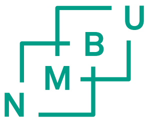

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Philosophiae Doctor (PhD)
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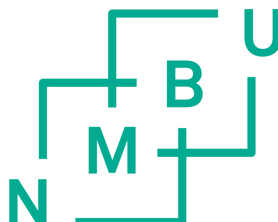

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May Linn Buberg

«For wisdom will enter your heart, and knowledge will be pleasant to your soul».

Proverbs; Chapter 2, verse 10

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1 Abbreviations and definitions

AIEC: Adherent-invasive *E. coli*
AMR: Antimicrobial resistance
APEC: Avian pathogenic *E. coli*
ARG: Antimicrobial resistance gene
CDT: Cytolethal distending toxin
CNF: Cytotoxic necrotizing factor
Cu: Copper
DAEC: Diffuse adherent *E. coli*
E. coli: *Escherichia coli*
EFSA: European food safety association
ESBL: Extended spectrum beta-lactamase
ESC: Extended spectrum cephalosporin
EHEC: Enterohemorrhagic *E. coli*
EIEC: Enteroinvasive *E. coli*
EPEC: Enteropathogenic *E. coli*
ETEC: Enterotoxiogenic *E. coli*
ExPEC: Extraintestinal pathogenic *E. coli*
GAP: Global action plan
HGT: Horizontal gene transfer
HIS: Heat inactivated serum
HS: Human serum
ICE: Integrative and conjugational elements
IMC: Inner membrane complex
IPEC: Intestinal pathogenic *E. coli*
LB broth: Luria-Bertani broth
LPS: Lipopolysaccharide
MDR: Multi-drug resistant
MGE: Mobile genetic elements
MIC: Minimum inhibitory concentrations
MLST: Multi locus sequence typing
MOA: Mechanism of action
MOI: Multiplicity of infection
NMBU: Norwegian University of Life Sciences
NORM-VET: Norwegian monitoring program for antimicrobial resistance in bacteria from food, feed and animals
OMP: Outer membrane protein
OriT: Origin of transfer
PBP: Penicillin-binding protein
PBS: Phosphate-buffered saline
PDR: Pandrug resistant

ROS: Reactive oxygen species
SBLE: Serine bound betalactamase
SEPEC: Sepsis associated *E. coli*
SGF: Simulated gastric fluid
SIF: Simulated intestinal fluid
SSF: Simulated salivary fluid
UPEC: Uropathogenic *E. coli*
UTI: Urinary tract infection
VAG: Virulence associated gene
VF: Virulence factors
VRE: Vancomycin-resistant *Enterococci*
WHO: World health organization
WGS: Whole genome sequence
XDR: Extensively drug resistant
Zn: Zinc

2 List of papers

<u>**Paper 1**</u>
Zinc and Copper Reduce Conjugative Transfer of Resistance Plasmids from Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli*.
Buberg May Linn, Witsø Ingun Lund, L'Abée-Lund Trine Marie, Wasteson Yngvild.
Microbial drug resistance (Larchmont, NY). 2020;26(7):842-9.

<u>**Paper 2**</u>
Population structure and uropathogenic potential of extended-spectrum cephalosporin-resistant *Escherichia coli* from retail chicken meat.
Buberg May Linn, Mo Solveig Sølverød, Sekse Camilla, Sunde Marianne, Wasteson Yngvild, Witsø Ingun Lund.
BMC Microbiology 2021 Mar 29;21(1):94.

<u>**Paper 3**</u>
In vitro digestion of ESC-resistant *Escherichia coli* from poultry meat and evaluation of human health risk.
Buberg May Linn, Wasteson Yngvild, Lindstedt Bjørn-Arne, Witsø Ingun Lund.
Frontiers in Microbiology 2023; 14:1050143.

3 Summary

Antimicrobial resistant microbes have become a global problem. In Norway, ESC-resistant *E. coli* was discovered on retail poultry meat through the Norwegian monitoring program for antimicrobial resistance in bacteria from food, feed and animals (NORM-VET) between 2012 and 2016 but has not been detected since. This finding was surprising as cephalosporins are not used for poultry in Norway. The work of this thesis aimed to fill in knowledge gaps about the ESC-resistant *E. coli* in the poultry-consuming chain, as their survival through digestion and the risk of these AMR isolates becoming ExPEC isolates in humans. Firstly, poultry receive higher amounts of Zn and Cu than what they biologically require, and as heavy metals are known to drive resistance development the first aim of this thesis was to investigate the effect of these metals on conjugation of ESC-resistance. In contrast to the hypothesis, it was concluded that exposure to the heavy metals Zn and Cu reduced the conjugation frequency between *E. coli* in a concentration dependent manner, possibly by downregulation of conjugation related genes. Secondly, the phenotypical and genotypical potentials of these isolates to become uropathogens were investigated as AMR *E. coli* from poultry have been documented to be genetically closely related to UPEC in humans. A total of 141 isolates was included in Paper 2 to evaluate their genetic population structure, and 18 isolates were selected for further analyses of phenotypical characteristics. The study concluded that the uropathogenic potential is highly isolate dependent. Finally, little knowledge about the fate of these ESC-resistant *E. coli* if ingested, and the possible alteration of their phenotypical traits, were available in the literature. These abilities were further studied in detail by using an *in vitro* digestion model (INFOGEST) with subsequent investigation of cell-association and conjugational properties after digestion. All 31 isolates included in the third study were able to survive digestion and displayed variable changes in ability to transfer their resistance plasmids as well as altered cell-association qualities. A virulence associated gene analysis of the total genome sequences concluded that two of the 31 isolates were to be considered UPEC isolates, while a third can be regarded a hybrid pathogen. In conclusion, this work does not change that Norwegian poultry is safe to eat despite the previous finding of ESC-resistant *E. coli* on retail chicken meat. Yet it is important to continue surveillance and monitoring to avoid the occurrence and spread of such resistance in the food-production chain to ensure food that is safe for consumers.

4 Norsk sammendrag (Summary in Norwegian)

Antimikrobielt resistente mikrober har blitt et globalt problem. I Norge har ESC-resistente *E. coli* blitt oppdaget på kyllingkjøtt gjennom Norsk overvåkning for resistente mikrober i mat, fôr og dyr (NORM-VET) mellom 2012 og 2016, men ikke blitt detektert siden. Dette funnet var overraskende siden cephalosporiner ikke blir brukt til kylling i Norge. Arbeidet i denne avhandlingen hadde som mål å fylle ut kunnskapshull om ESC-resistente *E. coli* i kyllingkonsum-kjeden, overlevelsen av disse gjennom fordøyelse og risikoen for at disse isolatene kan bli ExPEC isolater hos mennesker. For det første, kylling får høyere nivåer av Zn og Cu i fôret enn hva de biologisk krever, og siden tungmetaller er kjent for å drive resistensutvikling var det første målet for denne avhandlingen å undersøke effekten av disse metallene på konjugering av ESC-resistens. I kontrast til hypotesen ble det konkludert at eksponering til tungmetallene Zn og Cu reduserte konjugasjonsfrekvensen mellom *E. coli* i takt med konsentrasjonen, mulig ved nedregulering av gener relatert til konjugasjon. For det andre, det fenotypiske og genotypiske potensialet for disse isolatene for å bli uropatogener ble undersøk siden AMR *E. coli* fra kylling har blitt dokumentert å være genetisk nært beslektet med UPEC fra mennesker. Totalt 141 isolater var inkludert i artikkel 2 for å evaluere den genetiske populasjonsstrukturen, og 18 isolater var valgt ut for videre analyser av fenotypiske egenskaper. Studien konkluderte at det uropatogene potensialet er høyst avhengig av det aktuelle isolatet. Avslutningsvis, lite kunnskap var tilgjengelig om skjebnen til disse ESC-reisstente *E. coli* hvis de blir inntatt via munnen, og den mulige endringen av fenotypiske egenskaper i litteraturen. Disse egenskapene ble videre undersøkt i detalj ved å bruke en *in vitro* fordøyelsesmodell (INFOGEST) med påfølgende undersøkelse av celle-assosiasjon og konjugasjonsegenskaper etter fordøyelse. Alle 31 isolater inkludert i den tredje artikkelen var i stand til å overleve fordøyelse og demonstrerte varierende endringer i evne til å overføre resistensplasmider i tillegg til celle-assosierte egenskaper. En virulensassosiert gen-analyse av de totale gensekvensene konkluderte med at to av de 31 isolatene kan regnes som UPEC isolater, mens et tredje kan regnes som et hybrid patogen. For å konkludere, dette arbeidet endrer ikke at Norsk kylling er trygg å spise til tross for det tidligere funnet av ESC-resistente *E. coli* på kyllingfileter. Likevel er det viktig å fortsette overvåkning og monitorering for å unngå forekomst og spredning av slik resistens i matproduksjonskjeden for å sikre mat som er trykk for forbrukere.

5 Synopsis

When the first antimicrobials were discovered and it became known that these were something that could combat infectious diseases, it was considered quite a miracle. However, the discovery of antimicrobial resistance that shortly followed was not as miraculous. Microorganisms live in competitive environments where survival not only is dependent on being the fittest to the current environment, but the most fit to change. Being dynamic and adapting to constant changes is what has secured the evolutionary success of the bacterial kingdom, and is also what has startled, inspired, and frustrated researchers and clinicians for decades. As the development of resistance is rapidly moving forward, more information is needed regarding the spread of resistance in our food producing animals to be able to secure safe food production. This next chapter will introduce you to and guide you through the topics covered by this thesis on ESC-resistant E. coli from poultry meat in Norway.

5.1 Antimicrobial resistance

Antimicrobial resistance (AMR) has been referred to as "a silent pandemic" as it is estimated that the infections caused by resistant microbes account for 33 000 deaths in the European Union each year (European commision, 2022). Alexander Fleming, who in 1945 was awarded the Nobel Prize for his discovery of penicillin, foresaw the concept of antimicrobial resistance because of the widespread use of the new medicines and warned about it. The concern of the extensive and worrisome spread of resistance due to use of antimicrobials in animal husbandry and veterinary medicine was elaborated on in 1969 in The Swann report delivered to the parliament in the United Kingdom (UK Parliament, 1969). Since then, countless scientific papers have been published providing a large knowledgebase for the origins, development and spread of AMR, in addition to suggested measures to combat and evade further resistance development. In contrast to the recent SARS-CoV-2 pandemic where knowledge was very limited, but collected rapidly, symptoms relatively easy to recognize, and prevention possible in form of vaccines, the silent pandemic of AMR is already well-known and investigated but has proven itself much harder to strike down. The World Health Organization (WHO) defines AMR as; *"when bacteria, viruses, fungi, and parasites change over time and no longer respond to medicines making infections harder to treat and increasing the risk of disease spread, severe illness and death. As a result, the medicines become ineffective and infections persist in the body, increasing the risk of spread to others"* (WHO, 2022a) and announced a global action plan (GAP) against the spread of AMR in 2015 (WHO, 2015). This was closely followed up by the United States that formulated a national action plan for combatting AMR the same year (The White House, 2015). For the purpose of this thesis, AMR will from here on be referred to in the context of bacteria, even though many of the principles are true for other microorganisms the term "antibiotics" will be used from here on.

Historically, antibiotics have been used not only for treatment of infectious diseases, but also for growth promotion in food animal production and to treat non-infectious diseases that we today would consider inappropriate. It is this overuse and misuse of antibiotics that accounts for the highest proportion of resistance development (Levy and Marshall, 2004). In 2003, the EU decided to ban feed antibiotics as growth promotors as a measure to combat AMR development (EU, 2003), with a total ban entering into effect in 2006 (EU, 2005). Today most European countries do not feed food-producing animals antibiotics without diagnosing an infectious disease (Aidara-Kane *et al*., 2018). In China, 11 feed antibiotics used in food animal production were banned in 2020 (Wen *et al*., 2022). However, such use is still widespread on other continents, and actions limiting the usage of feed-antibiotics are highly recommended, especially in regions that are affected by the highest levels of AMR such as India, Brazil and Kenya (Van Boeckel *et al*., 2019). This misuse is problematic not only because it takes a joint effort if we are to successfully overcome AMR, but because some of these countries are responsible for a large proportion of international food export and may end up exporting AMR as well (George, 2018, Van Boeckel *et al*., 2019). Reasons for this high usage of antibiotics may be numerous and varies between the different countries. Limited prescription regulation, together with large volumes prescribed for agricultural purposes, or lack of knowledge about the global AMR situation might play a role in this overconsumption of antibiotics (Cuong *et al*., 2019, Cole, 2014).

Another challenge that contributes to making measures to combat AMR harder, despite global action plans, is the lack of new commercially available antibiotics. Between the 1960s and 2000s a large innovation gap was present, where the number of novel antibiotics patented was scarce (Walsh, 2003). This has continued into the 21st century and is currently going on, where the majority of "new" antibacterial drugs are modifications of already known substances. Examples of improvement of antibiotics include adding bacterial spectra, simplifying dosing regimen, improving the safety of usage of the drug, or adding additional compounds to encounter resistance mechanisms (Silver, 2011, Sutherland, 1991).
Silver described this phenomenon as "*a discovery void*", rather than a gap in 2011 (Silver, 2011, Hyun, 2021). The last decade has shown somewhat of an optimistic turn, as the G20 countries in 2017 agreed upon intensifying the global research and development of antimicrobial molecules, including existing antibiotics (Ministers, 2017). A review published by Terreni *et al*. in 2021 presented eight new antimicrobials approved by the FDA by 2017, including a treatment for MDR *Enterobacteriaceae* infections using a Meropenem and Vaborbactam combination (Terreni *et al*., 2021). Since then, an additional 12 new antibiotics have been approved, however, as many as 10 of them belong to classes where resistance mechanisms are already established (WHO, 2022c). One of the 12 new agents, cefiderocol, has proven effective towards both Extended spectrum beta-lactamase (ESBL)-producing and AmpC Gram-negative isolates, which is very promising. Other strategies to combat antimicrobial resistance, like the use of phages, antimicrobial peptides and nanomedicine are under investigation, and represent viable options for limiting the overuse of antibacterials (Terreni *et al*., 2021). However, these alternatives are beyond the scope of this thesis, and will not be discussed further.

