:B-	70, 625	ST155	*bla*TEM*, fosA3*
CTX-M-55	233-3a	3	DWOS	Feb	IncHI2	ST2	270, 799	ST8761	*tet(A), sul3, bla*TEM-1*, aadA22, cmlA1, dfrA14, floR, aph(3')-Ia, aac(3)-IId, qnrS1, mph(A), ARR-2*
CTX-M-55	344-2a	7	HTC	Aug	IncHI2/IncN	ST2*	267, 447	ST10	*tet(A), sul3, bla*TEM-1*, aadA22, dfrA14, floR, aph(3')-Ia, aac(3)-IId, qnrS1, mph(A), lnu(F), ARR-2*
CTX-M-55	279-2a	7	Raw	Apr	IncHI2/IncN	ST2*	261, 342	ST219	*tet(A), sul3, aadA23, dfrA14, floR, aph(3')-Ia, ant(3")-1a, qnrS1, mph(A), lnu(F), ARR-2*

CTX-M-55	181-3a	3	Raw	Dec	p0111	N/A	127, 570	ST224	*tet(A), sul2, aadA5, dfrA17, floR, aph(3'')-Ib, aph(6)-Id*
CMY-2	292-1b	3	DWOS	Jun	IncC	ST3	164, 259	ST657	*floR, tet(A), aph(6)-Id, aph(3'')-Ib, sul1, sul2, dfrA12, aadA2*
CMY-2	162-3b	3	DWOS	Nov	IncC	ST3	160, 272	STN2	*floR, tet(A), aph(6)-Id, aph(3'')-Ib, sul1, sul2, dfrA12, aadA2*
CMY-2	280-2c	7	DWS	Apr	IncC	ST3	155, 827	ST398	*floR, tet(A), aph(6)-Id, aph(3'')-Ib, sul1, sul2, dfrA12, aadA2*
CMY-2	259-2c	7	DWOS	Mar	IncC	ST3	116, 140	ST10	*floR, tet(A), aph(6)-Id, aph(3'')-Ib, sul1, sul2, dfrA12, aadA2*
CMY-2	259-1a	7	DWOS	Mar	IncY	N/A	162, 914	ST155	*floR, tet(A), aph(6)-Id, aph(3'')-Ib, sul2*
CMY-2	183-2b	3	DWOS	Dec	IncI1	ST12	102, 760	ST165	None
CMY-2	344-1a	7	HTC	Aug	IncI1	ST20	97, 674	ST162	None
CMY-2	331-2a	3	DWOS	Aug	IncI1	ST20	97, 660	ST162	None
CMY-2	290-1a	3	Raw	Jun	IncI1	ST20	95, 120	ST609	None

Abbreviations: isolate identification (ID); farm (F); sampled month (SM); multi-locus sequence type (MLST); digestate with solids (DWS); digestate without solids (DWOS); dewatered (DEW); heat-treated compost (HTC)

*the pMLST for IncHI2 was identified but not for IncN

2.6 FIGURES

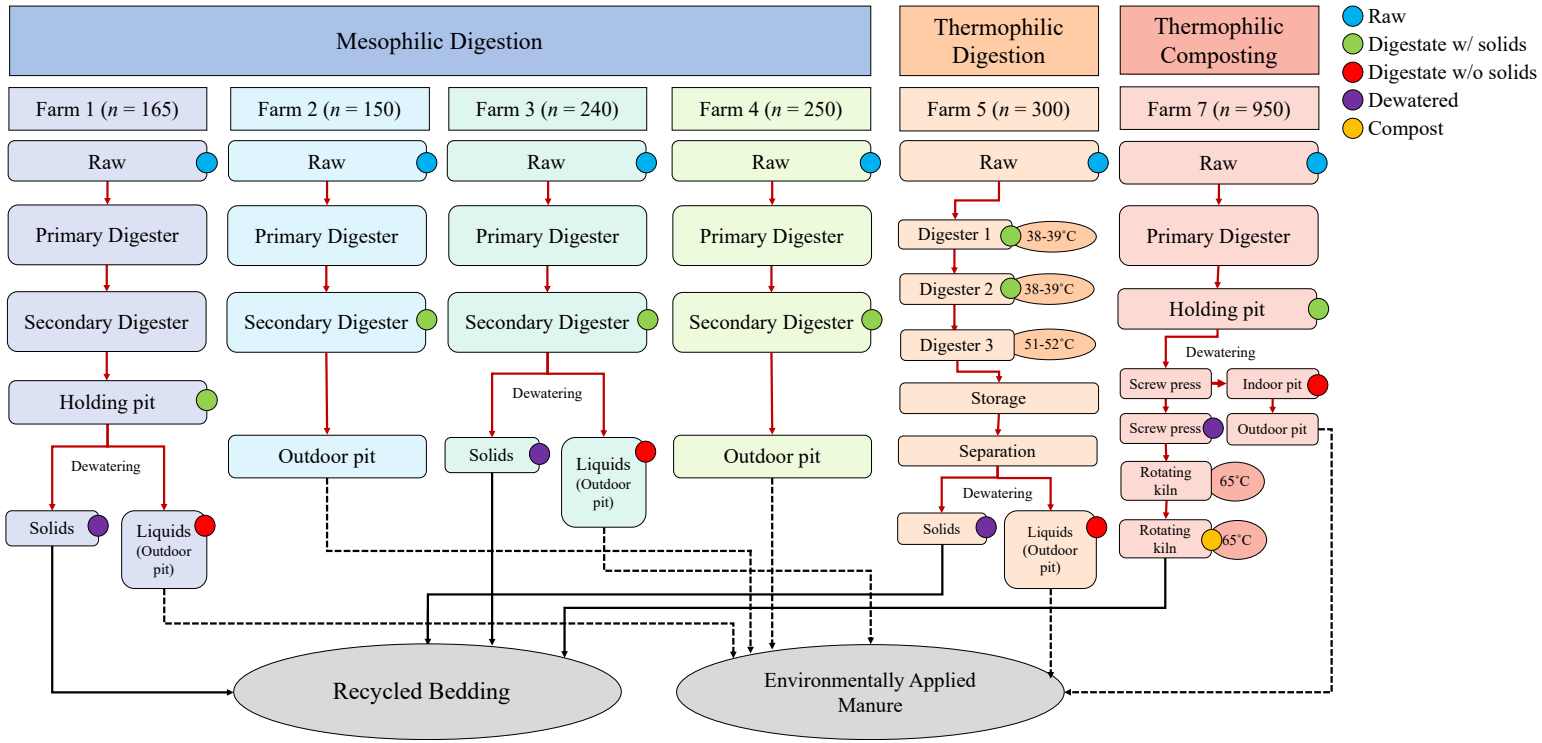

Figure 2.1 Flow[1] of manure treatment for each of the six farms from Southern Ontario sampled[2] for this study. Farms one through four use mesophilic temperatures (~35˚C) for anaerobic digestion, farm seven uses 65˚C for thermophilic composting in addition to mesophilic anerobic digestion and farm five uses thermophilic anaerobic digestion at 51-52˚C. Raw manure is exposed to the elements and is seasonal weather and temperature dependent. Co-products were added to primary digesters on each farm.

[1]Red arrows direct flow of manure to a proceeding process, black solid arrows indicate the final products destination being in the barn, black dashed arrows indicate the final product being applied to land as fertilizer

[2]The coloured circles in the top right corners of various manure treatment stages indicate where sampling occurred. The *n* values indicate the herd size on each farm.

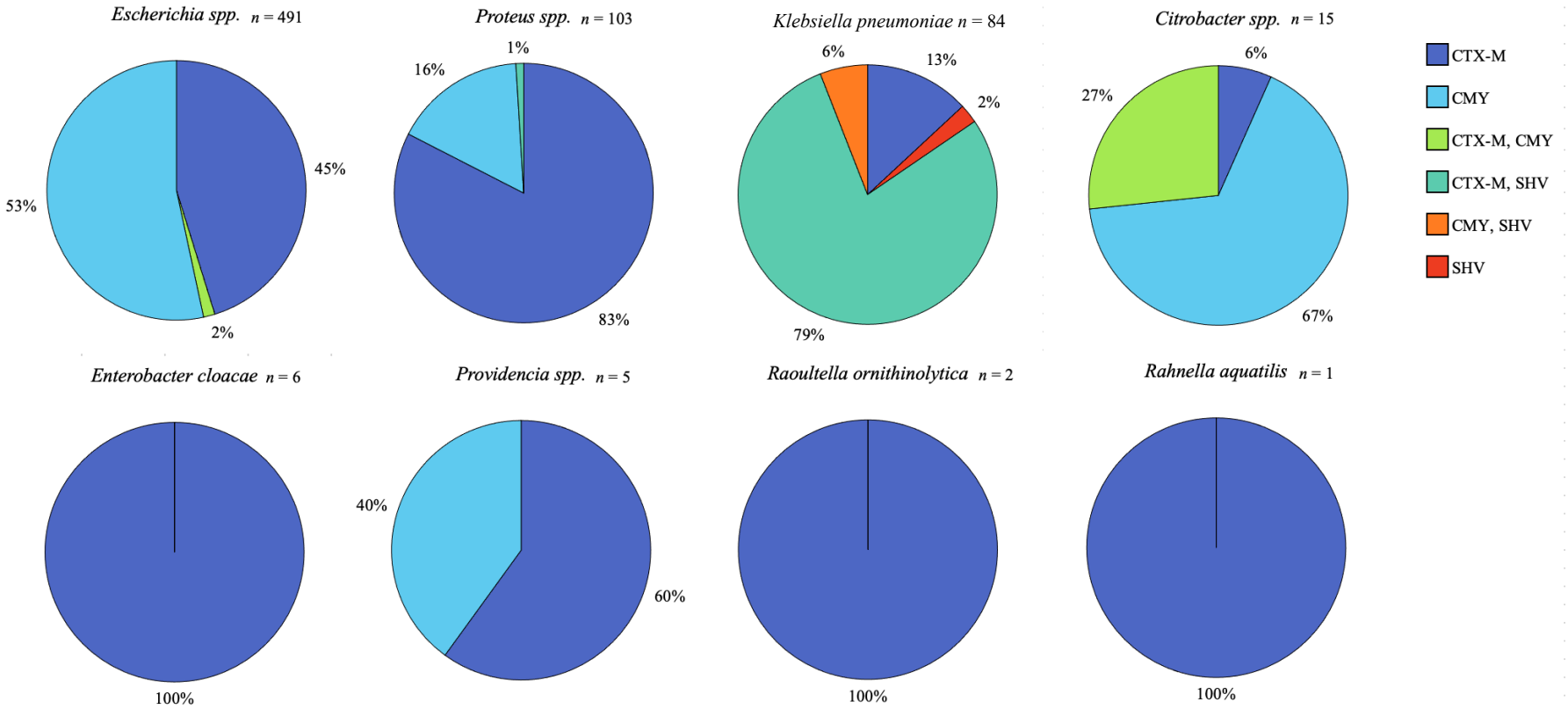

Figure 2.2 Prevalence of *Enterobacterales*[1,2] (*n* = 707) recovered at various manure treatment stages from six farms in Southern Ontario and distribution of ESC-resistance genes in which they carry.

[1]The *n* values represent the total number of each genus or species recovered from all samples, across all farms.

[2]For the genera with multiple species: *Escherichia spp*. includes *E. coli* (*n* = 493) and *E. fergusonii* (*n* = 1); *Proteus spp.* includes *P. mirabilis* (*n* = 73), *P. hauseri* (*n* = 17), *P. vulgaris* (*n* = 9) and *P. penneri* (*n* = 4); *Citrobacter spp.* includes *C. sedlakii* (*n* = 6), *C. braakii* (*n* = 4), *C. freundii* (*n* = 1), *C. gillenii* (*n* = 1), *C. koseri* (*n* = 1) and *C. youngae* (*n* = 1); *Providencia spp.* includes *P. rettgeri* (*n* = 4) and *P. stuartii* (*n* = 1).

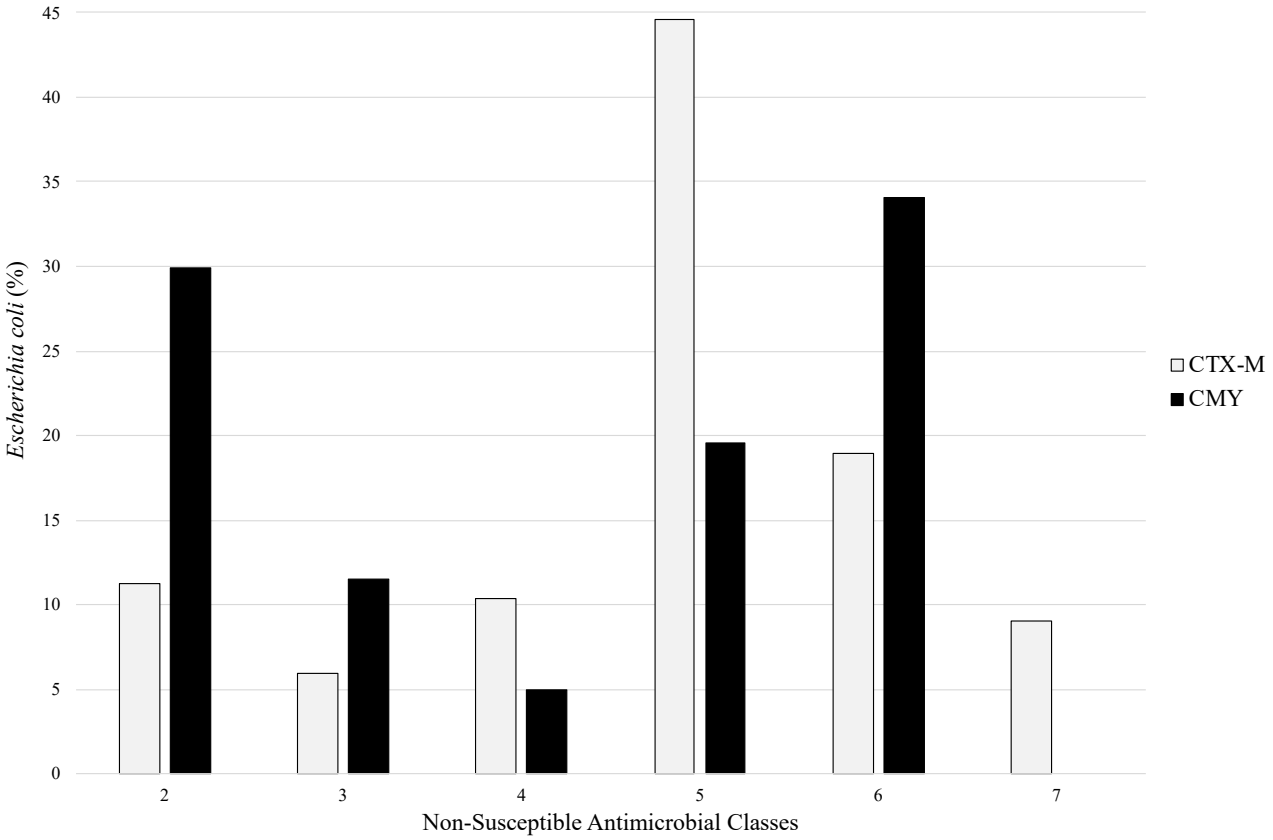

Figure 2.3 Antimicrobial resistance distribution[1,2] of *E. coli* carrying *bla*$_{CMY}$ or *bla*$_{CTX-M}$ from all sampled farms.

[1]Percentage of resistant *E. coli* were calculated based on totals for each group: *E. coli* carrying *bla*$_{CMY}$ (n = 261) and *E. coli* carrying *bla*$_{CTX-M}$ (n = 222).

[2]Number of antimicrobial classes with a resistant phenotype does not include zero, since those isolates would have been excluded during growth in selective media. Additionally, the scale does not include one class, since all the isolates were resistant to cephems (cefotaxime and cefoxitin) and other ESCs (ampicillin). Non-susceptible profiles include intermediate and resistant range.

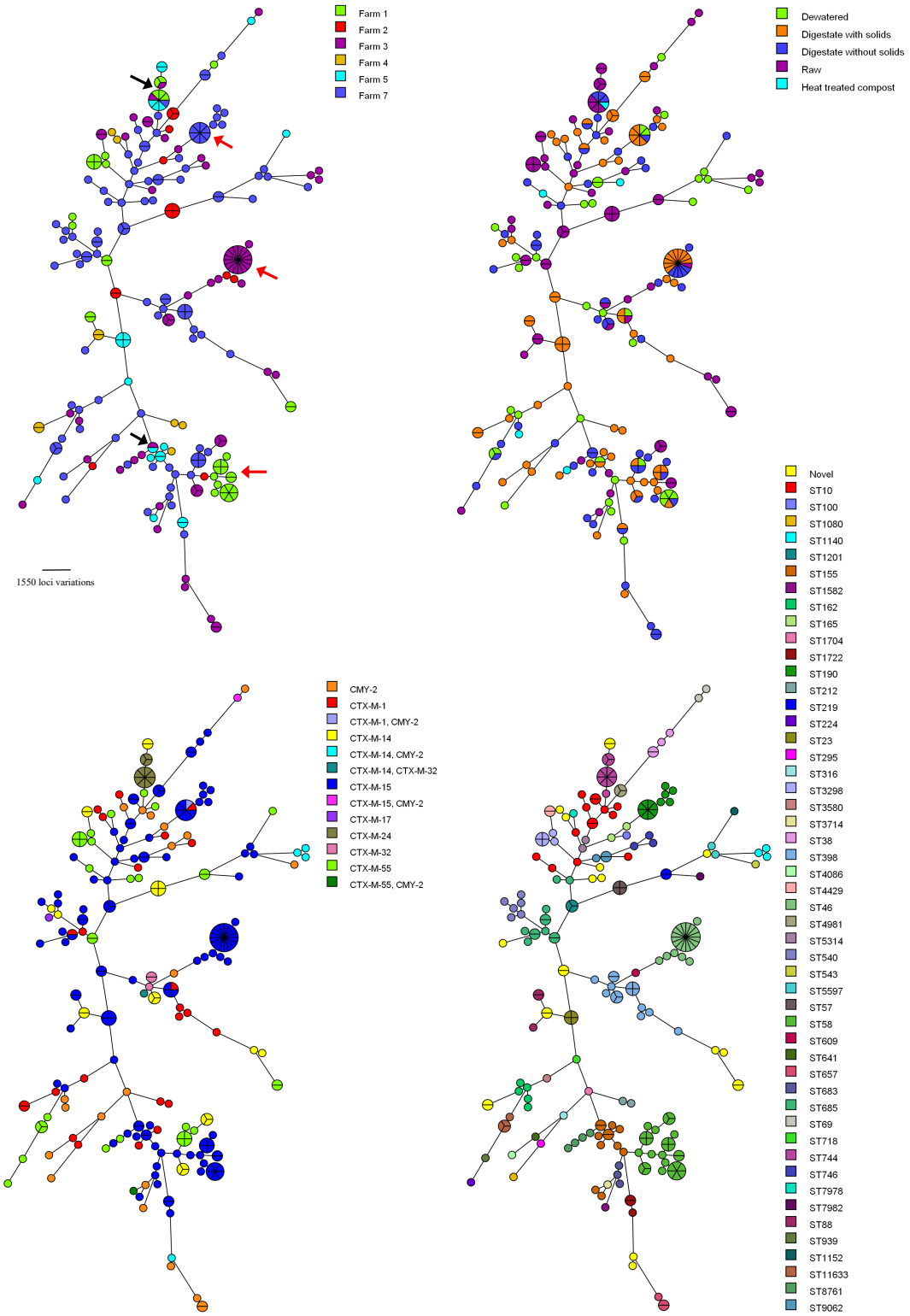

Figure 2.4 Minimum spanning trees using cgMLST comparison of 2,513 core genes among ESC-resistant *E. coli* carrying *bla*$_{\text{CTX-M}}$ and/or *bla*$_{\text{CMY}}$ Colour codes represent farm source, manure treatment source, ESC-resistance gene variant and MLST profile. Red arrows denote clusters of isolates from the same farm source, whereas black arrows denote clusters of isolates from different farm sources. Novel MLST profiles are further described in Table S2.2.

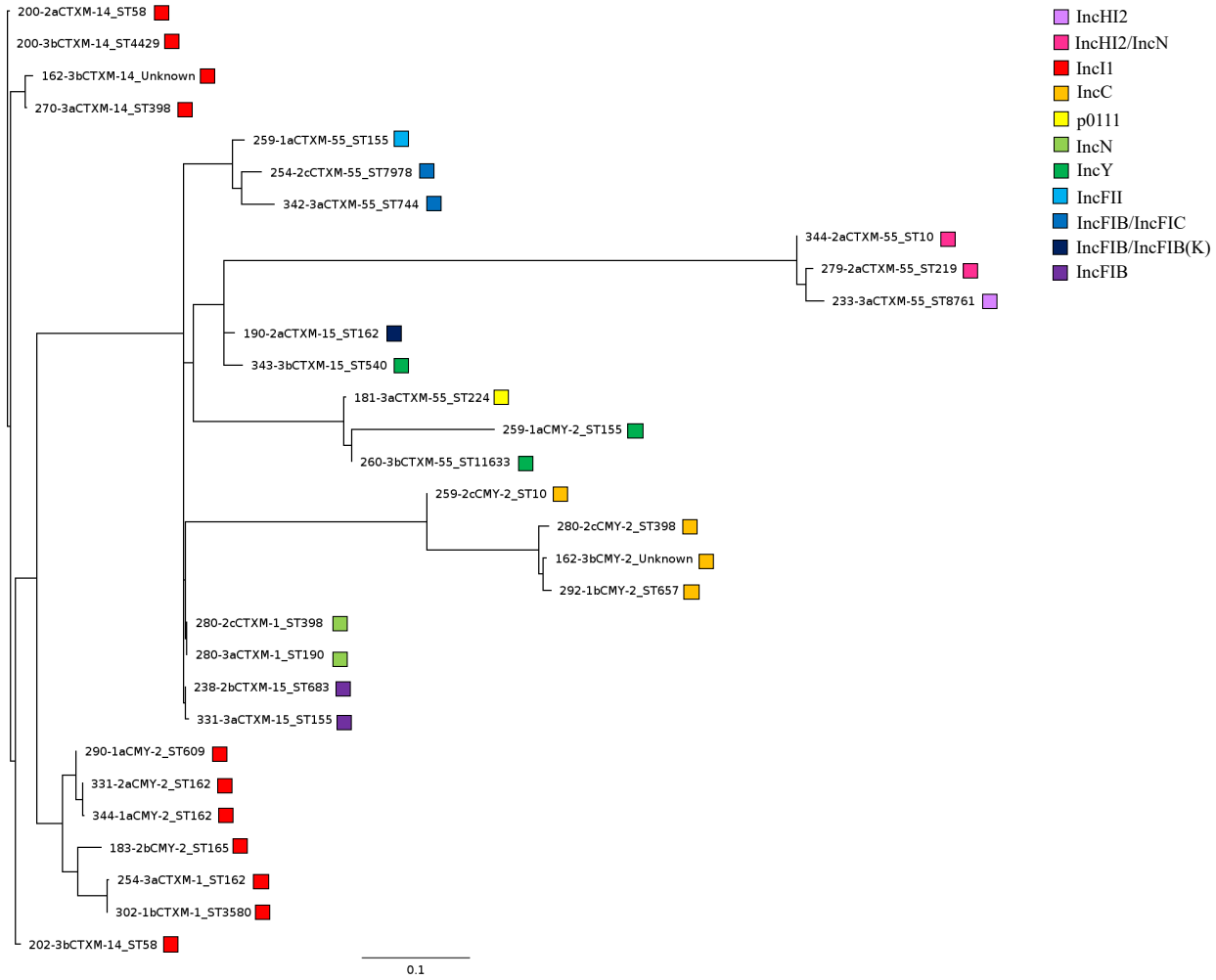

Figure 2.5 Similarity tree based on the presence and absence of 1,357 genes among ESC-resistance plasmids (*n* = 30) from *E. coli* isolates (*n* = 28) recovered from manure samples across two dairy farms in Southern Ontario. Coloured boxes to the right of each branch indicate the corresponding plasmid incompatibility groups. Each node includes the isolate ID, the ESC-resistance gene and variant, as well as the isolate MLST type.

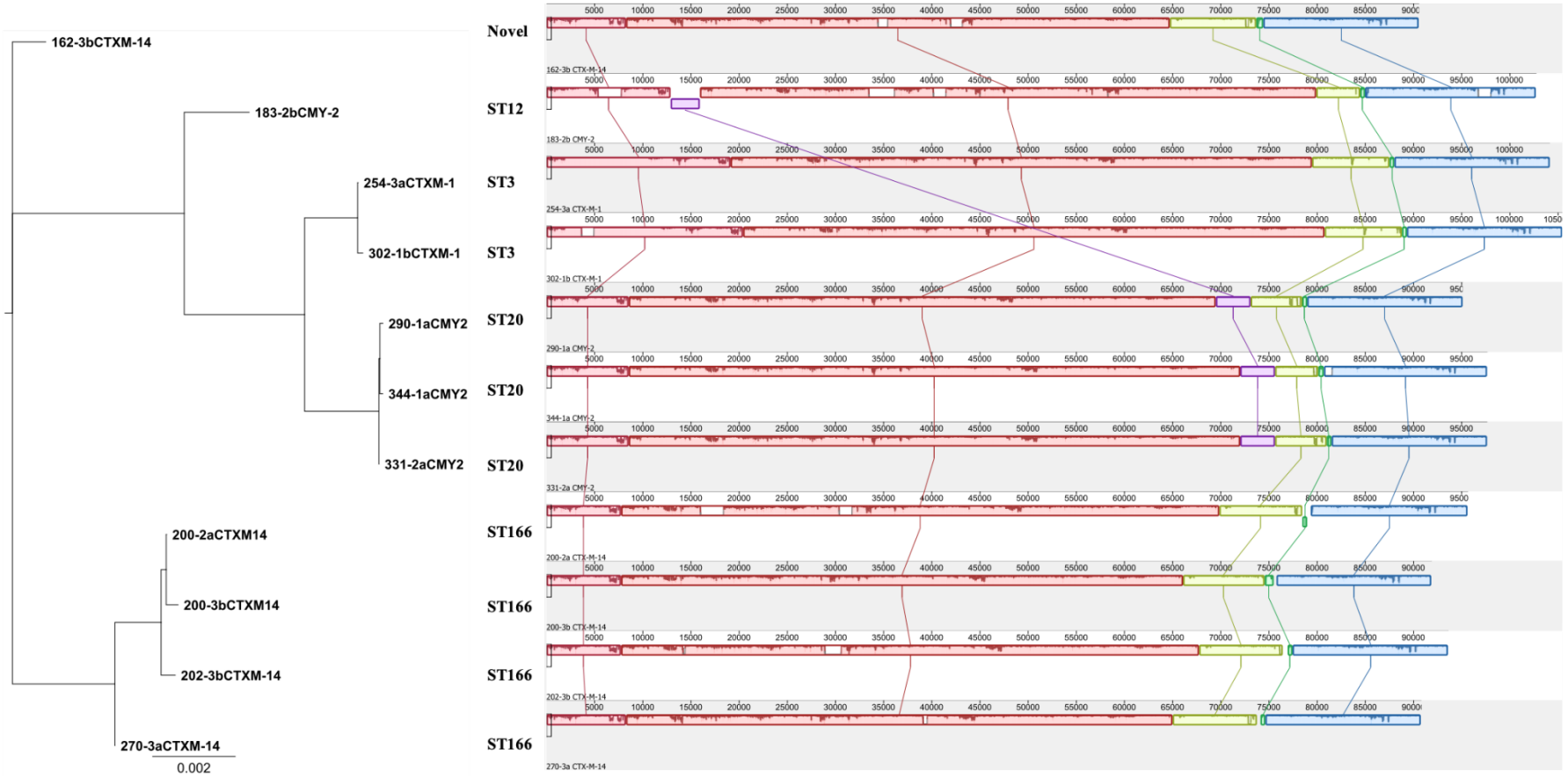

Figure 2.6 Phylogenetic maximum likelihood tree using SNP comparisons of IncI1 plasmids carrying *bla*$_{\text{CTX-M}}$ or *bla*$_{\text{CMY}}$ from *E. coli* using a total of 189 genes comprised of core genes (n = 63), shell genes (n = 90) and cloud genes (n = 36) on the left, pMLST profile in the middle and mauve alignments of the IncI1 plasmids on the right. Plasmid names are comprised of the isolate identification along with the ESC-resistance gene variant.

CHAPTER THREE: Extended-spectrum ß-lactamase-producing *Klebsiella pneumoniae* recovered from dairy manure in Southern Ontario, Canada

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ABSTRACT:

Extended-spectrum cephalosporin (ESC)-resistant *Klebsiella pneumoniae* from human clinical samples have been described in several publications, however, there is a paucity of information regarding ESC-resistant *K. pneumoniae* from livestock in general and in Canada in particular. This study investigated the epidemiology of ESC-resistant *K. pneumoniae* recovered from dairy manure and their ESC-resistance plasmids. *K. pneumoniae* isolates (n = 84) were previously screened by PCR for $bla_{\text{CTX-M}}$, bla_{CMY} and bla_{SHV} and underwent susceptibility testing using disk diffusion. Isolates carrying $bla_{\text{CTX-M}}$ (n = 74) underwent whole genome sequencing (WGS) using Illumina sequencing and a subset of these (n = 20) additionally with long read sequencing using Oxford Nanopore Technologies. Isolates were characterized based on core genomes, multi-locus sequence typing (MLST) and antimicrobial resistance (AMR) genes. Plasmids were characterized based on core gene SNPs, replicon types and AMR genes. For *K. pneumoniae*, a total of 25 known STs and four novel STs were identified. The most frequent CTX-M variant was $bla_{\text{CTX-M-15}}$ (n = 71), followed by $bla_{\text{CTX-M-1}}$ (n = 2) and $bla_{\text{CTX-M-32}}$ (n = 1). Isolates carrying $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-32}}$ were all ST3369, which in addition to ST2159, were the only two STs found on multiple farms. The $bla_{\text{CTX-M-15}}$ gene was located on replicons of the IncF, IncHI1 or IncY groups and on the chromosome, whereas $bla_{\text{CTX-M-1}}$ were harbored on the epidemic IncI1/ST3 plasmid. These results show a high diversity of ESC-resistant *K. pneumoniae* strains and ESC-resistant plasmids with a relatively low diversity of CTX-M variants in dairy manure.

3.0 INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen which is responsible for a range of human clinical infections. It is among the top five pathogens in Canadian hospitals (Zhanel et al., 2019). Common infections include nosocomial urinary tract infections (UTIs), septicemia, abdominal infections and bacterial pneumoniae (Meatherall et al., 2009; Vading et al., 2018). These bacteria belong to the ESKAPE group of pathogens and are a public health concern. An increase in the frequency of multi-drug resistant (MDR) *K. pneumoniae* strains have also been observed among human clinical infections (Denisuik et al., 2019; De Oliveira et al., 2020).

Extended-spectrum cephalosporin (ESC)-resistant *K. pneumoniae* strains are important for human health. *Enterobacterales,* including *K. pneumoniae*, are found in a variety of niches, including the feces of dairy and beef cattle, poultry, swine, horses and companion dogs (Munoz et al., 2006; Vikram and Schmidt, 2018; Kim et al., 2005; Mollenkopf et al., 2013; Vo et al., 2007; Marques et al., 2019). As a widespread opportunistic pathogen, *K. pneumoniae* is a cause of mastitis in dairy cattle, UTIs in dogs and a variety of clinical infections in horses (Massé et al., 2020; Pepin-Puget et al., 2020; Vo et al., 2007).

Recent studies have shown that ESC-resistance in *K. pneumoniae* is usually associated with $bla_{\text{CTX-M}}$, bla_{CMY} and the often intrinsic, bla_{SHV} (Wang et al., 2013; Denisuik et al., 2019; Ford and Avison 2004). The most frequently and globally identified variants of CTX-M in *K. pneumoniae* from both human and animal sources are CTX-M-14, -15 and -55 (Wyres et al., 2019; Hong et al., 2019; Melano et al., 2006; Lev et al., 2018).

The majority of research surrounding ESC-resistance has focused on *E. coli*. As a result, there is little literature regarding ESC-resistant *K. pneumoniae* from animal and environmental sources, including in Canada. Although *K. pneumoniae* are ubiquitous in the environment and may be playing an active role in dissemination of AMR genes through horizontal gene transfer (HGT) (Podshun and Ullman, 1998; Wyres and Holt, 2018), little is known about the potential role of *K. pneumoniae* in the circulation and transfer of ESC-resistance plasmids, particularly in dairy manure.

The objectives of this study were twofold. The first was to characterize ESC-resistant *K. pneumoniae* strains from dairy cattle in Canada and their *bla*$_{CTX-M}$-carrying plasmids. The second was to assess the distribution and persistence of *K. pneumoniae* strains and their *bla*$_{CTX-M}$-carrying plasmids through dairy manure treatments. These results as a whole will contribute to a better understanding of the epidemiology of ESC-resistance in *K. pneumoniae* within dairy manure in Southern Ontario and will provide a means of comparison for other Canadian regions.

3.1 MATERIALS AND METHODS

3.1.1 Sample processing and species identification

K. pneumoniae isolates (n = 84) were recovered from 34 of 164 dairy manure samples, from four of six farms investigated in Southern Ontario between November 2018 to August 2019 using the same methods as described in the previous chapter (Chapter 2, Section 2.1). Species identification was achieved using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) at the Animal Health Laboratory (AHL), University of Guelph, ON. All isolates were primarily identified as *K. pneumoniae* using MALDI-TOF, however further analysis using 16s rRNA read mapping identified eleven isolates as *Klebsiella quasipneumoniae* subspecies *quasipneumoniae*. These will be described accordingly throughout this study.

3.1.2 Characterization of ESC-resistant *K. pneumoniae*

K. pneumoniae (n = 84) isolates, as identified using MALDI-TOF, were screened using polymerase chain reaction (PCR) for *bla*$_{CTX-M}$, *bla*$_{CMY}$, and *bla*$_{SHV}$ as previously described (Chapter 2, Section 2.1). Their antimicrobial susceptibility profiles were determined using the disk diffusion method, following guidelines and interpretation criteria from CLSI (CLSI 2018; CLSI 2019) for cefoxitin (FOX), cefotaxime (CTX), ceftazidime (CAZ), ertapenem (ETP), gentamicin (GEN), ampicillin (AMP), amoxicillin-clavulanic acid (AMC), sulfonamide (SUL), sulfamethoxazole-trimethoprim (SXT), tetracycline (TET), streptomycin (STR), kanamycin (KAN), ciprofloxacin (CIP) and chloramphenicol (CHL).

Multi-drug resistance was defined as resistance to one or more antimicrobials from three or more antimicrobial classes, not including intermediate phenotypes (Chapter 2, Section 2.1).

Classes were categorized as follows: aminoglycosides (KAN, GEN and STR), tetracyclines (TET), quinolones (CIP), folate pathway inhibitors (SUL and SXT), phenicols (CHL), cephems (CTX, CAZ and FOX), ß-lactam/ß-lactamase inhibitor combinations (AMC), carbapenems (ETP) and other ß-lactams (AMP). AMC was not considered in the determination of multi-drug resistance since carrying *bla*$_{CMY}$ would naturally result in multi-drug resistance (i.e. resistance to cephems, other ß-lactams, and ß-lactam/ß-lactamase inhibitor combinations).

3.1.3 Illumina sequencing

K. pneumoniae (*n* = 63) and *K. quasipneumoniae* (*n* = 11) carrying *bla*$_{CTX-M}$, underwent whole genome sequencing (WGS). DNA were extracted using Epicentre MasterPure^{TM} DNA Purification kit (Epicentre, Madison, WI) following manufacturer's instructions. Sequencing was done with Illumina Miseq (PE300) (*n* = 20) (Illumina, San Diego, CA) at the Advanced Analysis Centre, University of Guelph, Guelph, ON, or Illumina NextSeq (PE150) (*n* = 54) (Illumina, San Diego, CA) at the National Microbiology Laboratory in Winnipeg, MB, (NML Winnipeg).