Despite promising results and signs of political interest, the rate of resistance is still higher than the rate of new development (Miethke *et al*., 2021). As of the most recent report, the pipeline includes 77 antimicrobials, with one of them being a novel agent, 45 traditional antibiotics and 32 non-traditional. Only six of these are considered innovative compounds (WHO, 2022). This lack of novelty is problematic because already existing resistance-mechanisms may easily adapt to the modified substance, limiting the usage of the antibiotics and risk wasting the effort and money invested in the development of the compounds (Silver, 2011). The reasons for this shift in priority is related to science moving away from the initial search for novel natural compounds and into more target-oriented research, and because of decreased profit for pharmaceutical companies (Plackett, 2020). Production of medication to treat chronic diseases is more profitable and the generic competitions have increased, in addition to the cost and time of clinical trials. Pressure is also being put on drug pricing, the industry faces consolidation and a crowded marketplace on top of a lack of industry productivity (Kraus, 2008, Livermore, 2004). The search for novel antibiotic agents is therefore currently driven by small and medium sized companies, and though several new antibiotics are expected to be approved in the years to come, many of them are likely to not make it past phase 2 and 3 due to increased costs (Terreni *et al*., 2021). Joined, these scientific, regulatory and economic barriers highly limit the development of new antibiotics, and with that our future treatment alternatives as AMR emerges (Dutescu and Hillier, 2021).

A comprehensive analysis of the global burden of AMR was published in The Lancet in 2022 (Murray *et al*., 2022). The number of deaths attributable to resistance in 2019 were estimated to 1.27 million in a scenario where AMR infections were susceptible, and 4.95 million deaths in a scenario of no infections. In both scenarios six pathogens were responsible for more than 70% of the infections (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii and Pseudomonas aeruginosa*) (Murray *et al*., 2022). All this taken into account and viewed in the light of the final report on AMR by O'Neill that estimated over 10 million fatalities by 2050 due to antibacterial resistance, largely due to Gram-negative bacteria (O'Neill, 2016), there is no doubt that this is a race against time. Further measures are urgent to slow down resistance development and prevent fatal outcomes of once treatable infections.

The following sections will provide insights to the different antibiotics and their resistance mechanisms with emphasis on the beta-lactams, information about how such resistance is able to spread, and their relation to food safety.

5.1.1 Antibiotics and antibiotic resistance

Antibiotics have, as their name implies, antibacterial effects, and are used to treat bacterial infections in humans and animals. They are not only wonderful clinical tools but have also shown to have growth promoting effects when added at subclinical levels in animal feed (Gonzalez Ronquillo and Angeles Hernandez, 2017). The negative impact of such usage in the light of resistance development will be drafted later. The origin of most known antibiotics are natural occurring compounds produced by for example fungi or other bacteria (Jakubczyk and Dussart, 2020). However, synthetically derived compounds have also been commercialized (Wright *et al.*, 2014). Antibiotics and similar substances produced by microorganisms are part of the neatly balanced microbial ecosystem. When produced by a microorganism antibiotics function as protection, providing advantages when competing over resources and contributing to the survival of the species. As stated by Bennet *et al.* *"...That bacteria have not only survived but adapted and proliferated impressively to colonize some of the most inhospitable parts of the planet attest to their powers of adaption. It should come as no real surprise that they have developed powerful DNA-modifying strategies that greatly facilitate the process of adaption and, hence, evolution."* (Bennett, 2008). This section will provide an overview of the most used classes of antibiotics, their function, spectrum, and indications.

Bacteria are single-cell organisms containing of a cell-membrane and/or cell-wall that surrounds the cytoplasm where all the essential cell machineries are located. Bacteria do not contain a core nucleus or large organelles but keep its genetic material either freely floating in the cytoplasm or attached to the cell wall. Like eukaryotic cells the cytoplasm contains ribosomes and other important cellular structures required for a fully functional cell-machinery and are thus capable of self-replication (Greening and Lithgow, 2020). Variations are found between species, and we usually distinguish between Gram-positive and Gram-negative bacteria. Together they account for approximately 90% of known bacteria and are surrounded by a cell-wall (the exception is *Mycoplasma* that does not have a cell-wall, only surrounded by a plasma membrane) (Kornspan and Rottem, 2012). The Gram-positive cell-wall is though and rigid while the Gram-negative is thinner but re-enforced by being surrounded by a lipid membrane called the outer membrane. This outer membrane is protective against environmental stressors and make cell-wall penetration hard for many of the known antibiotics, protecting the Gram-negative bacteria. Communication and transport through the cell-wall and outer membrane are secured through porins (Dörr *et al.*, 2019).

Antibiotics are grouped based on their targets and mechanisms of action (MOA), and sometimes based on their origin (Kohanski *et al.*, 2010). Bactericidal antibiotics directly kill the bacteria, while bacteriostatic antibiotics inhibit the growth of the bacteria. In general, the mechanisms can be summarized in three groups targeting either: protein synthesis, nucleic acid synthesis or the cell wall synthesis. In short, this is achieved by targeting e.g., folate synthesis, DNA gyrase or RNA polymerase, the 50S subunit, or the 30S subunit of the ribosome, or the cross linking of the cell-wall and thereby cell wall synthesis.

a) *Antibiotics that target protein synthesis*

The bacterial ribosome consists of two subunits; the 50S and 30S subunits, and antibiotics that inhibit protein synthesis may target either of the two. Antibiotics that target the 50S subunit include macrolides, lincosamides, streptogramins, amphenicols and oxazolidinones, while tetracycline and aminoglycosides target the 30S subunit (Katz and Ashley, 2005).

- Chloramphenicol inhibit translation by binding to the A site of the 50S subunit and is considered a broad-spectrum antibiotic effective against both Gram-positive Gram-negative bacteria (Schwarz *et al*., 2016).
- Lincosamides target translation of proteins causing truncated peptide chains and are used against Gram-positive infections, including effect against anaerobes and beta-lactamase producing Gram-positive bacteria (Spížek and Řezanka, 2017).
- Macrolides are bacteriostatic and inhibit translation by targeting 23S rRNA in the 50S subunit, causing truncated peptide chains (Vázquez-Laslop and Mankin, 2018). The spectrum is broad and target Gram-positive cocci and some Gram-negative pathogens including *Campylobacter, Haemophilus, Mycoplasma, Ureaplasma, Chlamydia, Legionella* and *Coxiella* (Aronson, 2016a).
- Streptogramins have a similar mechanism as the macrolides and bind reversibly to the 50S subunit, are therefore considered bacteriostatic and used to treat Vancomycin-resistant infections (Aronson, 2016b).
- Oxazolidinones limit translation by binding to 50S, preventing formation of a functional 70S subunit (Swaney *et al*., 1998). They are used to treat multi-drug resistant (MDR) Gram-positive infections including *Streptococci* resistant to penicillin, and Vancomycin-resistant *Enterococci* (VRE) (Foti *et al*., 2021).
- Tetracycline inhibits translation by binding to 16S rRNA of the 30S ribosomal unit, preventing tRNA binding to 30S at the A site (Markley and Wencewicz, 2018). Broad spectrum targeting both Gram-positive and Gram-negative bacteria, including *Chlamydiae, Mycoplasma, Rickettsiae* and even some protozoan parasites (Chopra and Roberts, 2001).
- Aminoglycosides (gentamicin, streptomycin and kanamycin) are bactericidal, concentration-dependent and targets the 30S subunit causing misreading and/or truncated proteins leading to cell death (Baquero and Levin, 2021). They are effective against both Gram-positive and Gram-negative bacteria, and were used as a first-line antibiotic before the discovery of cephalosporins (Krause *et al*., 2016).

b) *Antibiotics that target nucleic acid synthesis*

Sulfonamides, trimethoprim, rifamycin and quinolines including the fluoroquinolones interfere with DNA synthesis by targeting specific enzymes involved in the process (Cambau and Guillard, 2012).

- The quinolones, including fluoroquinolones target topoisomerase II and topoisomerase IV by trapping these enzymes at the DNA cleavage stage and prevent strand re-joining, leading to DNA breaks and cell death

(Drlica *et al*., 2008). They are used to treat infections with aerobic Gram-positive and Gram-negative bacteria. Frequently used for respiratory and urinary infections, septicemia, joint and bone infections, soft tissue infections, typhoid fever, anthrax, bacterial gastroenteritis and other severe infections (Pham *et al*., 2019).

- Rifamycin is a group of semi-synthetic antibiotics that inhibit the RNA synthesis by binding to RNA polymerase, leading to failed nucleic acid metabolism and subsequent cell death (Campbell *et al*., 2001). Spectrum includes both Gram-positive and Gram-negative bacteria and is often used to treat *Mycobacterium tuberculosis* (Maslow and Portal-Celhay, 2015).
- Trimethoprim affects one-carbon (C1) metabolism and folate synthesis by inhibiting dihydrofolate reductase, blocking production of tetrahydrofolate (Gleckman *et al*., 1981). It is almost always used in combination with sulfonamides which stop dihydrofolate acid synthesis by inhibiting dihydropteroate synthase and arresting cell growth (Sköld, 2000). This combination results in reduced mutation rate for resistance and is considered bacteriostatic.

c) *Antibiotics that target cell wall synthesis*

The group of antibiotics that target synthesis of the bacterial cell wall includes the beta-lactams, vancomycin, bacitracin and cycloserine. Because their target is not present in mammalian cells, they are considered very safe to use with few side-effects (Sarkar *et al*., 2017).

- Glycopeptides like vancomycin inhibit crosslinking and synthesis of peptidoglycan by binding to D-alanyl-D-alanine in the peptide chain (Zeng *et al*., 2016). They are considered a last-resort antibiotic to treat Methicillin-resistant *Staphylococcus Aureus* (MRSA) and have a narrow spectrum targeting the Gram-positive cocci.
- Colistin bind to lipid A in lipopolysaccharide permeabilizing the outer membrane causing cell death and is considered bactericidal (Andrade *et al*., 2020). The spectrum is narrow targeting mainly Gram-negative isolates, especially MDR isolates of *Acinetobacter, Pseudomonas* and *Klebsiella* (Lim *et al*., 2010, Alfei and Schito, 2022).
- Beta-lactam antibiotics inhibit transpeptidation by binding to PBPs, leading to lysis and cell death, and will be further explained later.

In addition to the three main groups of mechanisms we have the group of daptomycin that target the cell membranes. Daptomycin is inserted in the cell membrane, causing depolarization reducing the ability to create ATP and cell death. It is bactericidal and target Gram-positive bacteria (Heidary *et al*., 2018).

5.1.1.1 Beta-lactam antibiotics

The WHO have defined which antibacterial groups that are considered very important, highly important and critically important in the global perspective due to resistance development (AGISAR, 2019). The group of beta-lactams includes the penicillins, monobactams, carbapenems and the cephalosporins, all of which are considered highly or critically important antibiotics (AGISAR, 2019). Beta-lactams were the most frequently prescribed antibiotics for humans, both in hospitals and the community, in Europe in 2021 (ECDC, 2022). They are bactericidal and used to treat some of the most severe infections encountered with a spectrum ranging from narrow to broad, and the group is effective towards Gram-positive and Gram-negative bacteria alike. The peptidoglycans of the cell wall of Gram-positive and Gram-negative bacteria are crosslinked forming a structured network connected by penicillin-binding-protein (PBP). Beta-lactams target the PBP in the cell wall by covalently binding irreversibly to PBP, disturbing the network formation. As maintenance of the cell wall is a continuous process of breaking down and rebuilding the peptidoglycan network, the enzymes responsible for breaking down are still active leading to a form of self-destruction (Alfei and Schito, 2022). When disruption of the peptidoglycan layer occurs, the cell wall loses its structural integrity which results in lysis of the bacterium (Bush and Bradford, 2016). Differences in bacterial spectra are due to the antibiotic's relative affinity for different PBPs. PBPs varies between bacterial species and range from three to eight enzymes per species and are divided into classes on basis of molecular mass (Georgopapadakou and Liu, 1980, Bush and Bradford, 2016).

The beta-lactams have a mono- or bicyclic structure comprising a four-term beta-lactam nucleus. Each class was originally identified as a natural product, however, several modifications and semi-synthetic derivates have been developed since. Table 1 shows an overview over beta-lactam antibiotics as categorized by Alfei *et al*. (Alfei and Schito, 2022) and is inspired by Tooke *et al*. (Tooke *et al*., 2019).

Table 1: Schematic overview of the group of beta-lactam antibiotics, their structure, origin, indication and spectrum. Further subdivision of the groups exists but are omitted for simplicity. Categorized by the WHO as the following:
* Important, ** Highly important and *** Critically important.

Betalactams	Structure and origin	Indications and spectrum
Penicillins Narrow spectrum** - 1st generation - 2nd generation Extended spectrum*** - Aminopenicillins - Carboxypenicillins - Ureidopenicillins - Other Anti-staphylococcal** (Pandey and Cascella, 2022)	Beta-lactam ring is fused to a thiazolidine ring. Fungi Penicillin, described in 1929 by Alexander Fleming (Fleming, 1929).	Syphilis, *N. meningitides* infections, Skin and soft tissue infections, Endocarditis, *Streptococcal* pharyngitis, *S. pneumonia* and *S. meningitis.* Aminopenicillins: Upper respiratory tract infections, *E. faecalis, Listeria* infections, together with beta-lactamase inhibitors, intra-abdominal infections.
Carbapenems*** Ertapenem Doripenem Imipenem Meropenem *Etc.*	Bicyclic system completed by a five-membered pyrroline. Olicanic acid by Brown and coworkers in 1976 (Brown *et al.*, 1976).	Pneumonia, intra-abdominal infections, UTIs, Meningitis. Broad spectrum, effect towards many MDR-bacteria.
Monobactams Azetreonam Tigemonam Carumonam Nocardicin	Monocyclic systems where the beta-lactam ring is not fused to another molecular ring. Sykes and Imada in 1981 (Sykes *et al.*, 1981).	Nosocomial infections, UTIs. Aerobic Gram-negative pathogens.

Cephalosporins *Further described below* 1st generation** - Cefazolin, cefalexin, etc. 2nd generation** - Cefaclor, cefuroxime, etc. 3rd generation*** - Cefixime, ceftriaxone, cefotaxime, ceftazidime, etc. 4th generation*** - Cefepime, cepirome, etc. 5th generation*** - Ceftaroline fosamil, ceftolozane, ceftobiprole, etc.	Fusion partner is a six-membered dihydrothiazine. Fungi Cephalosporium, by Newton and Abraham in 1954 (Newton and Abraham, 1954).	Cephalosporins: 1st gen: Skin and soft tissue infections and perioperative prophylaxis. 2nd gen: Upper respiratory tract infections, perioperative prophylaxis. 3rd gen: Pneumonia, meningitis, urinary tract infections, Streptococcal endocarditis, Gonorrhoea, severe Lyme disease. 4th and 5th gen: Severe pneumonia, skin and soft tissue infections.

Beta-lactam antibiotics target PBP by mimicking D-alanyl D-alanine portion of peptide chain normally bound to PBP. PBP interacts with the beta-lactam ring leaving it unavailable for synthesis of new peptidoglycans which in turn leads to lysis of the cell. The cephalosporins belong to the beta-lactams and were historically further divided into three groups dependent on their spectra. 1st generation, 2nd generation and 3rd generation respectively, where 3rd generation has the broadest spectrum. 4th and 5th generation cephalosporins have also been produced in recent years (Tooke *et al*., 2019). They are bactericidal and kill bacteria by inhibiting cell wall synthesis. This is carried out by competitive inhibition of the transpeptidase enzyme, preventing the final crosslinking of the peptidoglycan layer causing rupture of the cell-wall and subsequent cell-death. 3rd, - 4th- and 5th-generation cephalosporins are also referred to as extended-spectrum cephalosporins, with increased hydrolytic stability to the common penicillinases SHV-1 and TEM-1 beta-lactamase (Martínez José, 2008).

Beta-lactamase inhibitors are substances developed as supplements to antibiotics to counter the bacterial production of beta-lactamases. In general, the beta-lactamase inhibitors have no effect alone, even though some antibacterial effects have been described for a few of them (Wise *et al*., 1978, Jacoby and Sutton, 1989). The first beta-lactamase inhibitor, Clavulanic acid, was introduced in the mid 1970’s (Reading and Cole, 1977). The clavam structured Clavulanic acid inhibits a broad spectrum of staphylococcal penicillinases, and that acts synergistically with penicillin. Others include sulbactam and tazobactam. Sulfone inhibitors like sulbactam, and non-betalactam-betalactamase-inhibitors, like avibactam, do not induce chromosomally mediated AmpC-beta-lactamases (Tooke *et al*., 2019).

5.1.1.2 Resistance mechanisms

Over the million years that bacteria have evolved, they have dealt with a large variety of substances and harsh conditions that have threatened their survival. It is therefore not hard to imagine that bacteria are designed to resist rapid changes in their environment, and adapt to threats, such as introduced antibacterial agents. Since bacteria lack cognitivity it is fair to assume that these genetic changes facilitating adaptation are made at random. Mutations that provide advantages are selected for, while less advantageous properties are eliminated from the population (Bennett, 2008). These mutations arise either by point mutations, insertions, deletions, duplications or repetitions of nucleotides in the bacterial genome, including extra-chromosomal genetic elements like plasmids (Munita and Arias, 2016). This evolutionary process is not only maintained by mutations in the presence of antibiotics, or spread by clonal transmission, but is highly driven by horizontal gene transfer (HGT) (Arnold *et al*., 2022). By HGT mechanisms bacteria acquire new genetic material, sometimes in large quantities, that plays a profound role in the dissemination of AMR genes. The mechanisms and elements involved in HGT will be elaborated in section 5.1.1.4 An important distinction when it comes to resistance is the difference between intrinsic and acquired resistance (see text box).

The different strategies bacteria use to resist killing by antibiotics can be divided into 1) modification of the antibiotic molecule, 2) decreased antibiotic penetration and efflux, 3) changes in target sites, and 4) resistance due to global cell adaptions. An additional strategy that bacteria may carry out is a locally limited access to target proteins, for example by Gram-positive bacteria that produce competitive proteins that occupy the binding-site for tetracycline on the ribosomes (Nikaido, 2009). While the formation of biofilm may not be regarded as a direct

Intrinsic vs. acquired resistance

Antibiotic resistance can be divided into *Intrinsic* and *Acquired* resistance. When bacteria develop or *acquire* resistance it is the process where previously effective antibiotic agents become ineffective due to adaption and evolution within the bacteria, either by mutations or by *acquiring* mobile genetic elements through horizontal gene transfer. *Intrinsic* resistance on the other hand is that the given antibacterial agent does not display any effect on the bacterial species. It is related to the general physiology of the organism, either by lack of target within the bacteria, inaccessibility into the cell or by active pumping out by chromosomally coded efflux pumps. Biofilm-formation has been regarded as a form of intrinsic resistance mechanism. (Kostyanev and Can, 2017)

mechanism of resistance, it is still another strategy that offers bacterial communities physical protection and increase their tolerance to antibiotics (Cadena *et al*., 2019), but this will not be further discussed. The following gives an overview of the mechanistic basis of antimicrobial resistance as described by Munita *et al* (Munita and Arias, 2016).

1) *Modification of the antibiotic molecule*.
   The first strategy to cope with antibiotics is to actively target the antibiotic molecule, either by chemical alteration or by enzymatic destruction of the molecule. Chemical alteration includes inactivation by phosphorylation (aminoglycosides, chloramphenicol), acetylation (aminoglycosides, chloramphenicol, streptogramins) or adenylation (aminoglycosides, lincosamides). Destruction of the antibiotic may also be carried out by production of specialized enzymes, often referred to as antibiotic destructases. This is a common mechanism in several bacterial species to gain resistance towards tetracyclines (Markley and Wencewicz, 2018). Another well-known example is beta-lactamases that hydrolyze the beta-lactam ring, which will be described in detail later. These enzyme-encoding genes are frequently found on plasmids.

2) *Decreased antibiotic penetration and efflux.*
   The second mechanism stops the antibiotic molecule reaching intracellular targets either by decreasing the permeability of the cell wall, or actively pumping out through efflux pumps. Increased efflux of lethal substances can be achieved by upregulating efflux pump genes, with a subsequent increase in number of efflux pumps located in the cell wall (Neuberger *et al*., 2018). Some efflux-pumps are substance-specific, but most of them are multidrug-transporters and contribute to multi-drug resistance (Alav *et al*., 2018) Gram-negative bacteria will also practice decreased influx with the same result of limiting the amount of cytoplasmic antibiotics. Changing the outer membrane permeability is also an effective measure to prevent diffusion of antibiotics that are not dependent on porins for cell-entry (Delcour, 2009).

3) *Changes in target site.*
   The third strategy is by altering the antibiotic target and making binding impossible. This is achieved either by modification of the target site, or by target protection. Target protection stops the antibiotic from reaching the target binding site, a mechanism that often affects tetracycline, fluoroquinolones and fusidic acid. Genes encoding for these mechanisms are commonly located on mobile genetic elements (MGE). Modification of the target site is achieved either by point mutations in the target-encoding genes, by enzymatic alteration of the binding site, or replacement or bypass of the target site. Even minor changes may result in improper binding and loss of activity. An example of this is the *Staphylococcal* acquisition of the *mecA* gene that encodes a PBP that has a lower affinity for all beta-lactam antibiotics (Hiramatsu *et al*., 2013).

4) *Resistance due to global cell adaptions.*
   This is a recently described mechanism, that is still the subject of extensive research to fully understand the underlying mechanism. As bacteria have co-existed with the host immune system and competed with residing microbiota, they have developed mechanisms to avoid disruption of important cellular processes to maintain metabolism and homeostasis. It is especially relevant for the expression of daptomycin resistance as daptomycin is an antibiotic closely related to cationic antimicrobial peptides produced by the innate immune system. It has been suggested that mutations in genes involved in cell wall biogenesis is responsible for phenotypic resistance towards daptomycin (Taylor and Palmer, 2016, Pogliano *et al*., 2012).

It is not uncommon that several of the mentioned mechanisms are present in one single bacterial strain, with the combined result providing clinical resistance to a given antibiotic (Hamed *et al*., 2018). Magiorakos *et al* suggested in 2012 definitions such as "multidrug resistant (MDR)", "extensively drug resistant (XDR)" and "pandrug-resistant (PDR)" to distinguish this further. MDR is used for bacterial isolates who have acquired non-susceptibility to at least one agent in three or more antimicrobial categories. XDR is used for acquired non-susceptibility to at least one agent in all but two or fewer antimicrobial categories. The last term PDR is used for bacteria non-susceptible to all agents in all antimicrobial categories (Magiorakos *et al*., 2012). Building on this, additional definitions have been proposed, such as "usual drug resistance (UDR)" for describing isolates who are not fully susceptible but can be treated with standard therapies (McDonnell *et al*., 2016), and "difficult-to-treat resistance (DTR)" defined as resistance to all of the typical first-line, lower toxicity antimicrobial agents. DTR include the fluoroquinolones and the beta-lactams, including carbapenems and beta-lactamase inhibitor combinations (Kadri *et al*., 2018).

5.1.1.3 Beta-lactam resistance

To resist killing by beta-lactam antibiotics bacteria have evolved to use all the previously mentioned mechanisms; changing the active site of PBP, downregulate the porin channels in the outer membrane of the Gram-negative cell wall, and actively pumping out the beta-lactams using MDR-efflux pumps (Tooke *et al*., 2019). The most common form of beta-lactam resistance is the production of enzymes that hydrolyze the beta-lactam ring making it ineffective and is more often produced by Gram-negative bacteria than Gram-positive (Götte *et al*., 2017). However, clinical resistance towards beta-lactam antibiotics is often multifactorial and consists of a combination of changes in permeability and/or efflux pumps, and production of beta-lactamases.

Beta-lactamases function by hydrolyzing the beta-lactam amide group, also called the beta-lactam-ring in the beta-lactam molecule. This breaks the structure of the molecule and make the antibacterial agents ineffective (Alfei and Schito, 2022).

These enzymes recognize all beta-lactam compounds consisting of the beta-lactam-ring. Newer synthetic beta-lactam alternatives have therefore been developed without the beta-lactam-ring to avoid this resistance mechanism. Two common classification schemes of the beta-lactamases are acknowledged; Ambler and Bush-Jacoby-Medeiros (Bush *et al*., 1995, Ambler, 1980). According to Ambler beta-lactamases are divided into four classes (A, B, C and D) based on the amino-acid structure (Ambler, 1980). Class A, C and D uses a serine-bound acyl ester intermediate (SBLEs) to hydrolyze the beta-lactam antibiotics, while class B require zinc for cleavage and are called metallo-beta-lactamases (MBLEs). Jacobys functional classification divide beta-lactamases into three groups based on the degradation of beta-lactam substrates and the effects of inhibitors; the cephalosporinases (corresponding to Ambler class C), the broad-spectrum, inhibitor-resistant and extended-spectrum beta-lactamases (Ambler class A and D) and the metallo-beta-lactamases (Ambler class B), all with respective subgroups (Bush and Bradford, 2016, Bush *et al*., 1995). Important enzyme families in class A are TEM, SHV, CTX-M and KPC, for class B NDM and VIM are included, class C have the CMY and ADC enzymes, while class D only contain OXA (Tooke *et al*., 2019). Genes that encode beta-lactamases are named *bla* followed by the name of the specific enzyme (Bradford *et al*., 2022). Enzymes have been named on the basis of molecular characteristics or functional properties (Jacoby, 2006). To begin with they were named after the bacteria or plasmid that produced them, but since then names have included discovery location, patient-names, or names of the discoverers. Recently a consensus on naming newly discovered beta-lactamases was published by Bradford and colleagues and more than 4300 unique beta-lactamases have been identified (Tooke *et al*., 2019, Bradford *et al*., 2022). Beta-lactamase genes may be located both on plasmids or MGE's and as an integrated part of the bacterial chromosome (Barlow and Hall, 2002). The key to the success of such enzymes is their ability to expand their spectrum as new substances are introduced as treatment, in addition to the excessive spread through MGEs.