3.1.4 MinION sequencing

A subset of isolates carrying *bla*$_{CTX-M}$ from dairy manure (*n* = 20), including two *K. quasipneumoniae*, were selected for sequencing using the MinION (Oxford Nanopore Technologies). Isolates were strategically selected using a phenetic tree created with the inhibition zone diameters obtained from antimicrobial susceptibility testing. This tree was created in BioNumerics v7.6 (Applied Maths, Austin TX. USA) using cluster analysis with Pearson correlation coefficient and the unweighted pair group method with arithmetic mean (*data not shown*). One or two isolates were selected from each cluster defined at a 94% cut off, with a goal of including the highest diversity of strains from a diversity of time periods and treatment processes. DNA extractions for these isolates were the same as above. Sequencing libraries and barcoding preparation were done using the SQK-LSK109 and EXP-NBD104/114 ligation and native barcoding kits (Oxford Nanopore Technologies) according to the manufacturer's instructions. Three flow cells were used (version FLO-MIN106 R9.4) and run for 48h each. Basecalling of fast5 files and demultiplexing was performed using Guppy Basecaller v3.3 (Oxford Nanopore Technologies) with barcode trimming enabled.

3.1.5 Sequencing analysis

Whole genome sequences of *K. pneumoniae* and *K. quasipneumoniae* with short reads only, were assembled in BioNumerics v7.6 (Applied Maths, Austin TX. USA) using the SPAdes *K. pneumoniae de novo* assembler, and assembly-free and assembly-based allele calling. Resistance genes and plasmid markers were identified using the *E. coli* functional genotype plug-in for BioNumerics at a 90% similarity which utilizes ResFinder and PlasmidFinder databases from the Center for Genomic Epidemiology, Technical University of Denmark, DTU. Multi-locus sequence types (MLSTs) were manually assigned using a MLST finder database from Center for Genomic Epidemiology, Technical University of Denmark, DTU (https://cge.cbs.dtu.dk/services/MLST/) with paired end Illumina reads. Any isolates with an unknown sequence type (ST) due to a novel allele or novel allelic configuration, were thoroughly analyzed to identify any errors in allelic regions by reference mapping of short reads. If no errors were present, and no ST was assigned, these strains were denoted as novel STs and given a temporary name for this study (Table S3.1). Additionally, all *K. quasipneumoniae* isolates were given temporary STs for this study using the MLST scheme for *K. pneumoniae* as a foundation for differentiation (Table S3.2). Minimum spanning trees (MSTs) were generated using the wgMLST (core enterobase) function for cgMLST trees and wgMLST (all loci) function for wgMLST trees in BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium) with 10x bootstrapping and no multithreading using the *Klebsiella pneumoniae* WGS scheme, using 634 core loci and 19,729 whole genome loci, respectively.

3.1.6 Plasmid assembly and analysis

Long and short reads were assembled using hybrid assembler Unicycler v0.4.8 (Wick et al., 2017), in parallel with Flye v2.6 (Lin et al., 2016) and were polished with short reads using Racon v1.4.0 (Vaser et al., 2017) and Pilon v1.23 (Walker et al., 2014). Unicycler assemblies were used for all downstream analysis. Assemblies were visualized using Bandage v0.8.1 (Wick et al., 2015) and annotated with Abricate (Seeman T, ResFinder; PlasmidFinder; VFDB). Sequenced genomes were analyzed using Geneious v9.1.8 (Biomatters, Auckland, New Zealand) and plasmids were aligned and visualized using Mauve plug-in v2.3.1 (Darling et al., 2004).

3.1.7 Phylogenetic analysis

Core gene phylogenetic analyses were performed on the whole genomes of *K. pneumoniae* isolates (*n* = 63) and on *K. quasipneumoniae* isolates (*n* = 11), separately. These analyses were completed using Snippy v4.4.5 according to developer's guidelines, under Core SNP Phylogeny (https://github.com/tseemann/snippy) with *K. pneumoniae* type strain ATCC 13883 (GenBank Accession #JOOW00000000.1) and *K. quasipneumoniae* subsp. *quasipneumoniae* strain 01A030 (GenBank Accession #GCA_000751755.1) as reference strains. The clean.full.aln files were analyzed with Gubbins v2.4.0 (Croucher et al., 2014) and the output clean.core.aln file was analyzed with SNP-sites v3.0 (Page et al., 2016). FastTree v2.2.11 (Price et al., 2010) was used to generate the trees which were analyzed in Geneious v9.1.8.

Phylogenetic SNP analyses were performed on the hybrid assembled, ESC-resistance plasmids using a pre-determined protocol (Cormier et al., *manuscript in preparation*). A gene presence/absence analysis was first conducted on all ESC-resistance plasmids independently of replicon types. The ESC-plasmids (*n* = 18) were analyzed with Prokka v1.14.6 (https://github.com/tseemann/prokka) and the output .gff files were analyzed with Roary v3.13.0 (Page et al., 2015). A SNP analysis was then performed on plasmids of the same replicon type. The same steps previously mentioned with Prokka and Roary were used, however SNP-sites were run on the core.gene.alignment output file from Roary. The SNP sites (IncFII plasmids: 17641, 19084, 19231, 21520; IncY plasmids: 15670, 16502, 17314, 17655), along with the core.gene.alignment file, were used to create a maximum likelihood tree with IQtree v2.0.3 (Minh et al., 2020). All trees were visualized in Geneious.

3.2 RESULTS

3.2.1 Antimicrobial susceptibility and multi-drug resistance

Based on the susceptibility testing of the *Klebsiella* spp. isolates carrying $bla_{\text{CTX-M}}$ (62/74 isolates), 83.7% were MDR. Of the ß-lactams tested, resistance to CTX (100%) and AMP (100%) and CAZ (90.5%) were common. Of these isolates 48.6% conferred an intermediate phenotype to AMC whereas only one isolate (1.3%) was fully resistant. No resistances to ETP or FOX were observed (Fig. 3.1). The most frequent resistances other than to ß-lactams were to

TET (77.0%), STR (63.5%), SUL (55.4%) and SXT (52.7%). The least common resistances were to CHL (9.5%), KAN (6.75%) and GEN (2.7%). No isolates were fully resistant to CIP, however 27.0% had an intermediate susceptibility profile.

Isolates carrying *bla*$_{CMY}$ (*n* = 5) were not investigated with WGS but did undergo susceptibility testing to the previously mentioned antimicrobials, and three of these were MDR. All isolates were resistant to CTX, CAZ, FOX, AMC and AMP. An increase in resistance to SUL, SXT, CHL and a decrease in resistance to KAN, GEN, STR, TET and CIP compared to isolates carrying *bla*$_{CTX-M}$ from dairy cattle were observed (Fig. 3.1). Of the remaining five ESC-resistant *K. pneumoniae* isolates recovered from dairy manure in our library two carried *bla*$_{SHV}$ only and the other three carried *bla*$_{CTX-M,}$ however the latter isolates were excluded from WGS analysis due to quantity of reads.

3.2.2 Recovery of *K. pneumoniae* carrying *bla*$_{CTX-M}$

Each of the four farms (two, three, five and seven) in which *K. pneumoniae* were recovered use different manure treatment pipelines (shown in the previous chapter Fig. 2.1). Recovery of isolates did not show a clear trend, neither increasing or decreasing through treatments, due to a low and inconsistent recovery rate. On farm two, ESC-resistant isolates were recovered in raw manure (*n* = 1/6) and digestate with solids (*n* = 2/6); farm three digestate with solids (*n* = 1/8) and digestate without solids (*n* = 1/8); farm five raw manure (*n* = 1/8) and dewatered manure (*n* = 1/7); farm seven raw manure (*n* = 8/8), digestate with solids (*n* = 7/8), digestate without solids (*n* = 4/8), dewatered manure (*n* = 7/8) and heat-treated compost (*n* = 1/8), with the proportion of positive samples in parenthesis. However, environmentally applied manure samples (digestate without solids farm three and seven) had slightly more positive samples than recycled bedding (dewatered farm three and heat-treated compost farm seven), with 31.2% (5/16) and 12.5% (2/16), respectively.

3.2.3 ESC-resistance gene diversity

K. pneumoniae (including *K. quasipneumoniae*) carrying *bla*$_{CTX-M}$ were recovered from four out of six farms, with farm seven having the highest recovery (27/40 samples) then farm two (3/12 samples), farm three (2/31 samples) and farm five (2/39 samples). Three *bla*$_{CTX-M}$

variants were identified using WGS, including *bla*$_{\text{CTX-M-15}}$ (*n* = 71), *bla*$_{\text{CTX-M-1}}$ (*n* = 2) and *bla*$_{\text{CTX-M-32}}$ (*n* = 1). Of the 71 isolates carrying *bla*$_{\text{CTX-M-15,}}$ 64 also carried *bla*$_{\text{TEM-1}}$, whereas the isolates carrying *bla*$_{\text{CTX-M-1}}$ or *bla*$_{\text{CTX-M-32}}$ did not (Table 3.1). All *K. pneumoniae* isolates carried a *bla*$_{\text{SHV}}$ gene, whereas the *K. quasipneumoniae* all carried *bla*$_{\text{OKP}}$. The *bla*$_{\text{SHV}}$ genes variants identified were *bla*$_{\text{SHV-1}}$ (*n* = 12), *bla*$_{\text{SHV-11}}$ (*n* = 24), *bla*$_{\text{SHV-25}}$ (*n* = 3), *bla*$_{\text{SHV-27}}$ (*n* = 4), *bla*$_{\text{SHV-28}}$ (*n* = 3), *bla*$_{\text{SHV-37}}$ (*n* = 1), *bla*$_{\text{SHV-60}}$ (*n* = 1), *bla*$_{\text{SHV-76}}$ (*n* = 1), *bla*$_{\text{SHV-119}}$ (*n* = 7) and novel variants (*n* = 7) (Table 3.1 and Table S3.1). Of these, the only known extended-spectrum ß-lactamase (ESBL) is SHV-27.

3.2.4 Strain diversity

Twenty-five STs were identified through multi-locus sequence typing (MLST) (Fig. 3.2 and Table 3.1). Five isolates were novel STs, with two of them being the same novel ST (Table S3.1). The eleven *K. quasipneumoniae* were of two different unknown STs (Table S3.2). Isolates of only two STs were found on more than one farm, ST2159 and ST3369 (Fig. 3.2). The most frequently recovered STs from dairy manure were all from farm seven, ST278 (6 samples), ST183 (4 samples) and ST219 (4 samples) (Table 3.1). Many other STs were only identified in one isolate, ST34, ST37, ST163, ST286, ST405, ST550, ST791, ST914, ST945, ST1655, ST2572 and ST4263 (Table 3.1).

3.2.5 Isolate relatedness between farm sources

The genomic similarities of isolates were visualized using both cgMLST and core genome SNP analysis (Fig. 3.2 and Fig. 3.3). Clusters of isolates originating from several samples, and therefore not likely to be replicates, are highlighted in Fig. 3.3. Clusters in both trees are supportive of one another, with the SNP analysis being more discriminatory. There are two clusters of isolates which are found on more than one farm (Fig. 3.2). The first cluster contains three ST2159 isolates from farm five, 246-3a and 246-3b, and farm seven 280-1b (dark purple cluster, Fig. 3.3). The other cluster includes two ST3369 isolates, 269-2e and 269-3b, from farm three which carry *bla*$_{\text{CTX-M-1}}$ and one from farm seven, 346-3b, which carries the closely related *bla*$_{\text{CTX-M-32}}$ (dark green cluster, Fig. 3.3). The three ST2159 isolates are closely related, whereas the ST3369 isolates are distinguishable by farm source (Fig. 3.2 and Fig. 3.3).

3.2.6 Strain persistence through dairy manure treatments

Using core genome SNP analysis on all sequenced *K. pneumoniae* isolates, excluding the eleven *K. quasipneumoniae*, ten tight clusters are evident (Fig. 3.3). Some of these clusters contain isolates from the same farm, but from different treatment stages and sampling periods such as the clusters highlighted in dark blue, orange, yellow, light green, red and violet, which are comprised of two STN3 isolates and one ST37 isolate in the first cluster followed by ST278, ST183, ST1966, ST706 and ST469, respectively. The two STN3 and one ST37 isolate in the dark blue cluster, along with the three ST1966 isolates in the light green cluster were recovered from the extremities of manure treatment, spanning the entire treatment process (Fig. 3.2 and Fig. 3.3). The dark blue cluster and the violet cluster containing two ST469 isolates are more closely related in the SNP core genome tree compared to the cgMLST trees, with the remaining clusters being identical in both trees (Fig. 3.3 and Fig. 3.2). The light blue cluster in Fig. 3.3 is comprised of three identical ST5108 isolates recovered from raw manure only, at different sampling periods (Fig. 3.2). Additionally, the magenta cluster in Fig 3.3, is made of seven isolates from farm seven with different sequence types, ST107 (n = 2) and ST219 (n = 5), recovered from various stages of the treatment process (Fig. 3.3). Within this cluster, two ST219 are recovered from heat treated compost and are distinguishable from the other three ST219 isolates recovered from digestate with solids and dewatered manure. However, similar to the previously mentioned cluster with two different ST profiles, this cluster is not as tight as those containing only one ST.

Although the eleven *K. quasipneumoniae* isolates were excluded from the Fig. 3.3, they were included in a separate core SNP analysis in reference to a *K. quasipneumoniae* subsp. *quasipneumoniae* strain 01A030. One *K. quasipneumoniae* isolate (280-3b) is distinguishably less related to the other ten in cgMLST MSTs (Fig. 3.2) and may be a different strain. This observation is supported by a different MLST allelic profile, with this isolate carrying two variants of *rpoB*, in contrast to the other ten isolates which only carry one *rpoB* with the same variant (Table S3.2). Despite this difference, the eleven isolates appear closely related based on SNP proportions in core loci (Table S3.3). These *K. quasipneumoniae* isolates were recovered from three treatment stages on farm seven including raw, digestate with solids and dewatered,

none of which are environmentally applied manure (Fig. 3.2). In addition, isolate 280-3b was the only isolate recovered from the anaerobically digested manure, digestate with solids (Fig. 3.2).

3.2.7 Plasmid diversity

A subset of isolates were sequenced with both long and short reads to enable ESC-resistance plasmid assembly (Table 3.2). These plasmids carried *bla*$_{\text{CTX-M-15}}$ on IncY (*n* = 6), IncFII (*n* = 5), IncFIB(K) (*n* = 2), IncFIB/IncHI1 (*n* = 1), IncFIB(K)/IncFII (*n* = 1) and a cointegrated IncR, IncFIA, Col (*n* = 1) (Table 3.2). Two of the isolates harbouring IncY plasmids carrying *bla*$_{\text{CTX-M-15}}$ were *K. quasipneumoniae*. Plasmid phylogeny using the presence and absence of genes shows the correlation between clusters and replicon types (Fig. 3.4). IncF plasmids were dominant and are dispersed throughout the tree (Fig. 3.4). Two *K. pneumoniae* isolates carried *bla*$_{\text{CTX-M-1}}$ on IncI1/ST3 plasmids and two *K. pneumoniae* isolates carried *bla*$_{\text{CTX-15}}$ on the chromosome. The two isolates carrying *bla*$_{\text{CTX-M-15}}$ on the chromosome (302-2b and 304-2b) were both ST219 from farm seven in June, from two different treatment processes. Additionally, they both carried *oqxB*, *oqxA*, *bla*$_{\text{SHV-1}}$, *mdf(A), fos* on their chromosomes and their core genomes had no SNP differences (Fig. 3.3). ISEcp1 insertion sequences were located upstream the *bla*$_{\text{CTX-M-15}}$ on both chromosomes, with no plasmid sequences flanking either ISEcp1 or *bla*$_{\text{CTX-M-15}}$. These are likely to be the same strain which has been recovered at two different treatments on the same sampling date.

3.2.8 Plasmid distribution in manure samples

The IncY (*n* = 6) and IncFII (*n* = 5) plasmids from isolates recovered in dairy cattle manure were further characterized based on core gene SNP analysis based on the proportion of SNP substitutions per site in core plasmid loci and Mauve alignments (Fig. 3.5 and Fig. 3.6). The IncY plasmids are from two different dairy farms. Two of these plasmids were from *K. quasipneumoniae* isolates from farm seven raw manure. Three other IncY plasmids were recovered from dewatered samples on both farms with very similar plasmids from two different strains, ST163 (238-1c) and ST4263 (343-2b), recovered in February and August, respectively (Fig. 3.6). These plasmids are the closest related of the IncY plasmids and are only dissimilar by 2.0 x 10^{-6} SNP substitutions per site. The plasmid recovered from an ST2159 (246-3a) in farm five is very similar to the two previously mentioned IncY plasmids with 3.0 x 10^{-6} SNP

substitutions per site to both replicons (Fig. 3.5 and Table S3.4). An IncY plasmid from *K. quasipneumoniae* isolate 279-3b is the least similar to the other IncY replicons with an average of 4.0 x 10^{-5} SNP substitutions per site (Table S3.4). Using mauve alignments, the IncY plasmids appear to be more dynamic with regards to potential recombinational events, as well as indels compared to the IncFII plasmids (Fig. 3.5 and 3.6)

The IncFII plasmids carried by isolates recovered from farm seven spanned the one-year sampling period, from December 2018 to October 2019 (Fig. 3.6). Isolates 187-1f (ST278) and 187-2g (ST5108) were recovered in raw manure from December 2018 and are dissimilar by only 9.0 x 10^{-6} SNP substitutions per site (Table S3.5). Whereas 345-1b (ST706) and 347-2b (ST183) were recovered from raw manure and digestate without solids, respectively, in October 2019 and are dissimilar by 3.0 x 10^{-6} SNP substitutions per site (Table S3.5). Isolate 261-1b (ST219) was recovered in thermophilically treated compost from a sample in March and appears to be the least related by an average of 2.6 x 10^{-4} SNP substitutions per site to the other IncFII plasmids (Fig. 3.6 and Table S3.5). Using mauve alignments, no recombinations or indels were observed amongst these plasmids, with the exception of 347-2b which has an insertion the other plasmids do not (Fig. 3.6)

3.3 DISCUSSION

Very little is known about the epidemiology of ESC-resistant *K. pneumoniae* in animals in Canada, despite the increase in human clinical infections caused by these bacteria (Denisuik et al., 2019). There is potential for transmission of ESC-resistance strains and their determinants between humans and animals, through close proximity or consumption of contaminated food sources via agricultural amendment or meat products (Hong et al., 2019; Marques et al., 2019; Gekenidis et al., 2020; Marti et al., 2013; Casella et al., 2017). Therefore, the main objectives of this study sought to further characterize ESC-resistant *K. pneumoniae* isolates in dairy cattle manure.

3.3.1 Recovery of *K. pneumoniae* carrying bla_{CTX-M} in manure treatments

The majority of isolates were from farm seven and were recovered at every treatment stage. In contrast, recovery on the other farms were sporadic and no isolates were recovered at all

in two of the six farms. This suggests that *K. pneumoniae* carrying *bla*$_{CTX-M}$ may not be endemic to all farms or are at least present in low concentrations. Overall, due to the low recovery rate of *K. pneumoniae* and *K. quasipneumoniae* isolates, it is difficult to assess the effectiveness manure treatments have on their prevalence and furthermore extensive analyses would be needed for this.