Enzymes with a phenotypic extended spectrum of activity (often termed ESBLs), in addition to carbapenemases, have been discovered within all classes, and further evolution is continuously ongoing (Castanheira *et al*., 2021). Strictly speaking the term ESBL includes beta-lactamases with extended spectrum within class A and class D, but as a working definition ESBL have also been used for the other beta-lactamase classes when activity beyond the defined spectrum have been discovered (Giske *et al*., 2009, Bush *et al*., 2009). This rapid development, together with inconsistent use of the term ESBL may cause confusion, but only highlights the clinical importance of focusing on such resistance development. Infections caused by ESBL-producing *Enterobacteriaceae* (including ESC-resistance) were in 2017 announced as critical by the WHO in their priority pathogens list for research and development of new antibiotics (WHO, 2017). *Enterobacteriaceae* have been shown to produce all classes of beta-lactamases (Alfei and Schito, 2022).

AmpC beta-lactamases are encoded by the *ampC* gene frequently carried on plasmids and in the chromosome of many *Enterobacteriaceae*. They hydrolyze cephalosporins, are resistant to clavulanic acid, but sensitive to cephamycins. Important for this group is that the resistance phenotype is inducible, meaning that

the gene is not expressed without the presence of an antibiotic or as a result of a mutation in the chromosome/plasmid. Expression of the gene will cause elevated MICs for the susceptible beta-lactam antibiotics. AmpC with extended spectrum have been described, and these are called Extended spectrum cephalosporinases. This is especially important for the families CMY, FOX and DHA, that have shown an increasing global dissemination. This thesis will focus on *E. coli* with and AmpC phenotype and the plasmid mediated resistance gene *bla*$_{CMY-2}$, in this setting called Extended spectrum cephalosporin-resistant *E. coli*. The *bla*$_{CMY2}$-gene originates from *Citrobacter freundii (Wu et al., 1999)* and often occur on plasmids (Ferreira *et al*., 2017). Pires *et al* documented a globally increased prevalence of *bla*$_{CMY-2}$, in both bovine, swine and poultry in publicly available genomes between 1980 and 2018 (Pires *et al*., 2022). Others have suggested that *bla*$_{CMY2}$ are more diverse when isolated from poultry than from other species (Carattoli, 2009).

5.1.1.4 Horizontal gene transfer

The process where bacteria share genes and potentially are provided with an evolutionary advantage is called horizontal gene transfer. It includes the mechanisms of transformation, transduction, and conjugation (Soucy *et al*., 2015). Even though mutations, genetic drift and selection occur vertically, and bacteria have short generation intervals leading to rapid evolution, it is HGT that gets accredited for the fast adaption to a changing environment. These mechanisms are also the main reasons for successful spread of MDR, and even if resistance and virulence traits often are related to HGT, its function extends far beyond the disease-causing organisms, and may represent the primary source of novel genetic material within the bacterial kingdom (Soucy *et al*., 2015).

The classical mechanisms of HGT will be explained briefly, while the process of conjugation will be elaborated in further detail for the purpose of this thesis. Common to all is the transfer of single-stranded or double-stranded DNA that moves from one bacterial cell to another, often code for virulence genes or AMR determinants and are called mobile genetic elements (MGE) (Arnold *et al*., 2022). The term "*mobile genetic elements*" is used for a wide range of elements that varies in size and mechanism; the jumping elements are not facilitated by active replication and cell division compared to for example the large plasmids which encode their own replication mechanism and can hold up to 2.5 Mb in length (Shintani *et al*., 2015). Transposable elements include IS elements, transposons, and transposing bacteriophages, which are generally small in size, ~2.5, ~5 and up to 500 kb in length respectively (Dion *et al*., 2020, Mahillon and Chandler, 1998). Resistance transposons are MGEs that holds a resistance gene and move from one site to another within a DNA molecule or to a separate DNA element within the same cell, for example from a plasmid to the chromosome, and vice versa (Khedkar *et al*., 2022). Integrons capture genes and utilize site-specific recombination in contrast to the transposition mechanisms. The integrase enzyme together with specific gene cassettes are integrated to new DNA strands. Integrative conjugative elements (ICEs) integrate to the host genome and are approx. the same size as phages (up to 500 kb) (Cury *et al*., 2017). In addition to horizontal sharing of genetic material,

successful resistance spread also includes clonal expansion of the successful species and selection pressure from the usage of antibiotics and other drivers.

Transformation

Transformation is the uptake of exogenous DNA from the environment and does not require cell-to-cell contact. Genetic information either from plasmids or dead bacteria is absorbed into the cell through endocytosis. A pre-requisite for this process to be carried out is that the recipient cell must be competent to receive such exogenous DNA. Natural competence is genetically regulated, involves specific competence proteins, and is often combined with a stagnation of growth to be able to carry out the DNA-uptake (Blokesch, 2016). The foreign genetic material is absorbed into the recipient cell, and integrated to the genome, either the chromosome or a plasmid. It is most common for bacteria to obtain DNA from similar bacteria due to limitations by homologous recombination.

Transduction

Transduction is dependent on bacteriophages, small viral particles, that act as vectors, transporting genetic material from one cell to another. This process is divided into two mechanisms: generalized transduction and specialized transduction. Generalized is when phages pick up any portion of the host's genome, while specialized includes only specific portions of the DNA. The DNA is then able to either recombine into the chromosome or replicate as a plasmid when transferred to the new host cell. A recent mechanism of lateral transduction has also been described that can transfer DNA at 1000-fold greater frequencies, that compared to generalized and specialized transduction, not necessarily include lysis of the host bacteria, but rather a more controlled copy and transfer mechanism (Chiang *et al*., 2019).

Conjugation

Conjugation is a much more complex mechanism of HGT and involves direct contact between two bacterial cells where genetic material is unidirectionally transferred. The genetic material comes in the form of MGE's, such as ICEs or conjugative plasmids. What separates the genetic elements transferred through conjugation from the other mechanisms is the large size of the material transferred, where the conjugative elements often code for the structures required for the transfer. The mechanism of conjugation and the respective structures involved in the process will be elaborated in the next section.

HGT resemble a bacterial version of sexual recombination as seen in eukaryotes However, it occurs not only within a single species, but also across species barriers. It has even been documented that HGT occurs between bacteria and fungi, plants or animals (Rancurel *et al*., 2017). Instead of being dependent on single point mutations, acquisition of single genes or combination of genes, that may be expressed quickly, allow for rapid evolution. The genetic material involved in HGT can provide novel genetic combinations, that may result in increased resistance,

virulence, or survival in the recipient strain (Hall *et al*., 2020). However, accepting foreign genetic material is a potential risky business for an organism as some selfish MGEs, e.g. transposons may be deleterious or cytotoxic (Baltrus, 2013). Beneficial resistance-genes more often gets incorporated into the genome (Vos *et al*., 2015). With the growing understanding of the underlying mechanisms of HGT it has become evident that "the tree of life" more likely resemble a "network of life" as these mechanisms have the capability to link distant branches of organisms together by sharing of genetic material (Rancurel *et al*., 2017).

Resistance- and virulence genes tend to accumulate on the same MGEs and have resulted in bacteria acquiring resistance genes active toward several classes of antibiotics at the same time. An example of this is the well-known Meticillin-resistant *Staphylococcus Aureus* (MRSA), that acquired a plasmid from *Enterococcus faecalis* and became resistant towards Vancomycin, the last resort treatment option used to combat MRSA (Gardete and Tomasz, 2014). When a bacteria receive a plasmid containing several resistance genes, these organisms with a combined resistance mechanisms are often called "superbugs" because effective treatment is absent or very limited. This is why HGT gets accredited for being the main driver of the rapid resistance development in bacteria. Mulvey *et al.* reconstructed a transmission event of the $bla_{KPC\text{-}3}$ gene through conjugation, from a patient infected with carbapenem-resistant *Klebsiella* and *Escherichia* (Mulvey *et al*., 2016). Strong selective pressures such as the use of antibiotics increases spread of MDR through HGT to ensure bacterial survival. Kröger *et al* demonstrated multiple examples of antibiotic-induced gene transfers, illustrating the rapid spread of ARGs through HGT, and the importance of understanding how clinical use, misuse and overuse affect this development (Kröger *et al*., 2018).

5.1.1.5 Conjugation

Conjugation allows bacteria to share genetic information to facilitate rapid spread of beneficial characteristics ensuring survival (Strahilevitz *et al*., 2009). The Gram-negative conjugation process will be described here; however, it is important to note that a similar process occurs in the Gram-positive organism. It involves direct contact between two bacterial cells, where communication is accomplished through a linkage between the cells using a sexpilus, mediated by the type 4 secretion system (T4SS), that together with the relaxosome are referred to as the mating apparatus (see below). Genetic material is then unidirectionally transferred from the donor-strain to the recipient-strain. The machinery required to facilitate this process is usually encoded on plasmids or ICEs, which in general tend to be smaller in size in the Gram-positive organism, compared to the Gram-negative, thus suggest differences in the cell-to-cell contact (Bennett, 2008). Conjugation may take credit for the wide genome plasticity displayed by bacteria, where especially the spread of AMR genes is highly related to conjugative mechanisms, and it has been thought to happen at a high rate among *Enterobacteriaceae* in the gut microbiota (Neil *et al*., 2021).

For a long time, the pilus was the only observable part of the conjugation machinery, but recently with modern methods even the smallest part of the machinery has been both purified and described (Amin *et al*., 2021). Particularly the review by Waksman explains the process of mating pair formation in detail (Waksman, 2019).

Relaxosome

The first step of the conjugation is binding of the relaxosome to the "origin of transfer" (OriT) on the plasmid DNA. It contains a key protein called "relaxase" that contributes by catalyzing a nicking reaction at the *nic* site and binds to the T4SS by a coupling protein. It is also responsible for terminating the conjugation.

Type 4 secretion system (T4SS)

The molecular structure of the T4SS in Gram-negative bacteria has been described in great detail by Ilangovan and colleagues (Ilangovan *et al*., 2015). It has been shown to have several functions and are able to transport both proteins and DNAs (Ilangovan *et al*., 2015, Waksman, 2019). It is one of six secretion systems in Gram-negative bacteria. The structure is made of the outer-membrane core complex (OMCC) that is connected to the inner-membrane complex (IMC) and powered by at least two ATPases. The pilus is also considered a part of the T4SS system.

Pilus

The last element is the pilus that assembles into a large helical filament, also termed the "sexpilus". It has been hypothesized to either serve as attachment, or a conduit for ssDNA transport, or both, and has been detected in most bacterial conjugation systems. Some authors have suggested that they are retractable, and that after achieving contact with the recipient cell, they pull the two cells tightly together. The assembly of the pilus requires energy, however the retraction does not, and has been suggested to happen spontaneously (Frost *et al*., 1985).

5.1.1.6 Plasmids

Plasmids are considered the most successful vectors of HGT. It has been defined by Waksman *et al* as "*A collection of genetic modules organized into a stable, usually circular, self-replicating replicon, which does not usually contain genes essential for cell functions*" (Waksman, 2019). Plasmids exist separately from the bacterial chromosome, but benefits from replication functions in the host cell, such as ribosomes etc. The genes they carry do not include genes essential for cell growth and replication, but those that are useful for the bacterial cell in specific (or extreme) environmental conditions. Extremes in the form of presence of antibiotics are considered for the purpose of this thesis.

The most common plasmids consist of circular double-stranded DNA (Bauernfeind *et al*., 1996). All from 2 to 400 genes or more have been described (Charlebois, 1999). The genetic material is structured in a backbone with additional accessory genes. Plasmid backbone consists of a core genetic locus, which is often used to group the plasmids, and is associated with key plasmid-specific functions such as replication (replicon-typing) and mobility (MOB typing). During conjugation the plasmids will promote their own transfer. Mobilizable plasmids with few genes are often small (> 10 kb) and are dependent on a conjugative element to be able to transfer. Conjugative plasmids are often larger (30-100 kb) because the functions of the T4SS also need to be included for conjugation to take place, as described previously.

The accessory genes code for clinically relevant traits. If a plasmid carries an AMR-gene, it is considered a resistance plasmid. A similar definition goes if the plasmid carries a virulence gene (virulence plasmid). Sometimes several genes coding different functions are carried on the same plasmid, if one such gene is selected for, other functions may follow facilitating co-selection. Another function that plasmids provide besides the spread of beneficial gene variants, is the reassembling and recombination of these genetic elements. This provides an even more rapid evolution than the one already carried out by bacteria with their short generation time, allowing a more active adaption to new niches, as previously mentioned (Bennett, 2008).

Plasmid typing is beneficial because it may provide epidemiological information essential to understand and potentially limit the rapid spread of AMR traits within bacteria (Coluzzi *et al*., 2022). Replicon and mobility (MOB) typing are the most common classification schemes for plasmids (Orlek *et al*., 2017). Replicon typing is based on the backbone loci encoding plasmid replication and have classically been divided by incompatibility (Inc) groups, either by incompatibility testing, by replicon probe hybridization, PCR-based replicon typing (PBRT), replicon subtyping or by *in silico* replicon typing/subtyping. Inc grouping is based on the principle that two plasmids that share common replication elements are unable to persist in the same cell. Within *Enterobacteriaceae* we have 26 known Inc groups (Johnson *et al*., 2007). Plasmids from IncF, IncI, IncA, IncC and IncH are the most abundant plasmids, especially found in bacteria isolated from human and animal sources (Rozwandowicz *et al*., 2018). Replicon probe hybridization was used prior to PBRT to classify the plasmids into their respective Inc groups by Southern blot hybridization using the replicons as probes. When PBRT took over, *Enterobacteriaceae* included 28 replicons, based on *rep* genes and regulatory sequences. Because these groups correspond somewhat to the established Inc groups, the nomenclature Inc is still used. Replicon subtyping is available for six of the common replicon types within *Enterobacteriaceae* and uses a pMLST scheme for subtype nesting (Orlek *et al*., 2017).

MOB typing is based on mobility functions and is performed either by PCR or *in silico*. This classification is based on determining the plasmid relaxase, the essential protein that initiates and terminates conjugative DNA processing. There are currently nine relaxase MOB classes, where the plasmids share similar genomic

traits, as it has been shown that various Inc-groups cluster within the MOB families (Garcillán-Barcia *et al*., 2009). MOB typing provides a lower resolution than replicon typing and is considered less complicated since a single plasmid may contain more than one replicon but only one relaxase. Yet, the detailed information available through replicon typing, especially if a pMLST scheme is present has made it the favorable method, especially for *Enterobacteriaceae*-associated plasmids (Orlek *et al*., 2017).

5.1.1.7 Potentially toxic metals and co-/cross-resistance

The mechanism of co-selection, known as co-resistance (two or more resistance genes present on the same genetic element) or cross-resistance (the same mechanism providing resistance against several substances) may cause resistance towards antibiotics to spread further due to selection pressure from for example metals in the absence of antibiotics (Pal *et al*., 2014, Yazdankhah *et al*., 2014, Chen *et al*., 2015). Heavy metals are in small fractions essential for both eucaryotic and procaryotic species. They contribute to a wide range of cellular processes, including protein synthesis, maintaining tissues such as skin and blood vessels, and also have important wound healing properties (Osredkkar, 2011). However, heavy metals in larger quantities are toxic to both cells, and the whole organism. It has been suggested that Zn and Cu have antibacterial effects, due to Zn's chemical affinity for thiol groups in biomolecules. Cu`s toxicity is based on hyperoxide radicals and cell wall interactions (Lemire *et al*., 2013). The presence of Cu increases the intracellular levels of reactive oxygen species (ROS) in bacteria (Lemire *et al*., 2013). Like ARGs, metal-resistance genes are often located on MGEs, and co-spread of metal and antibiotic resistance genes from food producing animals has been described (Fang *et al*., 2016). A recent study found that Zn and Cu influenced antibiotic resistance and transfer in agricultural soil more than doxycycline (Li *et al*., 2022). Tongyi *et al.* indicated that increasing amounts of Zn increased the abundance, diversity and mobility of ARGs in soil (Tongyi *et al*., 2020).

Both Zn and Cu have regularly been used as feed additives in agricultural production, where they may influence the gut microbiota of production animals, and also end up in manure and subsequently in the environment where they may act as bacterial stressors (Mazhar *et al*., 2021). Zn in the feed of food animals have been linked to an increase in MDR *E. coli* (Bednorz *et al*., 2013). Studies have also shown that the frequency of MGEs increased with increasing Cu, even in subtoxic levels (Hu *et al*., 2016, Malan *et al*., 2015, Knapp *et al*., 2017). Few studies are available regarding the effects of heavy metal in the feed of poultry. However, heavy metals in feed have been extensively investigated in pigs, and associations between livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) and resistance to Zn is one example of co-selection of AMR in food-producing animals (Dweba *et al*., 2018, Back *et al*., 2020). In 2017 the EU banned the use of zinc oxide as food supplementation for food-producing species. This was decided after the CVMP had concluded that the benefits (prevention of diarrhea in piglets) of such use did not outweigh the risks (environmental accumulation and co-selection for AMR), and is effective from June 2022 (EMA, 2017).

5.1.2 *Escherichia coli*

Escherichia coli (*E. coli*) belongs to the family *Enterobacteriaceae* and is a facultative anaerobic Gram-negative rod-shaped bacterium. It is found in the gastro-intestinal tract of vertebrates, and includes commensals, opportunistic bacteria, pathogens and what has most recently been referred to as a hybrid pathogen (Lindstedt *et al.*, 2018). It has been shown to be one of the first colonizers of the naïve intestinal microbiota in infants, and have important roles in the microbiota by e.g. producing vitamins (Eggesbø *et al.*, 2011). These commensal and beneficial strains, like their pathogenic and opportunistic cousins, have secretion systems and structural factors that allow them to interact with the gut epithelium (de Muinck *et al.*, 2013). It has also been shown that these *E. coli* actually are essential to establish the healthy function of the neonatal gut epithelium (Tomas *et al.*, 2015). Environmental isolates have also been described (Jang *et al.*, 2017). The genetic components of *E. coli* include a circular (double stranded) DNA in addition to various plasmids and ranges from 4.2 to 6.0 Mbp in size. The core-genome of *E. coli* consists of genes common for all *E. coli* strains (approximately 2000 genes) (Rasko *et al.*, 2008). The collection of genes that are possible to occur within the species *E. coli* is referred to as the "pan-genome" and include more than 23 000 genes (Tenaillon *et al.*, 2010, Tantoso *et al.*, 2022). The average core:pan-genome ratio found among *E. coli* is 1:5. It is likely to believe that this is accredited largely to HGT, as recombination has been shown to occur more frequently than mutation, still subsequent clonal evolution has allowed *E. coli* to adapt to various niches, ensuring the enormous success of the species (Yu *et al.*, 2021*). E. coli* is one of the most cultured and studied bacterial species and is known as fast- and easy growing and multifunctional (Vila *et al.*, 2016). It has been used as a model-organism in research and is frequently used as an indicator for fecal contamination. It has also been included among the ESKAPEE pathogens, an acronym for the seven most critically AMR infectious disease-causing bacteria defined by the United Nations (Llaca-Díaz *et al.*, 2012). *E. coli* is the bacterial species responsible for the highest counts of deaths globally that is attributable to and associated with AMR (Murray *et al.*, 2022).

5.1.2.1 Classification

The *E. coli* nomenclature is as dynamic as the bacteria itself and has gathered a considerable number of variants throughout the years. This diversification represents a high degree of host- and niche-specificity. Recently, Yu *et al* reviewed the taxonomy of the whole genus *Escherichia,* with emphasis on *E. coli* as the most important species in the genus (Yu *et al.*, 2021). Although *E. coli* is naturally occurring in the gastrointestinal tract of both mammals, birds and reptiles, the pathogenic subspecies have received, and will also in this thesis receive, the most attention.

Taxonomically, *E. coli* belongs in the genus *Escherichia,* family *Enterobacteriaceae*, order *Enterobacterales,* class Gammaproteobacteria, phylum Proteobacteria. Originally the name *Bacterium coli commune* was given with the discovery of the species in 1885 (Escherich, 1885). It was later renamed *Escherichia coli* in 1919 (Castellani and Chalmers, 1919). *E. coli* is the type species for the genus *Escherichia*

and has remained stable since the discovery, even if the genus has been subject to frequent changes. Until the use of genotypic methods for typing bacteria became easily available biochemical analyses were the golden standard method for determining taxonomic status for a given bacteria. The biochemical properties of *Escherichia* were described by Leclerc in 1962 (Leclerc, 1962) in the IMViC test (indole, methyl-red, Voges-Proskauer and citrate reaction profile). This led to grouping of the genus into Enteric groups based on the variations in the IMViC test. As the typing methods have progressed, several species have been separated from *E. coli,* either to become their own species within the genus, and some even separated from the whole genus of *Escherichia*. A standard reference collection of *E. coli* strains (ECOR) was established in the 1980s to gather the information about species diversity (Ochman and Selander, 1984).

Serotyping is traditionally performed by using antibodies to test for surface antigens. There are three types of antigens frequently used: somatic (O), capsular (K) and flagellar (H), where O and H are the golden standard. Over 700 *E. coli* serotypes have been described on this basis (Nataro and Kaper, 1998). With the progress of genetic assays, classical serotyping is less used as it is laborsome, but the molecular serotyping still focuses on the presence of the same antigens (Lacher David *et al*., 2014). PCR methods to distinguish between the different antigens have been developed. Multi-locus enzyme electrophoresis (MLEE) and multi-locus sequence typing (MLST), in addition to whole genome sequencing (WGS) provide more detailed information used to place the bacterium in question into the correct *E. coli* lineage. MLST obtains sequences from housekeeping genes in the bacterial chromosome distinct for different lineages. In addition to providing information about the serotype or MLST, the level of relatedness, core-genome MLST or whole-genome MLST may be defined by these methods (Maiden *et al*., 2013). Phylogenetic trees may also be built based on SNPs of the core genome, and thereby demonstrate the evolutionary history of the isolate. Another frequently used method to classify *E. coli* is the use of phylogenetic groups. The different phylogenetic groups display different genetic and phenotypic traits, and thus provide information about their properties. Traditionally this includes A, B1, B2 and D. This has been extended based on MLST and WGS data to include C, E, F, and *Escherichia* clade I, but which still remain rare, compared to the isolates included in the traditional groups (Jang *et al*., 2017). Recently new phylogroups have been added and have been named G and H (Lu *et al*., 2016). Today WGS allows for *in silico* typing for various typing schemes, including molecular serotyping, virulence-typing, and localization of resistance genes etc. (Bessonovo *et al*., 2021).

Further distinguishing of the species *E. coli* based on the host-and tissue specificity has resulted in separation into pathotypes. This goes for the non-commensal lineages within the species. The pathotypes are divided into two main groups: based on the disease they cause in humans. Intestinal Pathogenic *E. coli* (IPEC) cause disease in the gastrointestinal tract and Extraintestinal Pathogenic *E. coli* (ExPEC) cause diseases outside of the gastrointestinal tract. IPEC include Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), the Shiga toxin producing variant (STEC), Enteroaggregative *E. coli* (EAEC), Diffuse-adherent *E. coli* (DAEC), Enteroinvasive *E. coli* (EIEC) and Adherent-invasive *E. coli* (AIEC). ExPEC includes

Uropathogenic *E. coli* (UPEC), Neonatal meningitis *E. coli* (NMEC) and Sepsis-associated *E. coli* (SEPEC). The last commonly known abbreviation is the Avian pathogenic *E. coli* (APEC) responsible for causing infections in birds, especially hens (Stromberg *et al*., 2017). PCR and sequencing are used to determine pathotypes (Sora *et al*., 2021). If a strain has virulence determinants of more than one pathotype, it may be considered a hybrid strain, or a hybrid pathogen (Lindstedt *et al*., 2018).

The emergence of AMR has made it common to emphasize the resistance profile in addition to other characteristic when describing *E. coli* strains, clones or lineages. The relevant example for the purpose of this thesis is ESC-resistant *E. coli* providing resistance towards extended-spectrum cephalosporins.

5.1.2.2 Virulence factors

Structures produced by bacteria responsible for causing disease in a host are called virulence factors (VF) and are coded by virulence-associated genes (VAGs). These are primarily proteins that provide essential traits making pathogens able to for example invade the host organism, colonize, and adapt to a defined niche within the susceptible host (Wu *et al*., 2008). They can also provide evasion of the host's immune system to ensure survival of the pathogen (Zecconi and Scali, 2013). Both structural and secretory factors can be produced by bacteria, where membrane adhesins, biofilm forming abilities and fimbriae are examples of structural VFs, while secretory factors include toxins and vacuoles. *E. coli* VFs, and their respective VAGs are numerous, and often, but not exclusively, encoded on plasmids or other MGEs (Sarowska *et al*., 2019). Each of the *E. coli* pathotypes have distinctive combinations of virulence factors (Pakbin *et al*., 2021). The following demonstrate examples of abilities gained by VFs according to Kaper *et al.* (Kaper *et al*., 2004).

> *Immune evasion:*
> The host's immune system strives to beat off pathogens, and strategies to evade killing of the host immune system have therefore developed in bacteria. These include increased resistance to killing by serum, evolvement of a thick protective capsule, in addition to rapid change in surface proteins to avoid recognition by immune cells.
>
> *Iron acquisition:*
> To be able to survive outside of the gastrointestinal tract, the ability to obtain iron is important, as this is essential for normal cell function in the bacteria. Various mechanisms for iron acquisition have evolved in successful ExPEC strains. Especially survival in the harsh environment of the urinary tract requires access to iron.
>
> *Toxins:*
> Toxins are proteins that can target the cells of the host or competing microorganisms to cause damage or competitive advantages in the fight of resources available. Examples of toxins produced by *E. coli* include

hemolysins, LPS, cytotoxic necrotizing factor (CNF) and cytolethal distending toxin (CDT).

Cell wall associated:
The adhesins are surface structures that provides connection points to the epithelial surface in the tissues of the host, e.g., the intestinal mucosa, or the epithelial surface of the bladder. In addition to the previously mentioned strategies, structural proteins are also involved in biofilm formation and colonization. VFs such as fimbriae or flagella contribute to movement and establishment in the host tissue. Examples of cell wall associated VFs include outer membrane proteins (OMPs), curli fimbriae, P-fimbriae, invasins and the Type 1 fimbriae.