3.3.2 Susceptibility phenotypes and multi-drug resistance

A large proportion of *K. pneumoniae* isolates were MDR, 83.7% and 60% carrying *bla*$_{CMY}$ or *bla*$_{CTX-M}$, respectively. Of the ESC-resistant *Klebsiella* spp. isolates carrying *bla*$_{CMY}$ or *bla*$_{CTX-M}$ combined, 82.2% (65/79) are MDR, compared to 79% of ESC-resistant *E. coli* (388/491) from the same manure samples (Chapter 2, Section 2.2). The majority of phenotypic resistances were to TET, STR, SXT and SUL, which are some of the most commonly prescribed antimicrobials in veterinary medicine, including dairy cattle (Saini et al., 2012). This followed the same trend as in *E. coli* from the same samples (Chapter 2, Section 2.2). In ESC-resistant *Klebsiella*, the least common resistances were to CIP, CHL, KAN and GEN. Except for reduced susceptibility to CIP (intermediate range only) which seems more frequent in *Klebsiella* (27%) than in *E. coli* (3.4% resistant range only, 4.4% intermediate + resistant range), the frequency of resistance to these latter antimicrobial agents were similar in both species (Chapter 2, Section 2.2). Worth emphasizing, the proportion of *Klebsiella* which are non-susceptible to ciprofloxacin were still within intermediate ranges and have not yet crossed into the fully resistant category (Fig 3.1). All ESC-resistant isolates were susceptible to ETP, indicating the frequency of carbapenemase resistance is low or non-existent. This was expected since these critically important and expensive antimicrobials are normally not used in farm animals.

3.3.3 ESC-resistance gene variants

Three variants of *bla*$_{CTX-M}$ were identified, all belonging to enzyme group one. The most commonly recovered variant is *bla*$_{CTX-M-15}$, which is known to be widespread globally in *K. pneumoniae* from both human and veterinary clinical infections (Abbassi et al., 2008; Coelho et al., 2010; Lee et al., 2011; Haenni et al., 2011; Ewers et al., 2014). Similar to what has been observed in *E. coli* from the same manure samples, the *bla*$_{CTX-M-15}$ was often carried on the same plasmid as *bla*$_{TEM-1}$ (Chapter 2, Section 2.2; Tran et al., 2021). The overwhelming prevalence of *bla*$_{CTX-M-15}$ and the infrequent recovery of *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-32}$ among the CTX-M-positive

isolates suggest a generally low diversity of *bla*$_{\text{CTX-M}}$ variants in *K. pneumoniae* from dairy manure. This seems to contrast with the situation in *E. coli* (Chapter 2, Section 2.2). However, since the majority of isolates originated from one of the farms, studies on more farms would be needed to confirm this trend.

K. pneumoniae intrinsically carry *bla*$_{\text{SHV}}$ variants, which are typically *bla*$_{\text{SHV-1}}$ or *bla*$_{\text{SHV-11}}$, which are non-ESBL (Matthew et al., 1979; Bush et al., 1995). However, in rare cases the variant is an ESBL. This was the case in four isolates of two STs, ST1966 (n = 3) and ST914 (n = 1) carrying *bla*$_{\text{SHV-27,}}$ which were recovered from farm seven. These STs are not closely related, with the three ST1966 clustered tightly together (bright green cluster Fig. 3.3) and the singular ST914 (342-1b) opposite of the tree (Fig. 3.3). Since the prevalence of ESBL SHV variants is low in dairy manure niches, it may not be a cause of immediate concern, but some monitoring may be warranted.

3.3.4 Isolate diversity

There appears to be extensive strain and plasmid diversity within *Klebsiella* from dairy manure. The majority of *bla*$_{\text{CTX-M}}$ genes were carried on plasmids, however two isolates carried their *bla*$_{\text{CTX-M-15}}$ on the chromosome. In both cases, the chromosomal integration was due to an ISEcp1 insertion sequence and not integration of an entire plasmid. This insertion sequence is frequently associated with the movement and mobilization of *bla*$_{\text{CTX-M-15}}$ (Karim et al., 2001) and is not entirely surprising.

K. pneumoniae strains frequently associated with human clinical infections globally, include ST11, ST15, ST37, ST147, ST258, ST405 or ST512 (De Oliveira et al., 2020; Navon Venezia et al., 2017; López-Camacho et al., 2018). Of these, ST37 (raw manure) and ST405 (anaerobically digested manure) were identified in this study. ST37 is suspected to have spread globally through importation and trade and is considered a highly virulent strain (Zurfluh et al., 2015). The low frequency of these *K. pneumoniae* strains in dairy cattle manure is reassuring, but again this warrants further monitoring.

3.3.5 Plasmid diversity

The majority of plasmids carrying *bla*$_{\text{CTX-M-15}}$ were replicon type IncF and were diverse (Fig. 3.4). This is similar to what is found in *E. coli* from the same manure samples, with highly diverse IncF plasmids carrying *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-55}}$ (Chapter 2, Section 2.2). Similar IncF plasmids were found in multiple strains, suggesting these plasmids are mobile within the farm and manure treatment environments. This information suggests IncF plasmids carrying *bla*$_{\text{CTX-M-15}}$ may be an important driving force of *bla*$_{\text{CTX-M}}$ dissemination within dairy manure.

The only two isolates carrying *bla*$_{\text{CTX-M-1}}$ harbour the gene on the previously described epidemic plasmid *bla*$_{\text{CTX-M-1}}$/IncI/ST3 (Carattoli et al., 2018). This same plasmid is also present in *E. coli* from the same manure samples (Chapter 2, Section 2.2). This plasmid may have recently been introduced to the dairy cattle environment in Canada or Ontario, as indicated by its low frequency in both *K. pneumoniae* and *E. coli.* It will be worth monitoring the evolution of its spread in animal populations.

3.3.6 Strain persistence and plasmid distribution

Out of the 25 STs identified for *K. pneumoniae*, two were found on more than one farm. Of these, ST2159 carried an IncY plasmid (246-3a) which was highly similar to other IncY plasmids in different STs (Fig. 3.5 and Table S3.4). IncY is a phage-like plasmid and this may indicate that transduction is playing a role in the dissemination of *bla*$_{\text{CTX-M-15}}$ within dairy cattle niches. In this study ST3369 was found on two different farms. It is the only strain which carries CTX-M variants other than CTX-M-15, with two isolates carrying *bla*$_{\text{CTX-M-1}}$ on an IncI1/ST3 plasmid and the other isolate carrying the closely related *bla*$_{\text{CTX-M-32}}$. Further investigations would be needed to assess whether clonal expansion of *K. pneumoniae* ST3369 may be playing a role in the spread of these genes beside the horizontal transfer of the pandemic *bla*$_{\text{CTX-M-1}}$/IncI1/ST3 plasmid observed in *E. coli*.

Two strains were found to persist through treatments from raw to environmentally applied manure, including two novel STN3 isolates which were dissimilar by 7.9 x 10^{-5} SNP substitutions per site (dark blue cluster, Fig. 3.3) and three ST1966 isolates dissimilar by an average of 4.19 x 10^{-4} SNP substitutions per site (light green cluster, Fig. 3.3). In both cases, the

significant number of SNPs in these two clusters prevents an evaluation of whether the strains were introduced repeatedly in the system or persisted over long periods of time. More information and isolates as well as more comprehensive approaches such as metagenomic analysis would be required to assess this accurately.

Assessing distribution of ESC-resistance plasmids from *K. pneumoniae* in dairy manure showed that highly conserved IncFII plasmids could be recovered over a one-year sampling period, even though they were found in different strains. This illustrates the role of these plasmids in the spread of ESBL genes such as the important $bla_{\text{CTX-M-15}}$ not only in *E. coli*, but also in *K. pneumoniae* populations present in the dairy cattle environment and its manure.

Eleven *K. quasipneumoniae* isolates, were recovered from farm seven at three different treatment stages, suggesting potential persistence through treatments and over time. Two of these isolates were hybrid assembled and both carried $bla_{\text{CTX-M-15}}$ on IncY plasmids similar to those in *K. pneumoniae* (Table S3.4), suggesting HGT of IncY plasmids between *Klebsiella* species. Due to the recent description of *K. quasipneumoniae*, little research has focused on this new species, especially with regards to its role in ESC-resistance and transmission (Brisse et al., 2014). These findings may indicate that *K. quasipneumoniae* is also involved in the persistence and transmission of ESC-resistance plasmids and warrants further investigation.

3.3.7 Conclusion

Although diverse strains were recovered, ESC-resistant *K. pneumoniae* were not recovered as frequently as ESC-resistant *E. coli* amongst the sampled dairy farms in Southern Ontario. ESC-resistant *K. pneumoniae* were not recovered in every farm and one farm provided the majority of isolates, suggesting that these bacteria may play a small role in the broad dissemination of ESC-resistance but could under specific circumstances act as reservoirs of transmission within local bacterial populations. Despite a large proportion of recovered isolates being MDR, the frequency of MDR *K. pneumoniae* appears similar to ESC-resistant *E. coli* from the same sources. The most frequently recovered $bla_{\text{CTX-M}}$ variant was overwhelmingly $bla_{\text{CTX-M-15}}$, with $bla_{\text{CTX-M-1}}$ appearing only twice, and both times on the epidemic plasmid IncI1/ST3 known to circulate widely in *E. coli* populations (Carattoli et al., 2018). Thus, *K. pneumoniae* may be part of the bacterial populations regularly involved in the circulation of this plasmid. As

is known for *E. coli* in general, the majority of plasmids carrying *bla*$_{\text{CTX-M-15}}$ were IncF plasmids with diverse genetic structures. Despite the relatively limited number of isolates recovered, a few strains seemed to persist through the entirety of dairy manure treatments.

3.4 TABLES

Table 3.1 Epidemiological data for *Klebsiella* spp. carrying *bla*$_{CTX-M}$ recovered from dairy manure samples from four farms in Southern Ontario.

Farm	Manure Treatment	Isolate ID	MLST	CTX-M	SHV or OKP	TEM-1
2	Raw	327-1b	ST469	15	SHV-11	
	Digestate with solids	328-1b	ST405	15	SHV-76	Yes
		267-1a	ST469	15	SHV-11	
3	Digestate with solids	269-2e	**ST3369**	1	SHV-1	
		269-3b	**ST3369**	1	SHV-1	
	Digestate without solids	233-1c	ST1418	15	SHV U	Yes
		233-3b	ST1418	15	SHV U	Yes
5	Raw	335-2b	STN4	15	SHV U	Yes
	Dewatered	246-3a	**ST2159**	15	SHV-28	Yes
		246-3b	**ST2159**	15	SHV-28	Yes
7	Raw	187-2f	ST34	15	SHV-25	Yes
		301-1g	ST37	15	SHV U	Yes
		187-1f	ST278	15	SHV-11	Yes
		235-1d	ST278	15	SHV-11	Yes
		235-2b	ST278	15	SHV-11	Yes
		257-2c	ST278	15	SHV-11	Yes
		257-3b	ST278	15	SHV-11	Yes
		279-2b	ST278	15	SHV-11	Yes
		340-1b	ST278	15	SHV-11	Yes
		340-2c	ST278	15	SHV-11	Yes
		340-3a	ST278	15	SHV-11	Yes
		345-1b	ST706	15	SHV-25	Yes
		257-1b	ST1966	15	SHV-27	Yes
		187-2g	ST5108	15	SHV-11	Yes
		187-3e	ST5108	15	SHV-11	Yes
		345-2b	ST5108	15	SHV-11	Yes
		206-1a	STQ1	15	OKP	Yes
		206-3f	STQ1	15	OKP	Yes
		235-3a	STQ1	15	OKP	Yes
		279-1b	STQ1	15	OKP	Yes
		279-3b	STQ1	15	OKP	Yes
		301-1c	STQ1	15	OKP	Yes
		301-2b	STQ1	15	OKP	Yes
		301-3a	STQ1	15	OKP	Yes
		345-3b	STQ1	15	OKP	Yes
		206-2a	STN2	15	SHV-37	Yes
		301-3b	STN3	15	SHV-11	Yes
	Digestate with solids	280-2b	ST107	15	SHV-1	Yes
		302-3b	ST168	15	SHV-11	

	302-3c	ST168	15	SHV-11	
	258-1c	ST183	15	SHV-119	Yes
	302-2b	ST219	15	SHV-1	
	236-2a	ST278	15	SHV-11	Yes
	341-2b	ST286	15	SHV U	Yes
	302-1d	ST550	15	SHV-60	Yes
	346-1b	ST791	15	SHV-1	Yes
	258-1b	ST945	15	SHV-1	Yes
	280-1b	**ST2159**	15	SHV-28	Yes
	236-1b	ST2572	15	SHV-11	Yes
	346-3b	**ST3369**	32	SHV-1	
	280-3b	STQ2	15	OKP	Yes
	188-1a	STN1	15	SHV U	Yes
Digestate without solids	342-3b	ST107	15	SHV-1	Yes
	347-1b	ST183	15	SHV-119	Yes
	347-2b	ST183	15	SHV-119	Yes
	347-3b	ST183	15	SHV-119	Yes
	342-1b	ST914	15	SHV-27	Yes
	303-2b	ST1655	15	SHV U	Yes
	281-2c	ST1966	15	SHV-27	Yes
	281-3b	ST1966	15	SHV-27	Yes
	342-2b	STN3	15	SHV-11	Yes
Dewatered	238-1c	ST163	15	SHV-11	Yes
	190-3a	ST183	15	SHV-119	Yes
	348-2b	ST183	15	SHV-119	Yes
	348-3b	ST183	15	SHV-119	Yes
	282-2b	ST219	15	SHV-1	
	304-2b	ST219	15	SHV-1	
	209-1a	ST706	15	SHV-25	Yes
	190-1b	ST1517	15	SHV-11	Yes
	190-1c	ST1517	15	SHV-11	Yes
	343-2b	ST4263	15	SHV-11	Yes
	304-1b	STQ1	15	OKP	Yes
Heat treated compost	261-1a	ST219	15	SHV-1	Yes
	261-1b	ST219	15	SHV-1	Yes

Isolates carrying *bla*$_{OKP}$ instead of *bla*$_{SHV}$ are *K. quasipneumoniae* subsp. *quasipneumoniae* and bolded STs are those recovered in samples from more than one farm source.

Table 3.2 *Klebsiella pneumoniae* and *K. quasipneumoniae* isolated from dairy cattle manure on four dairy farms in Southern Ontario and characteristics of their plasmids carrying *bla*$_{CTX-M}$

Plasmid Characteristics				Strain Characteristics		Epidemiological Data		
Replicon Type	Plasmid size (bp)	*bla*$_{CTX-M}$	Additional resistance genes on CTX-M plasmid	Isolate ID	MLST	Farm	Manure Treatment[a]	Sample Month/Year
IncFIB, IncHI1	309, 626	15	*ant(3'')-Ia, bla*$_{TEM-1}$*, catB3, dfrA1,*	335-2b	STN4	5	Raw	Aug/19
IncFIB(K)	211, 537	15	*aadA2, ant(3'')-Ia, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id, catA2, dfrA12, mph(A), qnrS1, sul1, sul2, tet(A)*	267-1a	ST469	2	DWS	Apr/19
	205, 257	15	*aadA2, ant(3'')-Ia, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id, catA2, dfrA12, mph(A), qnrS1, sul1, sul2, tet(A)*	327-1b	ST469	2	Raw	Aug/19
IncFIB(K), IncFII	237, 827	15	*ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1}$*, dfrA14, qnrB1, sul2, tet(A)*	328-1b	ST405	2	DWS	Aug/19
IncFII	95, 484	15	*bla*$_{TEM-1}$	347-2b	ST183	7	DWOS	Oct/19
	94, 401	15	*bla*$_{TEM-1}$	261-1b	ST219	7	HTC	Mar/19
	94, 102	15	*bla*$_{TEM-1}$	187-1f	ST278	7	Raw	Dec/18
	94, 426	15	*bla*$_{TEM-1}$	345-1b	ST706	7	Raw	Oct/19
	94, 418	15	*bla*$_{TEM-1}$	187-2g	ST5108	7	Raw	Dec/18
IncI1	104, 823	1	*sul2*	269-3b	ST3369	3	DWS	Apr/19
	109, 290	1	*sul2, tet(A)*	269-2e	ST3369	3	DWS	Apr/19
IncR, Col, IncFIA	97, 075	15	*aac(3)-IIa, ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1}$*, catA2, dfrA14, sul2, tet(D)*	233-1c	ST1418	3	DWOS	Feb/19
IncY	100, 397	15	*ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1}$*, dfrA14, qnrS1, sul2, tet(A)*	238-1c	ST163	7	Dew	Feb/19
	88, 268	15	*ant(3'')-Ia, dfrA14, qnrS1, tet(A)*	302-3b	ST168	7	DWS	Jun/19
	99, 264	15	*ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1}$*, dfrA14, qnrS1, sul2, tet(A)*	246-3a	ST2159	5	Dew	Mar/19
	98, 488	15	*ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1}$*, dfrA14, qnrS1, sul2, tet(A)*	343-2b	ST4263	7	Dew	Aug/19
	98, 485	15	*ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1}$*, dfrA14, qnrS1, sul2, tet(A)*	279-3b*	STQ1	7	Raw	Apr/19
	92, 997	15	*ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1}$*, dfrA14, qnrS1, sul2*	301-1c*	STQ1	7	Raw	Jun/19

[a]digestate with solids (DWS), digestate without solids (DWOS), dewatered (Dew) and heat-treated compost (HTC).

**K. quasipneumoniae* subsp. *quasipneumoniae*

3.5 FIGURES

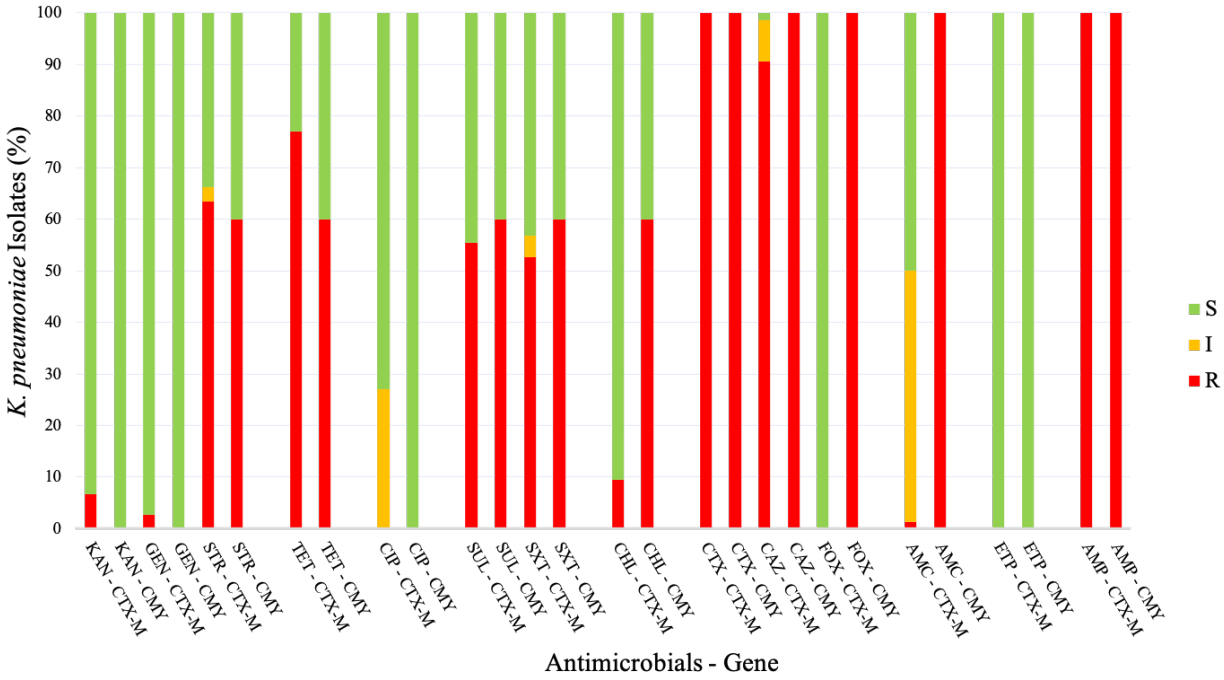

Figure 3.1. Distribution of susceptibility frequencies for *K. pneumoniae*, as determined by MALDI-TOF, carrying *bla*$_{\text{CTX-M}}$ (n = 74) and *bla*$_{\text{CMY}}$ (n = 5) from dairy cattle manure. Antimicrobial susceptibility profiles were based on disk diffusion using cefoxitin (FOX), cefotaxime (CTX), ceftazidime (CAZ), ertapenem (ETP), gentamicin (GEN), ampicillin (AMP), amoxicillin-clavulanic acid (AMC), sulfonamide (SUL), sulfamethoxazole-trimethoprim (SXT), tetracycline (TET), streptomycin (STR), kanamycin (KAN), ciprofloxacin (CIP) and chloramphenicol (CHL). Antimicrobials were grouped to determine multi-drug resistance; aminoglycosides (KAN, GEN, STR), tetracyclines (TET), quinolones (CIP), folate pathway inhibitors (SUL, SXT), phenicols (CHL), cephems (CTX, CAZ, FOX), carbapenems (ETP) and other ß-lactams (AMP). Intermediate and resistant categories were kept separate for the multi-drug resistance classification and AMC was excluded from multi-drug resistance classification.

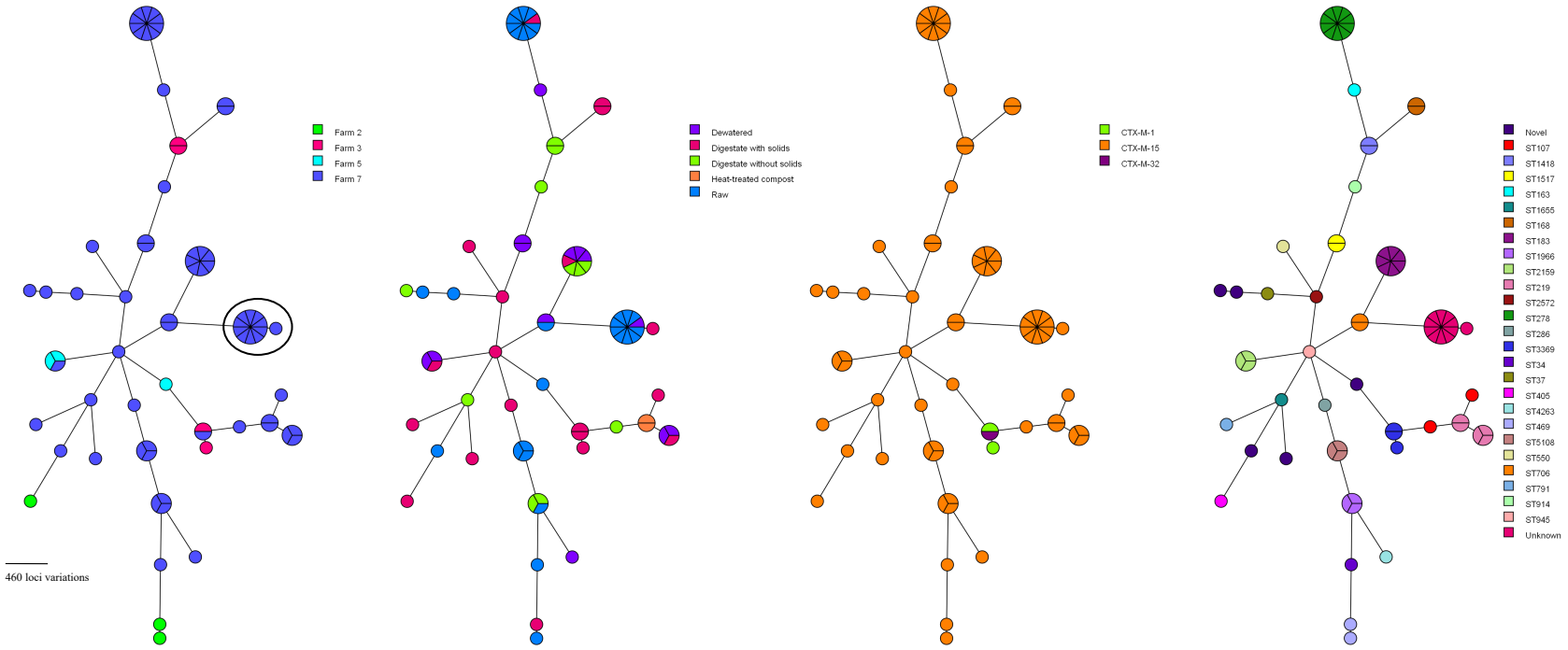

Figure 3.2. Minimum spanning trees created using cgMLST with 634 core genome loci for *K. pneumoniae* (*n* = 63*)* and *K. quasipneumoniae* (*n* = 11) isolates from dairy manure recovered from four dairy farms in Southern Ontario. Trees are colour coded based on farm source, treatment process, variant of *bla*$_{\text{CTX-M}}$ and MLST profile. The black solid circle encapsulates the *K. quasipneumoniae* isolates. On all farms, digestate with solids is the first anaerobically digested manure and digestate without solids is environmentally applied manure. On farm five, dewatered manure is used as recycled bedding whereas on farm seven, dewatered manure is thermophilically treated to create compost, which is then used as recycled bedding.

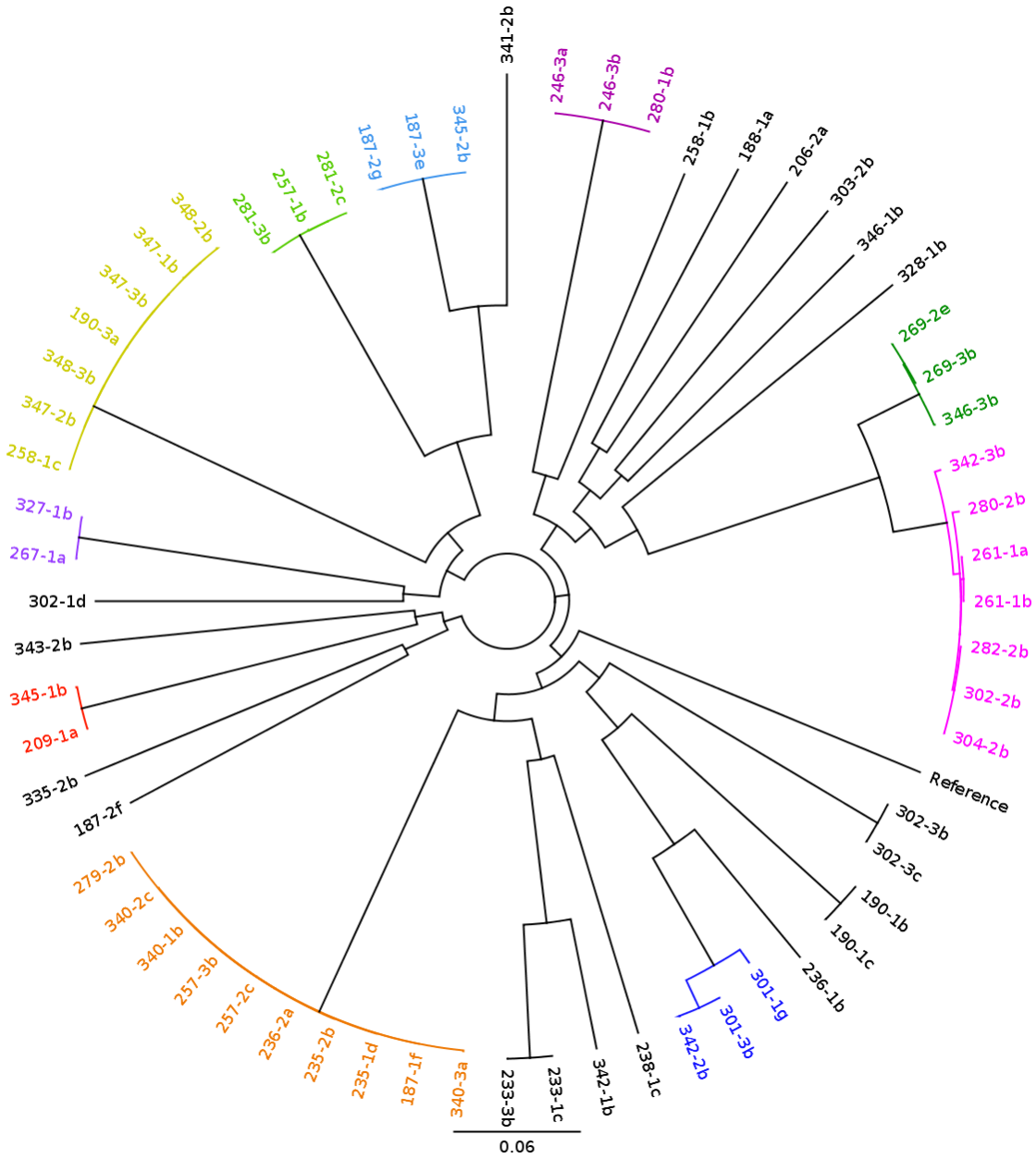

Figure 3.3. Single nucleotide polymorphism (SNP) maximum likelihood phylogenetic tree using 128,910 core SNP sites from *K. pneumoniae* isolates (n = 63) carrying *bla*$_{\text{CTX-M}}$ in reference to *K. pneumoniae* type strain ATCC 13883 (GenBank Accession #JOOW00000000.1). Clusters which contain isolates from several manure samples, suggesting that they represent different isolates of the same strain, are colour coded and described in further detail in the text.

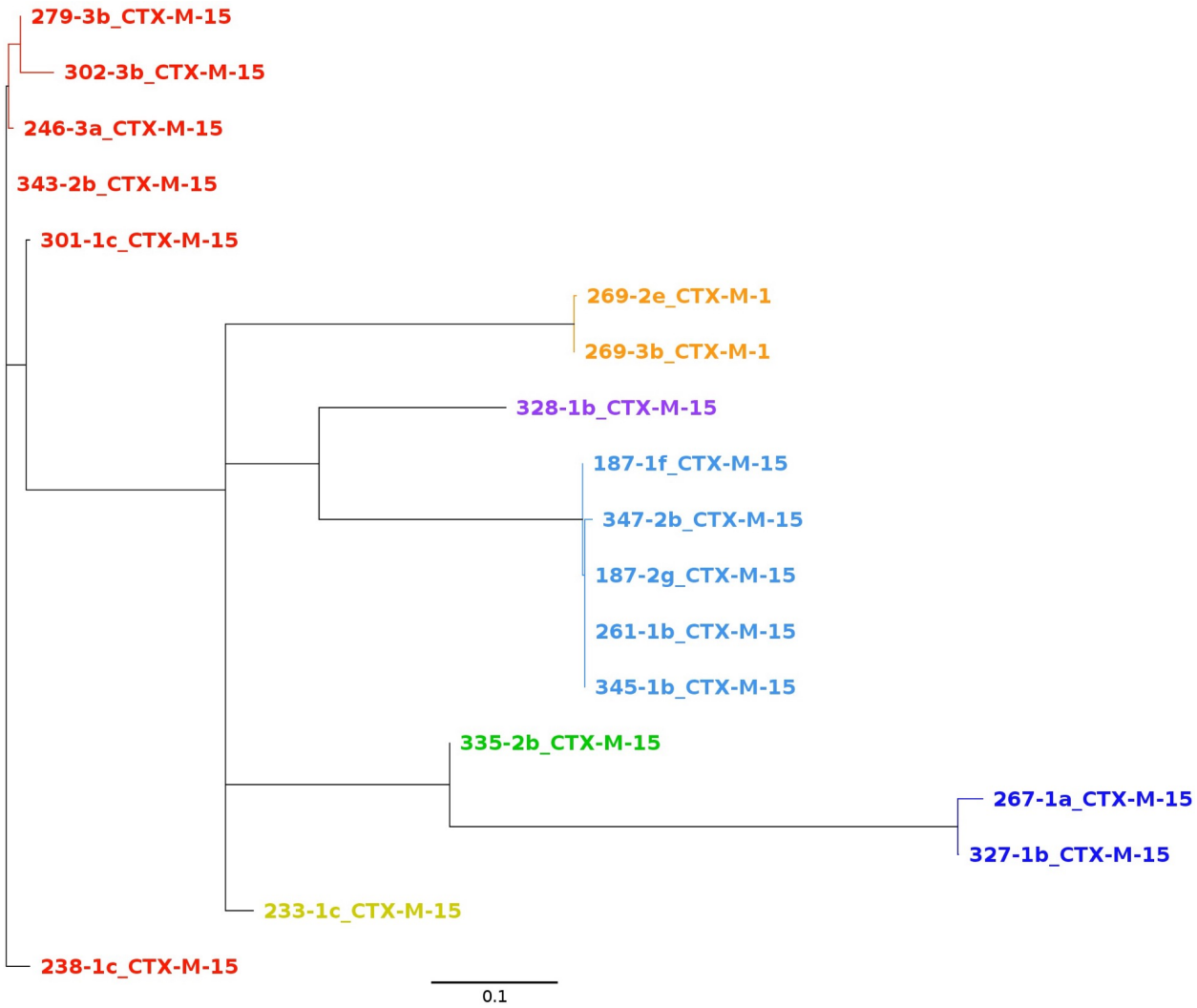

Figure 3.4 Phenetic tree based on presence and absence of 1,026 genes among plasmids carrying *bla*$_{\text{CTX-M}}$ in *K. pneumoniae* from dairy cattle manure sampled from November 2018 through to October 2019. Plasmid replicon types are indicated by the colour with red indicating IncY plasmids, orange IncI1, yellow IncR/IncFIA/Col, green IncFIB/IncHI1, light blue IncFII plasmids, dark blue IncFIB(K) plasmids and purple representing an IncFIB(K)/IncFII plasmid. Within the red cluster, IncY plasmids are carried in two *K. quasipneumoniae* isolates (301-1c and 279-3b).

Figure 3.5 Phylogenetic maximum likelihood SNP analysis among loci (n = 110) comprised of core genes (n = 74) and shell genes (n = 36), with epidemiological data including farm number, manure treatment and isolate MLST, with mauve alignments for plasmids carrying *bla*$_{\text{CTX-M-15}}$ on replicon types IncY harbored in *K. pneumoniae* and *K. quasipneumoniae* (301-1c and 279-3b) recovered from dairy manure.

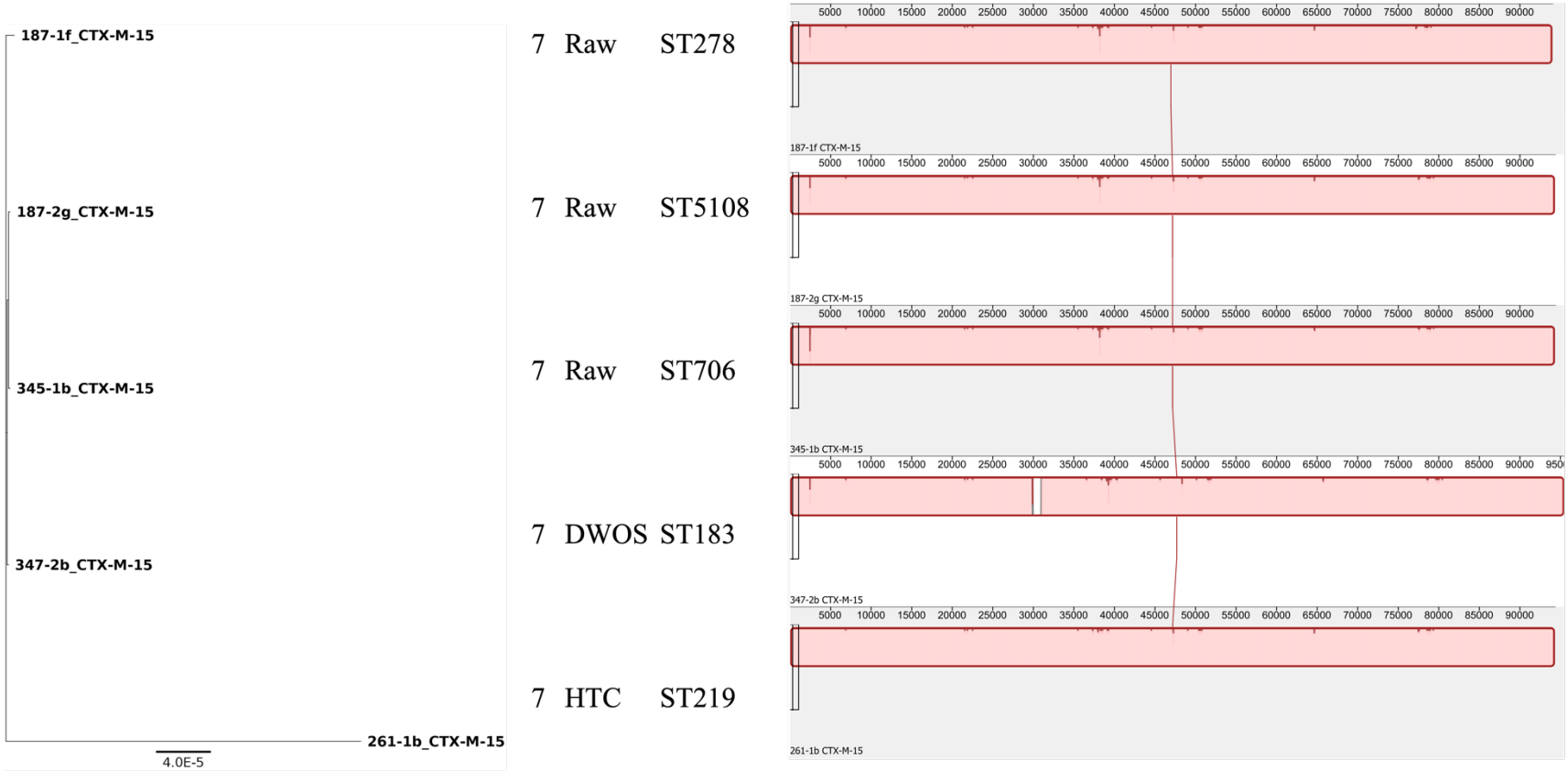

Figure 3.6 Phylogenetic maximum likelihood SNP analysis among loci (n = 123) comprised of core genes (n = 121) and shell genes (n = 2), with epidemiological data including farm number, manure treatment and MLST, with mauve alignments for plasmids carrying *bla*$_{\text{CTX-M-15}}$ on replicon types IncFII harbored in *K. pneumoniae* recovered from dairy manure.