5.1.2.3 ExPEC

According to Johnsons criteria, two or more of the following VAGs have to be present to qualify as an ExPEC; *papA/papC, sfa/foc, ada/dra, iutA* and *kpsMT II* (Johnson and Russo, 2005). The phylogroups of B2 and D more often cause ExPEC infections compared to the other *E. coli* phylogroups. Phylogroup B2 is especially associated with UPEC infections while D is associated with other ExPEC pathotypes (Rezatofighi *et al*., 2021). VFs of ExPEC include various fimbriae (Type 1 fimbriae, Dr fimbriae, S fimbriae, F1C fimbriae), adhesins (Afimbrial adhesin, *Iha*, *Mat*), invasins (*Ibe A, B, C*) , iron acquisition genes (Aerobactin, Salmochelin, *ChuA, Hma, Sit A, B, C*), toxins (serin protease autotransporter, secreted autotransporter toxin, vacuolating autotransporter, hemolysin A, cytotoxic necrotizing factor, cytolethal distending toxin) and protectins (transfer protein, capsule antigens, outer membrane protein and *iss*-gene). Even more VFs than what have been covered here have been described, and are responsible for everything from colonization, metabolism, competition against other microbes and production of toxins (Sarowska *et al*., 2019). As previously mentioned, APEC is considered an ExPEC due to causing salpingitis in birds. The genetic similarity between APEC and ExPEC isolated from humans have suggested that APEC isolates may have a zoonotic potential (Moulin-Schouleur *et al*., 2007). *Manges et al.* demonstrated in 2016 that APEC and UPEC displayed a close relation, despite expressing host-specificity (Manges, 2016). For the purpose of this thesis only the ExPEC pathotype UPEC will be covered in detail in the next section.

5.1.2.4 UPEC

Urinary tract infections (UTI) are regarded some of the most common infections in humans, both in the hospital setting, and among the community acquired infections (Flores-Mireles *et al*., 2015). UPEC have been confirmed to account for the majority of UTIs in humans (Hooton, 2012, George and Manges, 2010). To be classified as an UPEC three or more of the following VAGs must be present; *chuA, fyuA, vat* and *yfcV* (Spurbeck *et al*., 2012). UPEC virulence factors include Type 1 fimbriae, P-fimbriae, curli fimbriae for biofilm formation, LPS, outer membrane proteins, siderophores and toxins such as alpha-hemolysin, and are all regarded attractive targets for new diagnostics (Terlizzi *et al*., 2017).

An UTI can present everything from asymptomatic bacteriuria to cystitis and in severe cases pyelonephritis and urosepsis (Foxman, 2010). The process of a UTI starts with the pathogen, in this case UPEC, colonizing the distal parts of the urethra performed by the help of adhesins expressed by UPEC. Bacteria then ascend the urethra and colonize the epithelial surface of the bladder by the Type 1 fimbriae that enable invasion of the urothelium. UPEC also produce toxins that may destroy host cells, in addition to iron acquisition systems that allow for uptake of essential nutrients like iron. UPEC is also able to catabolize urinary serine into ammonium and pyruvate to satisfy carbon and nitrogen demands (Brzuszkiewicz *et al*., 2006). Biofilm forming abilities are important for the establishment of a bacterial community in the bladder, and in some cases the ascendation continues to reach the kidneys causing pyelonephritis (Terlizzi *et al*., 2017). Numerous fimbriae and flagella make this ascendence and colonization possible. It has been suggested that host-microbe metabolic interactions also may play an important role in the development of UTIs (Klein and Hultgren, 2020, Subashchandrabose *et al*., 2014). Relapsing events of UTIs are common, and may be due to UPEC colonization of the bladder, or by reinfection if the distal colon of a human gets colonized by a UPEC strain (Mysorekar and Hultgren, 2006). There is no consensus on how UPEC end up colonizing the intestinal mucosa but both food contamination and sexual transmission have been suggested (Wiles *et al*., 2008). Even though any bacterial strain colonizing the distal parts of the colon, if translocated to the urinary tract, may cause uncomplicated cystitis, the VFs found in UPEC are different from other intestinal *E. coli.* The more severe cases of UTIs described for UPEC witness a more specialized strain that thrives in the urinary tract (Terlizzi *et al*., 2017).

Antibiotics are frequently prescribed to treat UTIs, and in Norway the recommendations are broad-spectrum penicillins, nitrofurantoin, or trimethoprim (often combined with sulfamethoxazole), ideally based on a cultured- and resistance test for uncomplicated cystitis. While more complicated cases with e.g. pyelonephritis or risk for sepsis development often are treated with cephalosporins and gentamicin in addition to the ones previously described (Helsedirektoratet, 2022). With the increasing AMR development worldwide, these types of infections that start quite innocently may potentially end up in more severe sequela due to treatment failure (Wurpel *et al*., 2016).

5.1.3 Food Safety

The UN set out in their Sustainable Development Goal (SDG) number 2 to end world hunger by 2030 and ensure access by all people to <u>safe</u>, nutritious, and sufficient food all year round. They also specify that they aim to ensure sustainable food production systems and agricultural practices to increase productivity and production, in addition to maintain the genetic diversity of both seeds, plants and animals (United, 2015). Safe food may be defined at several levels, and could e.g., be *food without harmful elements*, suggesting food free from pathogens, pollutants and toxic substances. A large part of achieving food without harmful elements that is edible for the consumer is knowledge, both among food producers, but also how to

safely prepare food among consumers and knowledge about which substances or elements may be harmful in either a short- or long-term perspective. Safe managing of the food production line, safe handling, processing, preparation, and storage of the food are also key for achieving this goal. In general food quality is seldom a part of the food safety definition, however, good quality food is often regarded by consumers as safe food. Defining "*harmful elements*" in turn would also include the absence of elements that may be harmful in a short or a longer time frame. This would include the absence of e.g., carcinogenic elements, elements that may cause chronic diseases over long- time exposure, and AMR elements that may lead to failure of treatment in the case of an infection. Once again, knowledge and research are of the essence to provide information on what safe food is.

5.1.3.1 Food production chain

The food production chain starts with the breeding and production at the farms. This goes for both plant- and animal derived products. Further the food products move through harvesting or slaughter, transport, processing, and preparation, often also packaging, before they reach the stores or the market and end up in the homes of consumers.

Food borne pathogens can enter the food production chain at different levels, through contaminated water, foodstuffs, and equipment for harvesting, through slaughter, processing of meat, with handling of humans in the production facilities, and finally in the stores or at markets before the food products reach the consumers. Cross-contamination while preparation of food may also happen, both in restaurants, and in the homes of consumers. Each level of the chain represents different hazards, and different measures are required at different stages throughout the food-production. Legislation, monitoring, and control exhibited by food safety authorities ensure that producers follow rules and guidelines. Consumer education is mainly based on advising, either from producers through packaging (e.g., "*do not consume raw*"-warnings), or through public information campaigns informing consumers to for example not use the same knives for uncooked meat and salad.

5.1.3.2 Norwegian poultry production

Poultry meat production in Norway account for 70 million slaughtered chickens yearly (Animalia, 2020). In the rearing period they are kept inside isolated and heated houses bedded with wood shavings as free-range chicken. They have free access to food and water and are not fed any growth promotors or feed-antibiotics. Coccidiostatics such as Narasin have been used previously, but were phased out by the industry and replaced with a vaccine by 2016 (Nortura, 2016). When the chicken has reached approximately 1.2kg or 28-32 days of age they are sent to slaughter. Industrial slaughter of poultry is carried out at a high pace, and the facilities are moist environments. Contamination from removal of the intestines happens frequently, and even though measures are made to limit the contamination large amounts of fecal bacterial contamination have been documented to occur on poultry meat (Rouger *et al.*, 2017).

5.1.3.3 Zoonotic perspectives

A zoonosis is defined as a disease or a pathogen that can be contracted between animals and humans (Chomel, 2009). Even though humans may spread disease to animals, we are usually most concerned about agents that reside in various animal populations that humans may be exposed to. The classical zoonotic agents are often regarded as pathogens with animal reservoirs that spread to humans from time to time, e.g. *Campylobacter* infections (Kemper and Hensel, 2023). Some pathogenic agents may be widespread within one species without causing disease but may be of great concern when crossing species barriers. An example of this is STEC in cattle, that have caused fatal food borne outbreaks in humans (Aurass *et al*., 2011). Direct transmission of *E. coli* would happen through handling of animals or animal feces, while contamination of food stuffs, either from insufficient slaughter- or food preparing hygiene, may also cause exposure to humans.

E. coli does not fit the picture of a classic zoonotic agent as it is already widespread in the human population. However, as *E. coli* includes both pathogenic and apathogenic variants, and frequently distribute resistance- and virulence genes through HGT, it may be considered an opportunistic zoonotic agent despite the important roles played by the commensal *E. coli* strains (Braz *et al*., 2020). When it comes to opportunistic pathogens, one of the variables that determines if disease develop is the one accounting for the host factors, which will not be discussed in detail. Yet it is important to note that a susceptible host, e.g., with a compromised immune system, will be more prone to disease, even though the given *E. coli* isolate has limited VFs (Tao *et al*., 2020). Strict pathogenic *E. coli* may cause disease even in the case of an immunocompetent host (Köhler and Dobrindt, 2011). Regarding information about the exposure to, and colonizing of pathogenic or opportunistic *E. coli* strains, most research is available for pathogenic STEC (Lange *et al*., 2022). It has been documented that after ingestion colonization of pathogenic STEC happens in the colonic epithelium, where the bacteria produce toxins and other inflammatory stimulants causing disease (Pearson and Hartland, 2014).

5.1.3.4 Antibiotics in feed

Antibiotics have been exploited through their growth promoting effects as feed supplements for food-producing animals. Peng *et al* reports an estimated global average antibiotic use of 172mg, 148 mg and 45 mg per kilogram of meat produced from pigs, chickens and cattle respectively (Peng *et al*., 2021). In addition, humans consume plants, caught or bred fish from the oceans, drink purified water and eat hunted meat. All of these are entry points for AMR to human food consumption, and thus pose a risk for humans to contract AMR bacteria. UN’s goal of ending world hunger by 2030 could be achieved by using large amounts of feed antibiotics in production, thus increasing the production yield. However, when the microbiota of our food gets exposed to antibiotics, we risk selecting for resistant bacteria and driving resistance development in commensal or opportunistic bacteria. This resistance may in turn spread to pathogenic bacteria that can end up on the table of consumers (Madec *et al*., 2017). If we exclude growth promotors and at the same time increase food-animal production, the pressure of infectious diseases among

livestock will also increase. Usage of feed-antibiotics are therefore not compatible with the specifications in the sustainability goals that, as previously mentioned, the food produced also need to be safe and sustainable. To further complicate the equation, an increased production will require increased labors regarding management, breeding and other health preventing measures to ensure good animal and human health. This opens for some ethical issues and requires novel solutions for the UN to be able to reach the goals by 2030. Interventions to limit usage of antibiotics have been shown to limit development and spread of AMR (Tang *et al*., 2017). An important tool to be able to make educated decisions on which interventions to implement is surveillance.

5.1.3.5 Surveillance

As a measure to collect information about, control and limit unwanted use of antibiotics, surveillance systems for detection of antibacterial resistance have been implemented throughout the world. Another goal of surveillance is to keep an eye out for development of new resistance types. Traditionally culture-based methods have been used to determine clinical resistance, and this is very useful in the case of determining MIC for patient treatment. This method has a major drawback as not all bacteria are culturable, and it requires a tremendous amount of work to perform in a larger scale, such as national surveillance. With the great development of WGS techniques and metagenomic analysis, lowering the cost of such analysis, surveillance and detection of AMR have become more easily available (Boolchandani *et al*., 2019). In addition to determining the AMR genes present, WGS techniques also provide information useful in determining epidemiological relations and spread and development of resistance. All this information collected from surveillance is often seen in context with information collected about antibiotic sales and usage in the respective countries. The WHO coordinates surveillance through the Global Antimicrobial Resistance and Use Surveillance System (GLASS) (WHO, 2022b). In Europe, surveillance is performed in a national level harmonized by guidelines from EFSA. Data are then reported to EFSA and the ECDC that joins and analyses the data which gets published in a yearly report (European Commision, 2022). Since 2014 the surveillance of ESBL/AmpC *E. coli* from food animals has been a mandatory part of the general surveillance. In the Nordic countries the surveillance efforts are called Danmap (Denmark), Finres (Finland), Svarm (Sweden), while in Norway the efforts are named NORM and NORM-VET and are being carried out by The Public Health Institute (FHI) together with the University Hospital in Tromsø (UNN) and The Veterinary Institute (VI). Fortunately, the situation regarding resistance in Norway, together with the rest of the Nordic countries is still favorable with lower occurrence of resistance compared to the status worldwide (Brolund, 2014, NORM/NORM-VET, 2022, Swedres-Svarm, 2022). Reasons for this include geographical limitations, good animal health, beneficial economical situations, good healthcare systems, good vaccine status in the population and a high living standard. Another reason for the limited AMR spread in Norway can be accredited to a close relationship between the veterinary sector, food producing sector and the legislative sector. For instance, Norwegian veterinarians does not profit from selling antibiotics, eliminating economic

incentives for prescribing antibiotics. In addition to surveillance, the Norwegian Scientific Committee (VKM) is mapping out knowledge gaps, evaluating threats and providing information for decision makers. In 2015 they published their Assessment of antimicrobial resistance in the food chains of Norway, where they concluded that; *"the probability of human exposure of ESBL-producing Enterobacteriaceae, and their respective corresponding genes, from live poultry and poultry meat was considered non-negligible*" (VKM, 2015), and is the background for the work performed in this thesis.