CHAPTER FOUR: Discussion and Conclusion

4.0 STRAIN PERSISTENCE AND DIVERSITY

Strains persisting through treatment processing

One of our main objectives was to assess persistence of ESC-resistance strains through manure treatment pipelines. In order to address this, we first used statistical analyses to grasp an idea of how anaerobic digestion, and separation of liquids and solids influence the recovery of ESC-resistant *Enterobacterales* in general and *E. coli* specifically. We found that downstream the treatment pipelines, a reduction in ESC-resistant *Enterobacterales* and *E. coli* was evident. The reduction was more drastic in dewatered manure, used for recycled bedding than its liquid counterpart, digestate without solids, which is used as environmental fertilizer. Since *E. coli* was recovered at almost all treatment stages on each farm, downward trends were elucidated through recovery of these isolates. In contrast, *K. pneumoniae* were recovered less consistently and primarily on one farm, making general conclusions about trends in recovery difficult to draw based on these observations. In addition, conclusions based on thermophilic temperatures and recovery of ESC-resistant *Enterobacterales* cannot be made with the design of this study since only two of the six farms utilized thermophilic temperatures, and in different ways. Sampling from more farms which use the same thermophilic treatments would be needed to understand the effects these temperatures have on the aforementioned bacterial populations.

With regards to strains persisting through manure treatments, eight *E. coli* STs were found throughout treatment pipelines. Within these, some STs were clearly formed of loosely related isolates (e.g. ST10, ST58, ST155, ST398, ST685 and ST8761), whereas others remained more conserved throughout treatments and over time (e.g. ST46 and ST190). In addition, the STs which were found throughout treatments on the same farm were, in general, STs found on multiple farms. These findings indicate that some *E. coli* STs are widespread and genetically diverse, such as ST10 and ST58 and some are widespread but conserved like ST744. The relatively lower recovery of *Klebsiella* isolates makes the evaluation of the persistence of strains through treatments difficult. However, compared to the large number of *E. coli* STs which were

found on more than one farm, only two *K. pneumoniae* STs (ST2159 and ST3369) were found on more than one farm. This could indicate that ESC-resistant *Klebsiella* strains are not as widespread between farms, but more analyses with a larger sample set would be required.

Isolate diversity and phylogenetic divergence

A large diversity of both *E. coli* and *K. pneumoniae* STs were identified with 50 and 25 STs, respectively. In addition to these known STs, 21 novel *E. coli* STs and 4 novel *K. pneumoniae* STs were recovered. Although there were some parallels to human clinical STs such as *E. coli* ST10 and ST69 (Manges et al., 2015; Vincent et al., 2010), and STs found in dairy cattle from other studies including *E. coli* ST69, ST88, ST398 and ST744 (Ludden et al., 2019; Ohnishi et al., 2013; Madec et al., 2012; Afema et al., 2018; Awosile et al., 2020), the majority of STs were unique to this study or limited in their recovery elsewhere, especially for *K. pneumoniae* strains. The latter is largely due to the lack of research regarding these species in animal and human niches, and further illustrates the importance of characterizing ESC-resistance in a broad range of bacterial species from diverse hosts. Few STs found in dairy manure are reflective of those important for human clinical infections but include ST10 and ST69 (Manges et al., 2015; Vincent et al., 2010). ST10 were frequent and diverse, whereas the latter were found only twice. Major clones such as ST177 or ST131, typically found in poultry and humans respectively, were not found in these samples. Together this information suggests that although *E. coli* and *K. pneumoniae* strains were diverse, the strains which harbour ESC-resistance genes have not been frequently associated with human clinical infections and therefore there may not be a strong pathogenic potential or direct human threat downstream.

In both *E. coli* and *K. pneumoniae* some isolates with a known ST were related to a novel ST. The novel STs identified in this study were not investigated further due to time constraints but novelty could be due to an accumulation of mutation in the core genomes over time in these manure niches. Naturally, *E. coli* and *K. pneumoniae* strains undergo temporal genetic mutations and at different rates depending on the environmental conditions, potentially leading to the emergence of new STs. SNP distance matrices, derived from the core phylogenetic trees, can retrospectively predict the time it would take for accumulation of mutations to occur since the

dissociation of lineages. Mutation rates may be influenced by the environmental changes through treatment processing such as osmotic pressure, oxygen levels, pH, nutrient compositions, temperatures and stress response such as dormancy in VBNC states. Mutation rates for *E. coli* in anaerobic and aerobic conditions have been assessed using various models (Sakai et al., 2006; Shewaramani et al., 2017). These models could be fitted to better understand phylogenetic relationships between novel STs and closely related known STs, such as the case observed in *K. pneumoniae* isolates with two novel STN3 isolates and the closely related but transient ST37 (Fig. 3.2; dark blue cluster, Fig. 3.3). A large proportion of novel STs were identified in both *E. coli* and *K. pneumoniae* species, which could indicate dairy manure environments may be hot spots for evolutionary events and would require further analysis such as the models discussed here. Alternatively, this may indicate the long-term persistence of some strains or clonal lineages in manure treatment facilities.

Manure treatment influence on bacterial species and strain diversity

Previous studies have investigated shifts in bacterial phyla pre- and post-anaerobic digestion at various temperatures (Zou et al., 2019; Sun et al., 2016). However, shifts between clonal lineages and strains within a species have not been fully investigated. The models mentioned above could be used to further predict and understand relationships between similar strains among the same farm, better understanding how manure treatments influence bacterial population dynamics within species, *in situ*. The largest diversity and highest prevalence of *Enterobacterales* species were found in farm seven. This farm may be an ideal candidate to investigate these shifts at the species level pre- and post- treatments, at both mesophilic and thermophilic temperatures. Additionally, this farm utilizes the most complex treatment pipeline including two mesophilic anaerobic digestion stages, dewatering and composting at thermophilic temperatures.

4.2 PLASMID PERSISTENCE, DISTRIBUTION AND DIVERSITY

Our second main objective was to assess plasmid diversity and persistence throughout manure treatment pipelines. Some plasmids from both *E. coli* and *Klebsiella* were found in

different STs and at different treatment stages such as IncI1 in *E. coli* and IncY and IncFII in *Klebsiella*. Additionally, the most frequent plasmids carrying *bla*$_{\text{CTX-M}}$ in both species appear to be associated and specific for each species with IncI1 in *E. coli* and IncY in *Klebsiella*, and less specific but more ubiquitous subtypes of IncF plasmids being prevalent in both. This could indicate some level of adaptation of IncI1 and IncY plasmids to each bacterial species. IncI1 are considered to have a narrow host range and are primarily found in *E. coli* and *Salmonella* spp., whereas the host range of IncY is unclear (Rozwandowicz et al., 2018). Additionally, IncF plasmids are limited to *Enterobacterales* but have predominantly been found in *E. coli* (Rozwandowicz et al., 2018). This strengthens the need to investigate non-*E. coli* *Enterobacterales* with regards to ESC-resistance determinants and their associated MGEs.

In both *E. coli* and *K. pneumoniae,* as well as in *K. quasipneumoniae*, *bla*$_{\text{CTX-M}}$ was harbored on similar replicon types. This suggests an active circulation of plasmids between these species, particularly with IncI1 and IncY plasmids. Most frequently in both genera, IncF replicons carrying *bla*$_{\text{CTX-M-15}}$ were highly diverse and recovered in a variety of strains. This contrasts with plasmids carrying *bla*$_{\text{CTX-M-1}}$, which were most frequently IncI1, with the epidemic IncI1/ST3 being found in both *E. coli* and *K. pneumoniae*. IncY phage like-plasmids carrying *bla*$_{\text{CTX-M-15}}$ were found in all three species but seemed more frequent in *K. pneumoniae* than *E. coli*. IncY plasmids transfer through transduction rather than conjugation, but due to the objectives and design of this study, phages were not investigated in our manure samples. The link between IncY plasmids carrying *bla*$_{\text{CTX-M-15}}$ in *Klebsiella* spp. and other species warrants further investigations. These would complement other recent studies on the role of viruses in the dissemination of ESC-resistance and AMR genes (Raj et al., 2018; Shousha et al., 2015; Ross and Topp, 2015).

Eleven *K. quasipneumoniae* isolates were identified and two were hybrid assembled, both carrying *bla*$_{\text{CTX-M-15}}$ on IncY plasmids similar to those found in *K. pneumoniae.* These two isolates were found at different treatment stages and time periods, suggesting that *K. quasipneumoniae* strains and their plasmids are also able to persist through multiple manure treatment stages. Due to the recent description of *K. quasipneumoniae*, very little research has investigated ESC-resistance in this species, especially with regards to its role in ESC-resistance

plasmid circulation in dairy cattle. These findings contribute to the elucidation of the host range and adaptability of ESC-resistance plasmids and further investigations regarding plasmid transmission and their host spectrum are required.

K. pneumoniae isolates carrying *bla*$_{CMY}$ showed similar susceptibility profiles to those found in *E. coli* carrying *bla*$_{CMY}$, with about half of the *K. pneumoniae* isolates conferring resistance to ß-lactams only and the other half showing additional resistance to sulphonamides, tetracyclines, chloramphenicol and aminoglycosides. Although these *K. pneumoniae* isolates were not investigated with WGS, further genomic analysis of their *bla*$_{CMY}$-plasmids may show relatedness to *bla*$_{CMY}$-plasmids from *E. coli* (i.e. IncI1 and IncC plasmids), similar to what was found with plasmids carrying *bla*$_{CTX-M}$ in both species.

ESC-resistance plasmids from this study parallel and contrast what is found in beef cattle, as well as human clinical isolates. In ESC-resistant *E. coli* from both dairy and beef cattle, *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-55}$ are widespread and resistance to tetracyclines, sulphonamide compounds and streptomycin are frequent (Carson et al., 2008; Cormier et al., 2019b). Worth noting, *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-55}$ are also the most frequent in humans (Bevan et al., 2017). This contrasts with *bla*$_{CTX-M-65}$, which has been found in beef cattle from Alberta, and not in any isolates in this study. Although there are similarities between animal species, the differences support the need to characterize ESC-resistant *Enterobacterales* in a variety of animal sources and commodities. The plasmids found to carry *bla*$_{CTX-M}$ from both *E. coli* and *Klebsiella*, and *bla*$_{CMY-2}$ from *E. coli*, are broad spectrum plasmids with stable inheritance in a variety of hosts (Rozwandowicz et al., 2018). Together the information gained by our study provides new insight into the replicons responsible for the dissemination of ESC-resistance in the dairy cattle environment and strongly suggests that HGT of ESC-resistance plasmids between bacterial species is occurring regularly.

4.3 RESERVOIRS AND TRANSMISSION

Enterobacterales

Of the eight *Enterobacterales* genera recovered in this study, ESC-resistant *E. coli* were the most frequently isolated. They were found in every farm and every sample, except for dewatered manure from farm three which consistently had no recovery of any ESC-resistant bacteria. Although one cannot exclude that this higher recovery rate could be the result of a bias in our isolation methodology, this suggests that *E. coli* is a driving force of dissemination and persistence of ESC-resistance within the dairy cattle environment. In contrast, *K. pneumoniae* carrying *bla*$_{\text{CTX-M}}$ were recovered from only four of the six farms. They were not recovered on farms one and four, infrequent and sporadic on farms two, three and five, but widespread on farm seven. This suggests that *Klebsiella* may be less important in the overall dissemination of ESC-resistance but could be playing a key role in the persistence of ESC-resistance as a reservoir for transmission under specific circumstances. Although the other six genera are less frequently associated with human or veterinary clinical infections and have therefore been less investigated, they also may be acting as a reservoir for ESC-resistance plasmids. Our results support the need for research on a wider range of bacterial species, not only those responsible for clinical infections, to better understand the transmission of ESC-resistance. This would be for instance needed for *Proteus* species for which almost as many isolates were recovered as for *Klebsiella pneumoniae*.

Non-*Enterobacterales*

Enterobacterales were the primary interest for this study. Although the corresponding data were not presented here, non-*Enterobacterales* were frequently recovered as well. These included *Acinetobacter* spp., *Pseudomonas* spp. and *Shewanella* spp. carrying ESC-resistance genes. These three genera do not intrinsically carry *bla*$_{\text{CTX-M}}$ or *bla*$_{\text{CMY}}$ and these results suggest non-*Enterobacterales* are also part of the microbial community involved in transmission and circulation of ESC resistance plasmids. Since both *Acinetobacter* and *Pseudomonas* are ESKAPE pathogens (De Oliveira et al., 2020), this information may be worth noting and may also warrant investigating their roles in the maintenance and dissemination of *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY}}$.

Conjugation and transduction

It is clear that HGT is the primary route of dissemination of ESC-resistance determinants within a population. Conjugation between *E. coli* and *K. pneumoniae* has previously been observed and is considered to be the most important route of transmission for ESC-resistance determinants (Poirel et al., 2013; Donati et al., 2014). However, the role of transduction is becoming more evident and can be generalized or specialized, with the former taking up a large section of DNA, possibly including a string of AMR genes (Raj et al., 2018; Shousha et al., 2015; Volkova et al., 2014; von Wintersdorff et al., 2016). Since IncY plasmids were among the most frequent replicons carrying *bla*$_{\text{CTX-M}}$, further research considering transduction as a main route of dissemination of ESC-resistance genes should be considered. IncY plasmids were more prevalent in *K. pneumoniae* (including *K. quasipneumoniae*) isolates than in *E. coli* isolates, indicating these plasmids may be particularly important with regards to persistence and dissemination of ESC-resistance among *Klebsiella* spp.

4.4 FUTURE DIRECTIONS

It is evident that future research should not focus only on *E. coli*, but explore a broad range of bacterial species, both within the *Enterobacterales* order and outside, to better understand the dynamics of ESC resistance transmission in the complex bacterial populations found in manure. This could be done using similar cultural methods used in this study or with metagenomic approaches in addition to conjugation assays between *Enterobacterales* and non-*Enterobacterales* using plasmids recovered in this research. An investigation into the stability and transmission of IncY plasmids between species and identifying if similar plasmids are found in other *Enterobacterales* such as *Proteus*, *Citrobacter* or *Enterobacter* would also be warranted. In addition to further characterization of the mobility of these plasmids, their genetic structures and functional characteristics could be characterized further. To get a better picture of plasmid dynamics in manure, a larger sample set of hybrid assembled plasmids would be needed. The strain, plasmid and ESC-resistance gene diversity observed in our samples provide invaluable information to guide further analyses, particularly quantitative investigations and sampling strategies regarding the adaptation and persistence of ESC-resistant bacteria and their plasmids.

4.5 CONCLUSION

Among *E. coli* and *K. pneumoniae* isolates recovered in dairy manure, parallels to isolates from human and veterinary clinical samples were evident. Despite the recent increase in ESC-resistance and infections caused by MDR *E. coli* and *K. pneumoniae* carrying ESC-resistance determinants in human clinical infections, little has been done to characterize these bacteria and their ESC-resistance plasmids in Canadian animals and dairy cattle in particular. These animals are pivotal in the Canadian economy whether they are producing milk for the dairy product industry or their manure is being amended to the environment to increase crop yield. These products are used domestically and traded internationally. Therefore, if crops are being treated with manure contaminated with ESC-resistant *Enterobacterales*, dissemination to a wide geographical area and integration into the human gut microbiome through food consumption is possible. Identified in this study, were epidemic plasmids IncI1/ST3 which are highly prevalent in Europe and have only recent been described in North America. Their repeated recovery in both *E. coli* and *K. pneumoniae* indicates that these bacteria are already widespread in dairy cattle populations and their environment and it is only a matter of time before these plasmids become endemic to Canada. The results observed through this research highlight the importance of manure treatment at reducing not only the prevalence of ESC-resistance but also the diversity of ESC-resistant *Enterobacterales* in environmentally applied manure outputs. Despite these reductions, some strains are able to survive and persist and HGT is evident within and between bacterial species, re-iterating the importance of effective manure treatments. Together the information provided in this thesis is bridging a large gap in understanding the epidemiology of ESCs in Canadian livestock and sheds light on parallels to human health.

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APPENDIX ONE: CHAPTER TWO SUPPLEMENTARY DATA

Table S2.1 Manure sampling schedule in six farms from November 2018 to October 2019.