5.1.3.6 Aims of the thesis

The aim of this thesis was to provide further knowledge about ESC-resistant *E. coli* from poultry, and the health hazard represented by the presence of such bacteria in the food chain. This was performed by asking the following research questions and addressing the subsequent objectives:

- Does the presence of Zn and Cu in poultry feed contribute to spread of ESC-resistance between *E. coli*?
  - Assessing the phenotypical and transcriptional effect of Zn and Cu on conjugation of bla_{CMY-2} carrying plasmids in a broth model with different concentrations of Zn and Cu. (Paper 1)

- Do ESC-resistant *E. coli* survive digestion, and are they able to colonize and/or cause intestinal disease?
  - Investigate survival of ESC-resistant *E. coli* through *in vitro* digestion, their ability to adhere to and invade gastrointestinal epithelial cells. (Paper 3)

- Are ESC-resistant *E. coli* able to spread their resistance genes, and if so, does this ability change after being digested?
  - Investigate the spread of resistance genes through conjugation in broth. (Paper 3)

- Do ESC-resistant *E. coli* have the potential to become ExPEC and specifically UPEC?
  - Characterizing ESC-resistant isolates with regards to presence of virulence-associated genes and phenotypic properties to evaluate their pathogenic potential. (Paper 2 and 3)

5.2 Materials and Methods

The following provides a brief summary of the materials and methods included in the thesis. Detailed descriptions can be found in the respective articles.

5.2.1 Materials

All ESC-resistant *E. coli* isolated from retail chicken meat in Norway in 2012 (n=66), 2014 (n=58) and 2016 (n=17) were included in the selection population (total n=141). The isolates were collected in the Norwegian surveillance program for antimicrobial resistance in the veterinary sector (NORM-VET) and were known to carry the pAmpC gene bla_{CMY-2} (NORM/NORM-VET, 2013, NORM/NORM-VET, 2015, NORM/NORM-VET, 2017). NORM-VET is coordinated by the Norwegian Veterinary Institute and follows the EU guidelines for surveillance. The reader is directed to the respective reports for details regarding procedures analyzing samples. A total of 31 isolates were included in the phenotypic experiments of Paper 2 and Paper 3. For selection criteria see the respective Papers. Table 2 gives an overview of the included isolates, their ID, and which paper they are included in. Detailed information about their sequence types, phylogroups, year of isolation and original reference is presented in Table 1 in Paper 3. *E. coli* CFT073 (Mobley *et al*., 1990), a well characterized UPEC strain, was used as a positive control in Paper 2, while *E. coli* DH5α was used as recipient for conjugation studies and *E. coli* K12 for comparison of gene content in Paper 3.

Table 2: Overview of the isolates included in the respective papers, and an overview of the experiments performed in the various paper.

<table>
<tr><th>Whole ID</th><th>Short ID</th><th>Included in Paper #</th><th>Overview experiments</th></tr>
<tr><td>2012-01-3586</td><td>3586</td><td>2, 3</td><td rowspan="31">Experiments performed in Paper 1:
- Conjugation in Zn and Cu

Experiments performed in Paper 2:
- Expression of type 1 fimbriae
- Motility in semi-liquid agar
- Biofilm production
- Growth curves
- Serum resistance
- Adhesion and invasion of Vero-cells (kidney)
- Colicin production

Experiments performed in Paper 3:
- In vitro digestion
- Conjugation in LB-broth
- Conjugation in SIF after digestion
- Adhesion and invasion of HT-29 cells (colon)

All isolates are whole genome sequenced by the Norwegian Veterinary Institute. Metadata from all 141 isolates in the main dataset can be found in the supplementary material in Paper 2.</td></tr>
<tr><td>2014-01-3678</td><td>3678</td><td>2, 3</td></tr>
<tr><td>2016-22-832</td><td>832</td><td>2, 3</td></tr>
<tr><td>2014-01-5656</td><td>5656</td><td>2, 3</td></tr>
<tr><td>2014-01-7037</td><td>7037</td><td>2, 3</td></tr>
<tr><td>2016-22-220</td><td>220</td><td>2, 3</td></tr>
<tr><td>2014-01-1336</td><td>1336</td><td>2, 3</td></tr>
<tr><td>2012-01-1295</td><td>1295</td><td>2, 3</td></tr>
<tr><td>2012-01-707</td><td>707</td><td>2, 3</td></tr>
<tr><td>2014-01-3680</td><td>3680</td><td>2, 3</td></tr>
<tr><td>2014-01-4991</td><td>4991</td><td>2, 3</td></tr>
<tr><td>2014-01-5104</td><td>5104</td><td>2, 3</td></tr>
<tr><td>2012-01-771</td><td>771</td><td>2, 3</td></tr>
<tr><td>2014-01-7011</td><td>7011</td><td>2, 3</td></tr>
<tr><td>2014-01-4267</td><td>4267</td><td>2, 3</td></tr>
<tr><td>2012-01-1292</td><td>1292</td><td>1, 2, 3</td></tr>
<tr><td>2012-01-2798</td><td>2798</td><td>1, 2, 3</td></tr>
<tr><td>2016-22-1061</td><td>1061</td><td>2, 3</td></tr>
<tr><td>2012-01-1988</td><td>1988</td><td>3</td></tr>
<tr><td>2012-01-2350</td><td>2350</td><td>3</td></tr>
<tr><td>2012-01-1659</td><td>1659</td><td>3</td></tr>
<tr><td>2012-01-5334</td><td>5334</td><td>3</td></tr>
<tr><td>2012-01-5997</td><td>5997</td><td>3</td></tr>
<tr><td>2014-01-14</td><td>14</td><td>3</td></tr>
<tr><td>2014-01-1676</td><td>1676</td><td>3</td></tr>
<tr><td>2014-01-2452</td><td>2452</td><td>3</td></tr>
<tr><td>2014-01-7149</td><td>7149</td><td>3</td></tr>
<tr><td>2014-01-2454</td><td>2454</td><td>3</td></tr>
<tr><td>2016-22-75</td><td>75</td><td>3</td></tr>
<tr><td>2016-22-226</td><td>226</td><td>3</td></tr>
<tr><td>2016-22-1059</td><td>1059</td><td>3</td></tr>
</table>

5.2.2 Laboratory methods

In summary, the experiments performed in Paper 1 included growth curves, MIC testing for Zn and Cu, conjugation in broth, confirmation with PCR and qPCR for expression of conjugation genes. These were performed for two of the isolates (1292 and 2798). For Paper 2 a series of phenotypic tests were performed on 18 of the isolates to assess their potential as uropathogens. These were agglutination test for expression of fimbriae, motility in semi-liquid agar, production of biofilm, adherence and invasion of Vero-cells, serum-resistance, growth curves in urine and production of colicins, in addition to evaluation of population structure, and a mapping of virulence- and resistance genes. Paper 3 put emphasis on the effect of exposure to gastrointestinal digestion, and included a static *in vitro* digestion model, conjugation in broth and adherence and invasion of HT-29 cells, and finally an *in silico* extensive virulence-associated gene analysis.
The following gives a short description and summarizes the principles for each experiment. Overnight culture was prepared in Luria-Bertani (LB)- broth for all experiments. Blank controls were included in all experiments, and all experiments were conducted in triplicate.

Cecum samples
Determination of concentrations of Zn and Cu in the chicken cecum intestine was performed by analyzing cecum-content from ten 25-day old chicken from a commercial poultry farm. The animals were not subject to any experimental interventions prior to euthanasia. The cecum-content was collected and analyzed for Zn and Cu concentrations at Eurofins Food and Feed Testing Norway (Moss, Norway). Information of the concentrations of Zn and Cu were used to determine the experimental concentrations for conjugation in broth in Paper 1.

Minimum inhibitory concentration (MIC)
Stepwise dilution in LB-broth was used to determine the MICs for $ZnCl_2$ and $CuSO_4$ for the two included isolates in Paper 1. Concentrations tested ranged from 0,03 μg/mL to 3 mg/mL for $ZnCl_2$ and 0.01 μg/mL to 1 mg/mL for $CuSO_4$.

Growth curves
To evaluate growth in the presence of metals for Paper 1, overnight culture was diluted 1:1000 in LB-broth containing $ZnCl_2$ and $CuSO_4$ in a 96-well microtiter plate and incubated in a Tecan plate reader for 24 hrs at 37°C. OD600 was measured every 10 min during incubation. For Paper 2 the same protocol was followed without metals to obtain growth curves in LB-broth and human urine.

Plasmid stability
Transconjugants from the conjugation assay in Paper 1 were propagated by serial transfer. Stationary phase culture was transferred fresh LB-broth supplemented with 0.05 mg/mL $ZnCl_2$ or 0.01 mg/mL $CuSO_4$ every 12 hrs for five days, corresponding to ~300 generations. The bacteria were grown at 37°C under agitation. Overnight culture without metal supplement was used for negative control. To confirm the presence or absence of plasmids, each sample was diluted and plated on MH agar plates with or without antibiotics (20 mg/mL nalidixic acid and 0.5 mg/mL cefotaxime). Plates without antibiotics were incubated at 37°C for

24 hrs and plates containing antibiotics were incubated for 48 hrs. The number of CFU was counted manually. The presence of the bla_{CMY-2} plasmid in the transconjugants was confirmed by PCR.

qPCR

Expression of conjugational genes *nikB* and *traB* in the presence of experimental concentrations of $ZnCL_2$ and $CuSO_4$ was investigated using quantitative PCR for Paper 1. Overnight cultures were inoculated in broth containing $ZnCl_2$ or $CuSO_4$ and incubated for 4 hrs at 37°C, as was the timeframe for the conjugation experiment. RNA was isolated using PureLink RNA Mini Kit according to manufacturer's protocol. On-column PureLink DNase treatment was applied, and cDNA synthesized as recommended by manufacturer. Real time reactions were carried out using Power SYBR Green PCR Master Mix, and amplification using Step One Real Time PCR system. The primer sequences used for the qPCRs are presented in Table 1 of Paper 1. Results were analyzed using StepOne Software v2.3.

Agglutination FimH / Type 1 fimbriae detection

The ability to express a D-mannose-binding phenotype, characteristic for functional Type 1-fimbriae, was assayed by the ability to agglutinate yeast cells (*Saccharomyces cerevisiae)*. Overnight cultures were centrifuged, and the pellet was resuspended in PBS. A 1:1 ratio of the bacterial suspension was mixed with yeast cells dissolved in PBS with and without 1% D-mannose solution on a microscopy slide for observation of agglutination.

Motility

One colony from a fresh blood agar plate was perpendicularly inoculated into a tube containing 5 mL semi-solid LB agar, 0.03%, 0.2% and 0.7% respectively, and incubated for 24 hrs at 37°C. Isolates were defined as motile if bacterial growth clouded the medium and grew diffusely, while non-motile was limited to growth in the inoculation-canal (Tittsler and Sandholzer, 1936).

Biofilm

Overnight cultures (37°C) were diluted 1:200 in LB-broth without NaCl and added to 96 well microtiter plates. The plates were incubated at 37°C for 24 hrs and at 20°C for 48 hrs. After incubation, the plates were washed three times with PBS to remove planktonic and loosely adherent cells. Adhered bacteria were stained with 0.1% crystal violet for 15 min followed by washing three times with PBS. Ethanol was added to each well and OD_{600} was measured using a Tecan plate reader (Witsø *et al.*, 2014).

Serum-resistance assay

To investigate the ability to resist serum-killing, 1:4 ratio overnight cultures were added to 20% human serum (HS, diluted in PBS) or heat inactivated serum (HIS, 56 °C for 60 min). Incubation was done at room-temperature, and samples were taken every hour for three hrs. CFU/mL was determined by dilution and plating of the samples on LB-agar plates. The colonies from HS samples were calculated as a percentage of the HIS samples and results categorized as followed: < 1% - serum sensitive, > 90% - serum resistant, and all in between considered intermediate.

Colicin production

Colicin production was investigated by inhibition of *E. coli* DH5α growth. Overnight culture of *E. coli* DH5α was spread on LB agar plates. Overnight cultures of the respective isolates were centrifuged, and the supernatants were sterile filtered through a syringe filter. A volume of 10 µL of the filtrate was spot inoculated on the dried LB agar plates with *E. coli* DH5α and incubated in 37°C for 24 hrs. Production of colicin was determined by the presence of an inhibition zone around the inoculation spot.

In vitro digestion model

In vitro digestion was performed following the INFOGEST protocol with minor modifications to adapt to digestion of bacteria (Pettersen *et al*., 2019). The digestion consists of three separate phases: oral, gastric and intestinal. Enzymatic activity of the digestive enzymes was tested prior to the experiment according to Supplemental material from Brokorb *et al.* (Brodkorb *et al*., 2019). Briefly, the digestion was carried out as follows; Overnight culture was mixed with simulated salivary fluid (SSF) and mixed for two minutes. Simulated gastric fluid (SGF) was then added, pH adjusted to 3.0, and pepsin and rabbit gastric extract were put in. The gastric step was incubated with mixing for 40 min, as has been described as the gastric passing time for liquid boluses, at 37°C. Simulated intestinal fluid (SIF) was then added, pH adjusted to 7.0, followed by adding bile and pancreatin, before incubating for another two hrs at 37°C with mixing. After incubation bacterial mixture was diluted and plated on selective agar plates (Müeller-Hinton agar containing 0.5 mg/L cefotaxime) and incubated for 24 hrs at 37°C. Colonies were counted manually and CFU/mL determined.