Farm	Manure Process	Nov	Dec	Jan	Feb	Mar	Apr	Jun	Aug	Oct	Total
1	Raw	X	X	X	X	X	X	X	X		8
	Digestate with solids	X	X	X	X	X	X	X	X		8
	Digestate without solids					X	X	X			3
	Dewatered		X	X	X	X	X	X	X		7
2	Raw			X	X	X	X	X	X		6
	Digestate with solids			X	X	X	X	X	X		6
3	Raw	X	X	X	X	X	X	X	X		8
	Digestate with solids	X	X	X	X	X	X	X	X		8
	Digestate without solids	X	X	X	X	X	X	X	X		8
	Dewatered		X	X	X	X	X	X	X		7
4	Raw	X	X	X	X	X	X	X	X		8
	Digestate with solids	X	X	X	X	X	X	X	X		8
5	Raw	X	X	X	X	X	X	X	X		8
	Digestate 1 with solids	X	X	X	X	X	X	X	X		8
	Digestate 2 with solids	X	X	X	X	X	X	X	X		8
	Digestate 3 without solids	X	X	X	X	X	X	X	X		8
	Dewatered		X	X	X	X	X	X	X		7
7	Raw		X	X	X	X	X	X	X	X	8
	Digestate with solids		X	X	X	X	X	X	X	X	8
	Digestate without solids		X	X	X	X	X	X	X	X	8
	Dewatered		X	X	X	X	X	X	X	X	8
	Heat Treated Compost		X	X	X	X	X	X	X	X	8

Table S2.2 Temporarily assigned MLST profiles for *E. coli* isolates carrying *bla*$_{CTX-M}$ or *bla*$_{CMY}$ with either a novel allele or a novel configuration of the seven alleles.

Isolate ID	Novel allele	Novel configuration	MLST alleles for *Escherichia coli*							MLST ID*
			adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	
274-1a	yes		10	SNV1	135	8	8	8	2	N1
274-2a	yes		10	SNV1	135	8	8	8	2	N1
162-3b		yes	92	466	87	96	70	58	2	N2
182-3c		yes	92	466	87	96	70	58	2	N2
284-1e	yes		423	SNV2	167	506	136	40	339	N3
284-3d	yes		423	SNV2	167	506	136	40	339	N3
241-1b	yes		10	SNV3	4	8	8	8	2	N4
241-3b	yes		10	SNV3	4	8	8	8	2	N4
273-2d	yes		6	95	4	1	9	SNV1	6	N5
273-2c	yes		6	95	4	1	9	SNV1	6	N5
259-1b	yes		10	SNV4	975	91	8	524	892	N6
329-2a	yes		6	SNV5	4	8	8	SNV2	2	N7
160-1c		yes	431	204	535	319	345	284	393	N8
160-2c		yes	431	204	535	319	345	284	393	N8
260-3a	yes		168	371	SNV1	30	442	22	2	N9
240-1b		yes	6	4	12	1	20	12	489	N10
240-2b		yes	6	4	12	1	20	12	489	N10
209-1c		yes	10	929	4	205	8	2	2	N11
238-1b		yes	10	929	4	205	8	2	2	N11
328-1a	yes		8	7	57	SNV1	6	18	6	N12
328-3a	yes		8	7	57	SNV2	6	SNV3	6	N13

*MLST identifications were assigned based on isolates MLST allelic profiles and were grouped based on confirmed locus variants and the allele containing a SNP. Each novel allele containing a SNP was denoted with prefix SNV to indicate a different variation of SNP within that loci group.

Table S2.3 Comparison of the recovery of four categories of ESC-resistant *Enterobacterales* and *E. coli* isolates from raw input manure to manure post treatment with anaerobic digestion at mesophilic temperatures individually and both manure outputs on four farms, individually and pooled.

Fa	Process[a,b]	ESC-Resistant *Enterobacterales* (CTX-M, CMY or SHV)			ESC-Resistant *E. coli* (CTX-M or CMY)			ESC-Resistant *E. coli* (CTX-M)			ESC-Resistant *E. col* (CMY)		
		Sp	Rp	Is	Sp	Rp	Is	Sp	Rp	Is	Sp	Rp	Is
1	RM	100 (8/8)	87.5 (21/24)	45.2 (38/84)	87.5 (7/8)	79.2 (19/24)	32.1 (27/84)	75.0 (6/8)	54.1 (13/24)	23.8 (20/84)	37.5 (3/8)	25.0 (6/24)	8.3 (7/84)
	TM	75.5 (6/8)	62.5 (15/24)	71.4 (25/35)	62.5 (5/8)	58.3 (14/24)	60.0 (21/35)	12.5 (1/8)	12.5 (3/24)	22.9 (8/35)	50.0 (4/8)	45.8 (11/24)	37.1 (13/35)
	EAM	50.0 (2/4)	50.0 (6/12)	100 (11/11)	50.0 (2/4)	50.0 (6/12)	81.8 (9/11)	25.0 (1/4)	25.0 (3/12)	45.5 (5/11)	25.0 (1/4)	25.0 (3/12)	36.4 (4/11)
	RB	57.1 (4/7)	38.1 (8/21)	80.0 (12/15)	42.9 (3/7)	33.3 (7/21)	73.3 (11/15)	14.3 (1/7)	14.3 (3/21)	26.7 (4/15)	28.6 (2/7)	19.0 (4/21)	46.7 (7/15)
2	RM	83.3 (5/6)	55.5 (10/18)	79.0 (15/59)	83.3 (5/6)	50.0 (9/18)	20.3 (12/59)	50.0 (3/6)	27.8 (5/18)	8.5 (5/59)	66.7 (4/6)	27.8 (5/18)	11.9 (7/59)
	TM	100 (6/6)	100 (18/18)	80.0 (36/45)	100 (6/6)	100 (18/18)	73.3 (33/45)	83.3 (5/6)	55.5 (10/18)	24.4 (11/45)	100 (6/6)	77.8 (14/18)	51.1 (23/45)
3	RM	100 (8/8)	100 (24/24)	59.0 (49/83)	100 (8/8)	100 (24/24)	45.8 (38/83)	100 (8/8)	62.5 (15/24)	22.9 (19/83)	87.5 (7/8)	66.7 (16/24)	26.5 (22/83)
	TM	100 (8/8)	83.3 (20/24)	60.1 (42/69)	100 (8/8)	83.3 (20/24)	46.4 (32/69)	75.5 (6/8)	58.3 (14/24)	24.6 (17/69)	75.0 (6/8)	54.2 (13/24)	21.7 (15/69)
	EAM	100 (8/8)	87.5 (21/24)	77.4 (41/53)	100 (8/8)	87.5 (21/24)	67.9 (36/53)	87.5 (7/8)	58.3 (14/24)	30.2 (16/53)	100 (8/8)	62.5 (15/24)	39.6 (21/53)
	RB	0 (0/7)	0 (0/21)	0 (0/0)	0 (0/7)	0 (0/21)	0 (0/0)	0 (0/7)	0 (0/21)	0 (0/0)	0 (0/7)	0 (0/21)	0 (0/0)
4	RM	100 (8/8)	100 (24/24)	47.19 (42/89)	100 (8/8)	100 (24/24)	30.3 (27/89)	12.5 (1/8)	8.3 (2/24)	2.2 (2/89)	100 (8/8)	100 (24/24)	28.1 (25/89)
	TM	87.5 (7/8)	79.2 (19/24)	59.3 (32/54)	87.5 (7/8)	70.8 (17/24)	48.1 (26/54)	37.5 (3/8)	20.8 (5/24)	13.0 (7/54)	87.5 (7/8)	58.3 (14/24)	35.2 (19/54)
5	RM	100 (8/8)	95.8 (23/24)	45.1 (37/82)	100 (8/8)	95.8 (23/24)	40.2 (33/82)	25.0 (2/8)	12.5 (3/24)	3.7 (3/82)	100 (8/8)	95.8 (23/24)	37.8 (31/82)
	TM	87.5 (7/8)	75.0 (18/24)	35.3 (24/68)	87.5 (7/8)	75.0 (18/24)	32.4 (22/68)	37.5 (3/8)	16.7 (4/24)	5.9 (4/68)	87.5 (7/8)	58.3 (14/24)	26. 5 (18/68)
	EAM[c]	50.0 (4/8)	41.7 (10/24)	66.7 (18/27)	50.0 (4/8)	41.2 (10/24)	66.7 (18/27)	37.5 (3/8)	25.0 (6/24)	29.6 (8/27)	37.5 (3/8)	29.2 (7/24)	37.0 (10/27)

	RB	43.0 (3/7)	33.3 (7/21)	57.1 (8/14)	28.6 (2/7)	28.6 (6/21)	42.9 (6/14)	14.3 (1/7)	4.8 (1/21)	7.1 (1/14)	28.6 (2/7)	23.8 (5/21)	35.7 (5/14)
7	RM	100 (8/8)	100 (24/24)	57.3 (74/129)	100 (8/8)	70.8 (17/24)	15.5 (20/129)	100 (8/8)	58.3 (14/24)	12.4 (16/129)	37.5 (3/8)	16.7 (4/24)	3.1 (4/129)
	TM	100 (8/8)	95.8 (23/24)	62.2 (61/98)	100 (8/8)	91.7 (22/24)	29.59 (29/98)	100 (8/8)	83.3 (20/24)	24.5 (24/98)	50.0 (4/8)	25.0 (6/24)	8.2 (8/98)
	EAM	100 (8/8)	91.7 (22/24)	66.7 (56/84)	100 (8/8)	91.2 (22/24)	33.3 (28/84)	100 (8/8)	83.3 (20/24)	29.0 (24/84)	37.5 (3/8)	20.8 (5/24)	6.0 (5/84)
	RB[d]	37.5 (3/8)	25.0 (6/24)	46.7 (7/15)	37.5 (3/8)	20.8 (5/24)	33.3 (5/15)	37.5 (3/8)	16.7 (4/24)	26.7 (4/15)	12.5 (1/8)	4.2 (1/24)	6.7 (1/15)
P	RM	97.8 (45/46)	91.3 (126/138)	48.5 (255/526)	95.7 (44/46)	84.1 (116/138)	29.8 (157/526)	60.9 (28/46)	37.7 (52/138)	12.4 (65/526)	71.7 (33/46)	56.5 (78/138)	18.3 (96/526)
	TM	91.3 (42/46)	81.9 (113/138)	59.6 (220/369)	89.1 (41/46)	78.9 (109/138)	44.2 (163/369)	56.5 (26/46)	40.6 (56/138)	19.2 (71/369)	73.9 (34/46)	52.2 (72/138)	26.0 (96/369)
	EAM	78.6 (22/28)	70.2 (59/84)	72.0 (126/175)	78.6 (22/28)	70.2 (59/84)	52.0 (91/175)	67.9 (19/28)	51.2 (43/84)	30.3 (53/175)	53.6 (15/28)	35.7 (30/84)	22.9 (40/175)
	RB	34.5 (10/29)	24.1 (21/87)	61.4 (27/44)	27.6 (8/29)	20.7 (18/87)	50.0 (22/44)	17.2 (5/29)	9.2 (8/87)	20.5 (9/44)	17.2 (5/29)	11.5 (10/87)	29.5 (13/44)

[a]Each sample was processed in triplicate to equate three biological replicates per sample.

[b]All raw samples were the manure input, treated manure from all six farms were digestate with solids samples, environmental manure on farms one, three, five and seven were digestate without solids, recycled bedding on farms one, three and five were dewatered manure whereas on farm seven this was heat treated compost.

[c]Digestate three without solids represents manure taken after the third digestion process on farm five after the solids have been removed. This farm utilizes two mesophilic digesters with the third digester performing at thermophilic temperatures.

[d]Heat-treated compost is unique to farm seven and is dewatered material that has been treated at thermophilic temperatures and left to mature under composting conditions

Numerical values within each cell represent the percentage, with the proportions in brackets.

Abbreviations: Farm (Fa); Samples (Sp); Replicates (Rp); Isolates (Is); Pooled data (P); Raw manure (RM); Manure after mesothermic anaerobic treatment (TM); Environmentally applied manure (EAM); Manure solids used for recycled bedding in the farm (RM). Isolates represent the selected colonies with unique morphologies from each plate, one plate per replicate, therefore three plates per sample (triplicate).

Numerical values in each cell are percentages followed by proportions in parenthesis

Table S2.4 Frequency of resistance to twelve antimicrobials in ESC-resistant non-*E. coli Enterobacterales* isolates from dairy manure.

Species (# of isolates)	*Enterobacterales* with a resistant phenotype (%)											
	CHL	CIP	SUL	SXT	KAN	GEN	STR	TET	AMP	AMC	CTX	FOX
Klebsiella pneumoniae (*n* = 84)	11.9	0	58.3	55.9	7.1	3.5	64.2	77.3	100	7.14	100	5.9
Citrobacter spp. (*n* =15)	60	0	60	60	6.6	6.6	60	60	100	100	100	100
Proteus spp. (*n* =103)	83.4	0	84.4	81.5	8.7	1.9	64	100	100	26.2	100	17.4
Enterobacter cloacae (*n* = 6)	33.3	0	100	100	16.6	16.6	100	100	100	100	100	100
Providencia spp. (*n* = 5)	100	0	100	100	0	0	80	100	100	100	100	60
Rahnella aquatilis (*n* =1)	0	0	0	0	0	0	0	0	100	0	100	0
Roultella ornithinolytica (*n* = 2)	0	0	100	100	0	100	100	100	100	50	100	0
Escherichia fergusonii (*n* = 1)	0	0	0	0	0	0	0	0	100	100	100	100

Abbreviations: Ampicillin (AMP); amoxicillin-clavulanic acid (AMC); cefotaxime (CTX); cefoxitin (FOX); sulfonamide (SUL); sulfamethoxazole-trimethoprim (SXT); tetracycline (TET); streptomycin (STR); kanamycin (KAN); gentamicin (GEN); ciprofloxacin (CIP) and chloramphenicol (CHL)

Table S2.5 Sources and MLST diversity of *E. coli* strains carrying *bla*$_{CTX-M}$ and/or *bla*$_{CMY}$ among all six dairy farms.

Farm	ESC-resistance determinant	Input	Other[c]	Output	
		Raw		EAM[a]	Bedding[b]
1	*bla*$_{CTX-M}$	**ST38 (n =1)**			
		ST58 (n =1)	**ST58 (n = 1)**	**ST58 (n = 1)**	**ST58 (n = 1)**
			ST88 (n = 1)		
		ST540 (n = 1)			
		ST685 (n = 1)			
		ST744 (n = 2)			
		ST3298 (n = 2)			
		STN3 (n = 1)			
2	*bla*$_{CTX-M}$	**ST10 (n = 1)**	N/A		N/A
				ST46 (n = 1)	
		ST57 (n = 2)			
				ST58 (n = 1)	
				ST165 (n = 1)	
				ST295 (n = 1)	
				ST4981 (n = 1)	
				STN12 (n = 1)	
				STN13 (n = 1)	
3	*bla*$_{CTX-M}$	**ST10 (n = 1)**	**ST10 (n = 2)**	**ST10 (*n* = 1)**	N/A
		ST46 (n = 2)	**ST46 (n = 5)**	**ST46 (*n* = 4)**	
		ST58 (n = 1)	**ST58** (n = 1)	**ST58 (*n* = 1)**	
				ST155 (*n* = 1)	
			ST162 (n = 1)		
		ST224 (n = 1)			
		ST398 (n = 1)		**ST398 (*n* = 1)**	
			ST641 (n = 1)		
		ST744 (n = 2)			
				ST746 (*n* = 2)	
		ST1140 (n = 2)			
		ST4429 (n = 1)			
			ST7978 (n = 1)		
		ST8761 (n = 1)	**ST8761** (n = 1)	**ST8761 (*n* = 1)**	
		STN8 (n = 1)			
		STN7 (n = 1)			
	bla$_{CMY}$, *bla*$_{CTX-M}$	ST1140 (n = 2)			N/A
				STN2 (n = 1)	
	bla$_{CMY}$		**ST10 (n = 2)**		N/A
		ST69 (n = 1)			
				ST162 (*n* = 1)	

				ST165 (*n* = 1)	
		ST609 (n = 1)			
				ST657 **(*n* = 2)**	
				ST746 (*n* = 2)	
			ST1582 (n = 1)		
		ST3714 (n = 1)			
			STN2 (n = 1)		
4	*bla*CTX-M		N/A	**ST155** (n = 1)	N/A
				ST212 (n = 2)	
				STN4 (n = 1)	
				STN5 (n = 1)	
		STN10 (n = 1)			
5	*bla*CTX-M		ST23 (n = 2)		
			ST155 (n = 3)	**ST155** (n = 1)	**ST155** (n = 1)
			ST718 (n = 1)		
				ST744 (n = 1)	
				ST939 (n = 1)	
				ST1152 (n = 1)	
			ST1722 (n = 1)	**ST1722** (n = 1)	
		STN1 (n = 1)			
	*bla*CMY, *bla*CTX-M	**ST69** (n = 1)			
7	*bla*CTX-M		**ST10** (n = 2)	**ST10** (n = 4)	**ST10** (n = 2)
			ST38 (n = 2)		
			ST58 (n = 2)	**ST58** (n = 3)	
		ST88 (n = 1)			
			ST100 (n = 1)		
		ST155 (n = 1)	**ST155** (n = 1)	**ST155** (n = 2)	
			ST162 (n = 2)		
		ST190 (n = 1)	ST190 (n = 7)	ST190 (n = 2)	
		ST219 (n = 1)			
		ST398 (n = 2)	**ST398** (n = 7)	**ST398** (n = 2)	
			ST540 (n = 3)	**ST540** (n = 1)	
		ST683 (n = 1)	ST683 (n = 2)		
		ST685 (n = 3)	**ST685** (n = 2)	**ST685** (n = 3)	
				ST744 (n = 1)	ST744 (n = 1)
		ST1201 (n = 2)			
			ST1722 (n = 1)		
			ST3580 (n = 1)		
		ST5314 (n = 2)			
			ST5597 (n = 2)		
			ST7982 (n = 1)		
					ST8761 (n = 1)
			ST9062 (n = 2)	ST9062 (n = 1)	

		ST11633 (n = 1)	ST11633 (n = 2)	
			STN6 (n = 1)	
		STN9 (n = 1)		
		STN11 (n = 2)		
*bla*CMY, *bla*CTX-M			**ST155** (n = 2)	
		ST398 (n = 7)		
*bla*CMY		**ST10** (n = 2)	**ST10** (n = 4)	
				ST162 (n = 1)
			ST316 (n = 1)	
		ST543 (n = 1)		
	ST746 (n = 1)			
		ST1080 (n = 1)		
		ST1704 (n = 1)		
		ST4086 (n = 1)		

[a]Other treatments include any other manure process on that farm. This includes anaerobically digested manure (digestate with solids) on farms one, three, five and seven. Two digestate with solids processes were sampled on farm three, in addition to dewatered manure on farm seven.

[b]EAM represents environmentally applied manure (digestate without solids) on farms one, three, five and seven, and digestate with solids from farms two and four.

[c]Bedding represents dewatered manure from farms one, three and five and heat-treated compost from farm seven.

[d]ESC-resistant isolates were not recovered from farm three dewatered samples at any time period.

Bolded MLST profiles are found in more than one farm, and any profile beginning with STN, has a novel MLST profile and was given a temporary name for this study. Number of samples in which each ST were identified are represented in parenthesis

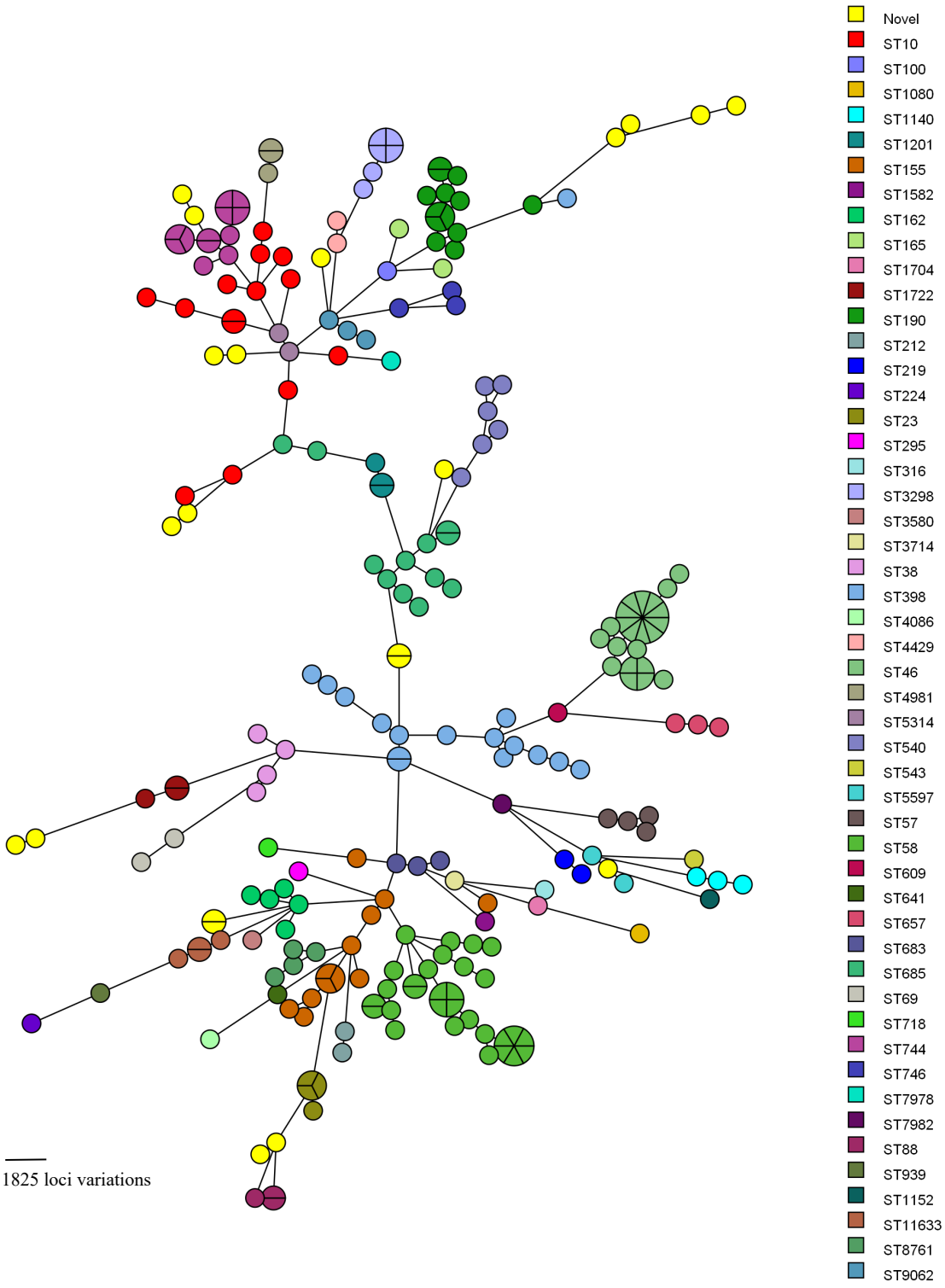

Figure S2.1 Minimum spanning tree made using wgMLST comparison of 17,350 genes and colour coded based on MLST profile for ESC-resistant *E. coli* (n = 248).

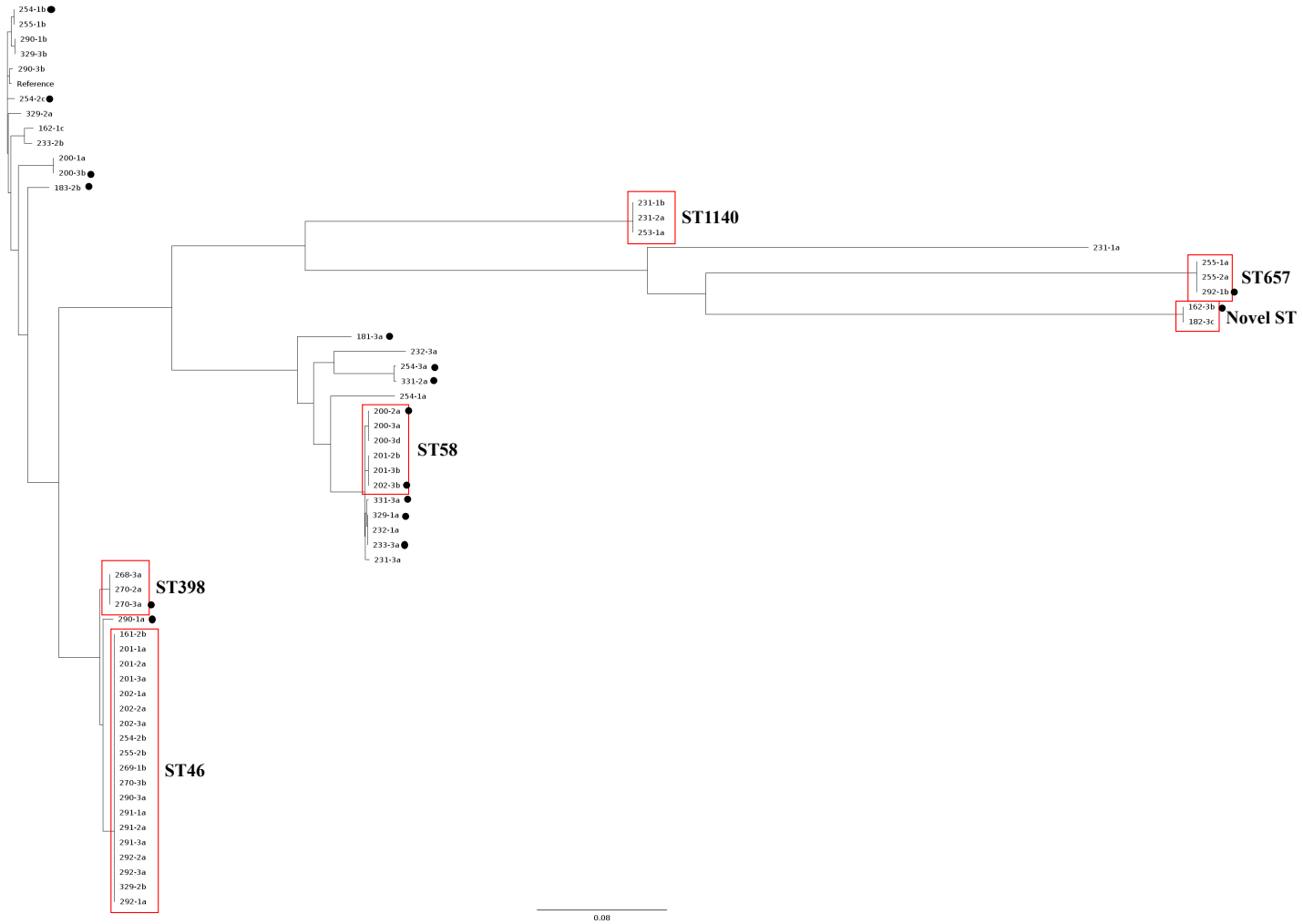

Figure S2.2 Maximum likelihood phylogenetic analysis based on proportional SNP differences among ESC-resistant *E. coli* (n = 60) from farm three using 190,646 SNP sites. Black dots indicate isolates which have been sequenced with both short and long reads and hybrid assembled. Clusters of closely related isolates are highlighted in red boxes with their MLST profiles to the right of each box.

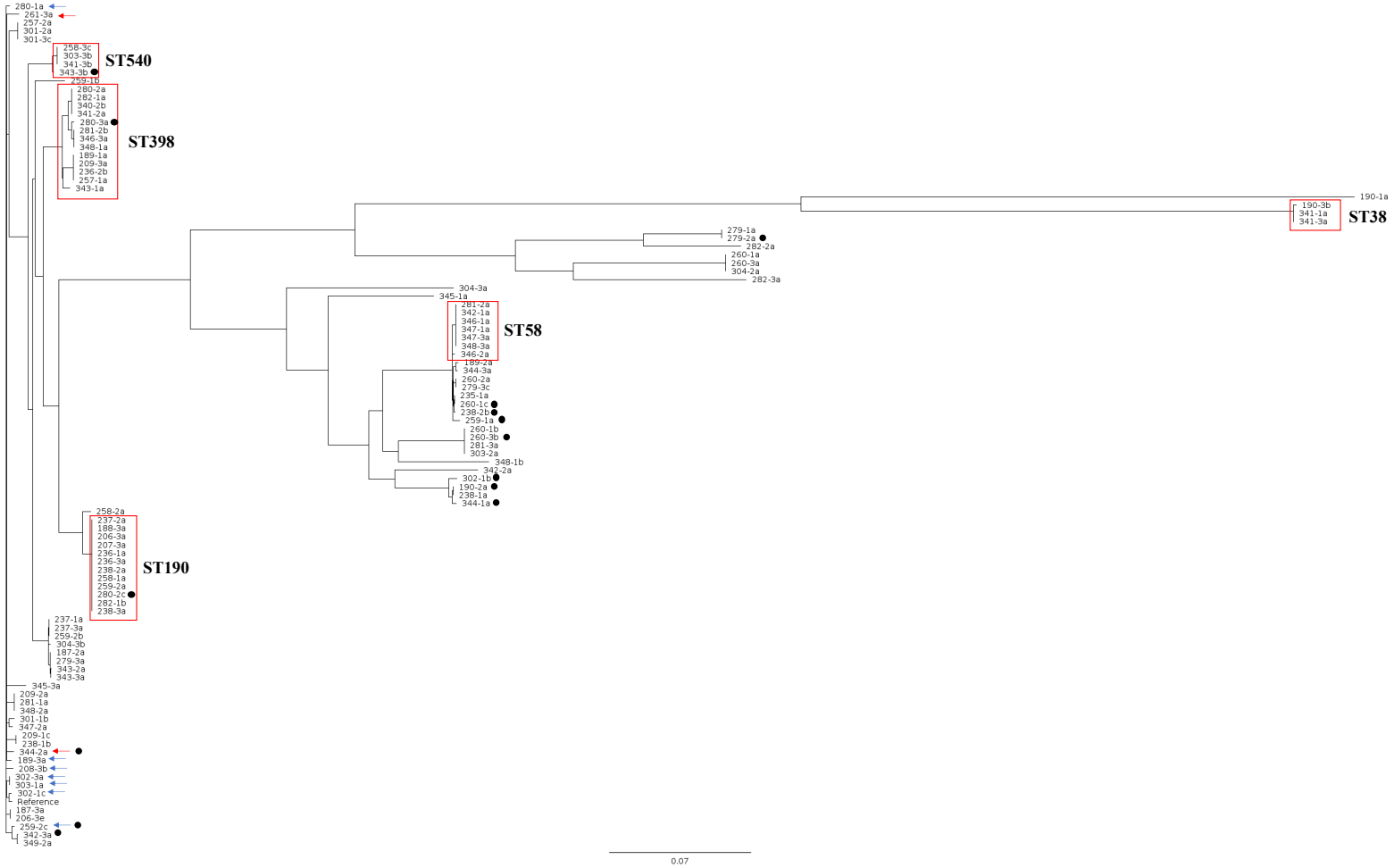

Figure S2.3 Maximum likelihood phylogenetic analysis based on proportional SNP differences among ESC-resistant *E. coli* (n = 101) from farm seven using 192,856 SNP sites. Black dots indicate isolates which have been sequenced with both short and long reads and hybrid assembled. Clusters of closely related isolates are highlighted in red boxes with their MLST profiles to the right of each box. Blue arrows indicate ST10 isolates and red arrows indicating ST10 recovered from heat treated compost.

APPENDIX TWO: CHAPTER THREE SUPPLEMENTARY DATA

Table S3.1 Temporary multi-locus sequence typing identifications for *K. pneumoniae* isolates with novel alleles or novel allelic configurations.

Isolate ID	Novel Allele	Novel Allelic Configuration	MLST alleles for *K. pneumoniae*							Novel MLST ID
			gapA	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*	
188-1a		yes	2	1	2	1	1	4	9	STN1
206-2a		yes	3	1	1	3	3	28	39	STN2
301-3b		yes	2	9	2	1	13	1	100	STN3
342-2b		yes	2	9	2	1	13	1	100	STN3
335-2b	yes		2	1	13	26	21	5	SNP	STN4

Table S3.2 Temporarily multi-locus sequence typing (MLST) identifications for *K. quasipneumoniae* isolates based off the MLST scheme for *K. pneumoniae* to enable strain differentiation.

Isolate ID	MLST alleles for *K. pneumoniae*							MLST ID
	gapA	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*	
206-1a	SNP	19	SNP	39	SNP	21	162	STQ1
206-3f	SNP	19	SNP	39	SNP	21	162	STQ1
235-3a	SNP	19	SNP	39	SNP	21	162	STQ1
279-1b	SNP	19	SNP	39	SNP	21	162	STQ1
279-3b	SNP	19	SNP	39	SNP	21	162	STQ1
280-3b	SNP	19	SNP	39	SNP	21 and 1	162	STQ2
301-1c	SNP	19	SNP	39	SNP	21	162	STQ1
301-2b	SNP	19	SNP	39	SNP	21	162	STQ1
301-3a	SNP	19	SNP	39	SNP	21	162	STQ1
304-1b	SNP	19	SNP	39	SNP	21	162	STQ1
345-3b	SNP	19	SNP	39	SNP	21	162	STQ1

Table S3.3 Single nucleotide polymorphism (SNP) distance matrix according to proportion of SNP differences between *K. quasipneumoniae* isolates carrying *bla*$_{\text{CTX-M}}$ in reference to *K. quasipneumoniae* subsp. *quasipneumoniae* strain 01A030.

ID	301-3a	345-3b	Ref	206-3f	206-1a	279-3b	301-1c	279-1b	304-1b	280-3b	301-2b	235-3a
301-3a		0.000301	5.829724	0.000386	0.000815	0.000773	0.000601	0.000986	0.000858	0.000601	0.000858	0.000472
345-3b	0.000301		5.829423	0.000343	0.000772	0.00073	0.000558	0.000943	0.000815	0.000558	0.000815	0.000429
Ref	5.829724	5.829423		5.829766	5.830195	5.830153	5.829981	5.830366	5.830238	5.829981	5.830238	5.829852
206-3f	0.000386	0.000343	5.829766		0.000515	0.000473	0.000301	0.000686	0.000558	0.000301	0.000558	0.000172
206-1a	0.000815	0.000772	5.830195	0.000515		0.000644	0.000472	0.000857	0.000729	0.000472	0.000987	0.000601
279-3b	0.000773	0.00073	5.830153	0.000473	0.000644		0.000172	0.000815	0.000687	0.00043	0.000945	0.000559
301-1c	0.000601	0.000558	5.829981	0.000301	0.000472	0.000172		0.000643	0.000515	0.000258	0.000773	0.000387
279-1b	0.000986	0.000943	5.830366	0.000686	0.000857	0.000815	0.000643		0.0003	0.000557	0.001158	0.000772
304-1b	0.000858	0.000815	5.830238	0.000558	0.000729	0.000687	0.000515	0.0003		0.000429	0.00103	0.000644
280-3b	0.000601	0.000558	5.829981	0.000301	0.000472	0.00043	0.000258	0.000557	0.000429		0.000773	0.000387
301-2b	0.000858	0.000815	5.830238	0.000558	0.000987	0.000945	0.000773	0.001158	0.00103	0.000773		0.000644
235-3a	0.000472	0.000429	5.829852	0.000172	0.000601	0.000559	0.000387	0.000772	0.000644	0.000387	0.000644	

Table S3.4 Single nucleotide polymorphism (SNP) distance matrix according to proportion of SNP differences among core loci in IncY plasmids carrying *bla*$_{\text{CTX-M-15}}$ from *K. pneumoniae* and *K. quasipneumoniae* isolates recovered from dairy manure.

Isolate ID	238-1c	246-3a	279-3b*	301-1c*	302-3b	343-2b
238-1c		0.000003	0.000041	0.000006	0.000006	0.000002
246-3a	0.000003		0.00004	0.000005	0.000005	0.000003
279-3b*	0.000041	0.00004		0.000039	0.000039	0.000041
301-1c*	0.000006	0.000005	0.000039		0.000002	0.000006
302-3b	0.000006	0.000005	0.000039	0.000002		0.000006
343-2b	0.000002	0.000003	0.000041	0.000006	0.000006	

**K. quasipneumoniae* subsp. *quasipneumoniae* isolates

Table S3.5 Single nucleotide polymorphism (SNP) distance matrix according to proportion of SNP differences among core loci in IncFII plasmids carrying *bla*$_{\text{CTX-M-15}}$ from *K. pneumoniae* isolates recovered from dairy manure.

Isolate ID	187-1f	187-2g	345-1b	347-2b	261-1b
187-1f		0.000009	0.000009	0.000008	0.000264
187-2g	0.000009		0.000002	0.000003	0.000261
345-1b	0.000009	0.000002		0.000003	0.000261
347-2b	0.000008	0.000003	0.000003		0.00026
261-1b	0.000264	0.000261	0.000261	0.00026	