Adherence and invasion assay

Cell-adhesion and invasion were performed for Paper 2 and 3 using Vero monkey kidney epithelial cells (Vero C1008, ECACC, Item number 85020206) and HT-29 human colorectal cells (RRID: CVCL_0320) respectively (Govorkova *et al*., 1996, Fogh *et al*., 1977). Cells were grown according to manufacturer's protocol (Ammerman *et al*., 2008) and the assay was carried out as follows: The cells were grown to 80% confluency, and cells in fresh medium were transferred to a microtiter plate in duplicate and grown to 80% confluency in the plate. Overnight cultures of bacteria were diluted, centrifuged, and resuspended in cell medium. For Paper 3 both digested and non-digested bacteria were exposed to the assay. Bacterial suspension was then diluted and added to the confluent cells with fresh cell medium equivalent to multiplicity of infection (MOI) 30:1. The plates were centrifuged to increase contact between bacteria and cells and incubated for two hrs at 37°C. To address adhesion to cells, the cells were washed three times with PBS to remove non-adherent bacteria and lysed with 1% Triton X for 10 min. The lysates were diluted in PBS and plated on LB agar for Paper 2 and MH agar containing cefotaxime for Paper 3. Bacterial invasion was assessed as described for the adhesion assay, however fresh medium with antibiotics (0.1 mg/mL gentamicin, and 20 mg/mL nalidixic acid) was added to the cells and incubated for 2 hrs to kill adherent bacteria. The cells were lysed and plated as described before.

Conjugation in broth
Conjugation in broth for Paper 3 was performed by mixing overnight culture of recipient (*E. coli* DH5-α) and donor (one tube for each respective isolate) at a 50:1 ratio in 4 mL LB-broth and incubated for 4 hrs at 37°C. The mating mixture was then plated on recipient- and transconjugant-selective plates containing nalidixic acid, or both nalidixic acid and cefotaxime. Colonies were counted, and CFU/mL calculated. The same protocol was performed with donors that had gone through *in vitro* digestion. To further simulate the gastrointestinal environment LB-broth was supplemented with simulated intestinal fluid. Mating and incubation were then performed for 4 hrs at 37°C as for non-digested isolates, and transconjugants and recipients counted on selective plates. In Paper 1, the same protocol was followed, but with three different concentrations of $ZnCl_2$ and $CuSO_4$ diluted in LB-broth respectively. The conjugation frequencies were compared between the two included isolates, and the 6 different additives.

In both Paper 1 and 3 transconjugants were confirmed by plating on bromothymol lactose blue agar. Transconjugants were identified by their ability to ferment lactose, while the donors did not. In addition, the presence of bla_{CMY2} was confirmed with standard PCR. See Paper 1 and 3 for the respective primers used.

Bioinformatics
Whole genome sequencing and the following bioinformatic analysis was performed by the Norwegian Veterinary Institute. Total genomic DNA were extracted using DSP DNA Mini Kit (Qiagen, Hilden, Germany) or Qiagen Blood and Tissue kit (Qiagen) followed by quality control testing. Samples were prepared with either the Nextera XT or Nextera Flex library preparation kit (Illumina, San Diego, CA, USA). Whole genome sequencing was performed using Illumina sequencing technology (Illumina HiSeq and Illumina NextSeq). Raw reads were available for inclusion in Paper 2 and 3. Quality control and assembly of samples was performed using the Bifrost pipeline, in addition to identification of resistance, virulence genes and *in silico* MLST-typing (Lagesen, 2020). The ARIBA (Antimicrobial Resistance Identification By Assembly) software (Hunt *et al*., 2017) was used to determine multilocus sequence type (MLST) according to the Achtman scheme (Wirth *et al*., 2006). Resistance and virulence genes for paper 2 was detected using the ResFinder (Zankari *et al*., 2012) and VirulenceFinder (Joensen *et al*., 2015) and vfdbcore (Chen *et al*., 2005) databases. Serotypes were determined using SerotypeFinder (Joensen *et al*., 2015). Genetic relationship was investigated using the cgMLST scheme available from Enterobase (https://enterobase.warwick.ac.uk/) in the chewBBACA suite (Silva *et al*., 2018). The cgMLST tree was visualized using the ggtree package (Yu *et al*., 2017) in R version 3.5.2 (Team, 2014). Sequence data is available at European Nucleotide Archive (ENA). Accession numbers are given in Table 2 of Paper 2.

Virulence associated gene analysis
An extended virulence associated gene analysis was carried out for Paper 3 for a deeper virulence analysis than what was performed in Paper 2. The sequences were scanned (i.e. BLAST search) against a custom database previously used for characterizing environmental isolates (Finton *et al*., 2020) now expanded to include

1191 genes/gene variants or genetic markers. The database contains genes related to both ExPEC and IPEC as well as loci suspected to contribute to virulence e.g., the ETT2 locus. Only matches with 95% nucleotide identity or more with 60% or more query coverage were included in the results.

5.2.3 Statistical methods

For analysis of data from Paper 1 a nonparametric regression analysis was performed in Stata (Stata MP/16 for Windows) to evaluate effect of heavy metals in the experiments. Strain and biological replicate were adjusted for. One-way ANOVA was used for evaluation of data in the plasmid stability experiment and analysis of gene expression. Level of statistical significance was set to $p < 0.05$.
Paper 2 and 3 were considered descriptive studies, thus extensive statistics were not performed. All experiments were performed in triplicate with three biological replicates. Standard deviations are provided in the figures showing quantitative data in the papers.

5.3 Summary of papers

For clarification, Papers are numbered in the chronological order they have been published in.

5.3.1 Paper 1

Norwegian poultry is not treated prophylactically with antibiotics, yet ESBL-producing bacteria were found on poultry meat in the surveillance program NORM-VET. Chicken receives higher amounts of heavy metals in their feed than what they biologically require. The aim of Paper 1 was to investigate the effect of Zn and Cu on the conjugational spread of resistance genes, and if excess amounts of Zn and Cu would act as resistance drivers. Two *E. coli* isolates from retail chicken meat carrying beta-lactamase encoding plasmids were used as plasmid donors in a liquid conjugation assay containing different concentrations of $ZnCl_2$ and $CuSO_4$. The experimental concentrations of Zn and Cu were determined by MIC testing and growth curves. The study found that Zn and Cu reduced the conjugation frequency between *E. coli* in a concentration dependent manner. The plasmids that were transferred remained stable in the host without any interference of the metals. qPCR was applied to show that Zn and Cu may inhibit the bacterial conjugation frequency by interfering with expression of the conjugational genes *nikB* and *tra*B.

5.3.2 Paper 2

Food-producing animals and their products have been suggested as possible sources for human acquisition of ESC-resistant *E. coli,* and poultry are considered the most probable source of ESC-resistant extra-intestinal pathogenic *E. coli* (ExPEC) obtained from food. In Paper 2, the aim was to characterize ESC-resistant *E. coli* from retail chicken meat regarding their population genetic structure, the presence of virulence-associated geno- and phenotypes, in order to evaluate their uropathogenic potential. Phenotypic virulence testing was performed on a selection of 18 isolates to evaluate possible potential of these strains to cause UTIs in humans. Methods included evaluation of motility, biofilm formation, ability to resist serum killing, growth in urine, colicin production and cell-adhesion and -invasion. In addition, *in silico* typing was performed. Furthermore, the population structure of all ESC-resistant *E. coli* isolated from retail chicken meat from 2012-2016 in the Norwegian monitoring program on AMR in the veterinary sector (NORM-VET) was explored. The study concluded that the uropathogenic potential of resistant *E. coli* from poultry meat is highly strain-dependent, and that they are unlikely to pose high risk to Norwegian consumers. It is, however, still important to continue monitoring to ensure that pathogenic strains do not establish themselves in the food-production chain.

5.3.3 Paper 3

Exposure to ESC-resistant *E. coli* may result in consumers becoming carriers if these bacteria colonize the human gut or their resistance genes spread to other bacteria in the gut microbiota*.* We hypothesized that ESC-resistant *E. coli* from poultry can survive digestion and thereby cause infections and/or spread their respective resistance traits within the gastrointestinal tract. A selection of 31 ESC-resistant *E. coli* isolates from retail chicken meat was exposed to a static *in vitro* digestion model (INFOGEST). Survival, alteration of colonizing characteristics in addition to conjugational abilities were investigated before and after digestion. Whole genome sequencing data from all isolates were screened through a custom-made virulence database of over 1100 genes for virulence- and colonizing factors. All isolates were able to survive digestion. Most of the isolates (23/31) were able to transfer their bla_{CMY2}-containing plasmid to *E. coli* DH5-α, with a general decline in conjugation frequency of digested isolates compared to non-digested. Overall, the isolates showed a higher degree of cell adhesion than cell invasion, with a slight increase after digestion compared non-digested, except for three isolates that displayed a major increase of invasion. These isolates also harbored genes facilitating invasion. In the virulence-associated gene analysis two isolates were categorized as UPEC, and one isolate was considered a hybrid pathogen. Altogether the pathogenic potential of these isolates is highly dependent on the individual isolate and its characteristics. Poultry meat may represent a reservoir and be a vehicle for dissemination of potential human pathogens and resistance determinants, and the ESC-resistance may complicate treatment in the case of an infection.

5.4 Discussion

5.4.1 Material and methodological considerations

5.4.1.1 Study population

The isolates included in the thesis came from a population of 141 isolates collected through NORM-VET in 2012, 2014 and 2016 coordinated by the Norwegian Veterinary Institute. The sampling schemes for NORM-VET are designed for obtaining samples representative for the entire populations of the different animal species or food stuffs being analyzed. Samples from poultry are collected every other year. However, only one ESC-resistant *E. coli* was detected from poultry meat in 2018, and none in 2020. These years are not included in the study.

Prior to 2012 only few isolates with the ESC-resistance profile were detected in the Norwegian poultry population. All isolates have been shown to have the AmpC phenotype, which requires the presence of antibiotics to be expressed. The finding of such resistance in the poultry population was alarming, and the industry together with the research- and decision-making communities quickly took action to combat further development and spread of resistance. Although ESC-resistant *E. coli* have not been detected in Norway the last years, the results detected in this study are still relevant and can be extrapolated to apply for other countries with a similar AMR-burden in their food-production.

Thirty-one of the 141 isolates were selected for experiments to further describe their characteristics. For Paper 1, two isolates previously included in the work of Mo *et al.* (Mo *et al*., 2016) were selected due to their plasmid-content for the conjugation assays. When expanding to evaluate the uropathogenic potential of the poultry isolates, 16 additional isolates were selected to represent the spread in phylogroups within the sample population of 141 isolates. Meaning that since group D was the largest group among in the population, the majority of the selected isolates belonged to phylogroup D too. Lastly, when performing the experiments for Paper 3, an additional 13 isolates were included to increase the power of the experiments as we expected the variation in these results to be more subtle. Table 1 gives an overview of the respective isolates and their contributions to the respective papers.

5.4.1.2 Conjugation experiment

Conjugation experiments can be carried out in different ways. The choice to use a liquid conjugation protocol was based on the doctoral work of Mo (Mo *et al*., 2016), in which the isolates from Paper 1 were included, and showed the highest rate of conjugation. The liquid protocol was also better adapted to addition of heavy metals in different concentrations.

During the conjugation experiments in Paper 1, various concentrations of Zn and Cu were used to evaluate their effect on the conjugation rate of the included isolates.

MIC values were determined to ensure that the chosen concentrations did not surpass the toxic limits for the included isolates and did not exceed the amount of heavy metal found in the poultry feed and in the poultry intestines. One challenge was that the higher concentrations of metals used in the pilot study precipitated in the LB-broth. This problem was solved by incubating the broth at 37 °C with shaking to properly dissolve the metals and ensuring even metal concentrations in the conjugation broth.

The laboratory strain *E. coli* DH5-α was chosen as a recipient strain due to its promiscuous nature, and frequent use in conjugational studies in the literature. For the first pilot study a Donor:Recipient ratio of 1:1 was used, but no conjugation was detected. The final ratio used for the conjugation in broth, both for Paper 1 and Paper 3 was a ratio of 1:50 Donor:Recipient. With lower ratio than this, no conjugation was observed. During the experimental work of Paper 2, we observed that several of the isolates used as donors produced colicins which may explain that no conjugation was observed in the presence of higher amounts of donor.

5.4.1.3 Phenotypical virulence experiments

To evaluate the uropathogenic potential of the ESC-resistant *E. coli* from poultry in Paper 2 more extensively, several phenotypical tests were considered for the study presented in Paper 3. The spectrum of tests to evaluate the uropathogenic potential ranges from human clinical infection studies, that are considered the most accurate, but requires volunteers, to *in vitro* evaluation of single characteristics that are simplified tests to evaluate single phenotypical traits. As an alternative to human clinical infection studies representative murine models to simulate UTIs exist. The included isolates were not considered likely to become uropathogens, thus the decision was therefore made to initially evaluate the phenotypic traits by different *in vitro* experiments, and to compare the results to the genomic analyses, before considering the need for further use of animal models. In Paper 2, emphasis was put on assays demonstrating ability to survive in the urinary tract, such as motility, expression of Type 1 fimbriae and serum survival amongst others. For Paper 3, survival through digestion was evaluated as an indicator for the oral infection route, with subsequent colonizing ability by evaluating adherence and invasion after digestion. Table 3 gives an overview of the included tests in Paper 2 and Paper 3.

Table 3: Strengths and weaknesses of the phenotypical assays included in the thesis and how they were performed.

Assay	Strengths	Weaknesses
Yeast agglutination	The test confirms expression of Type 1 fimbriae that provides attachment to urinary tract epithelium.	It is a qualitative test and does not consider the degree of expression.
Motility	Demonstrates the ability to move in semi-liquid agar. Motility is required to be able to ascend to other tissues than the gastrointestinal tract.	The test is qualitative and does not confirm expression of specific flagella.
Biofilm in microtiter plates	This assay evaluates the biofilm forming abilities. It was performed for several timepoints and temperatures, that represents survival in the environment and in the body.	The test was not performed in urine, or on gastrointestinal cells, only plastic surface material.
Growth curves	The test was performed in LB broth and in urine, with shaking which representing high velocity from the UT and the GIT	The test was limited to 24 hrs cultures, and only performed in "healthy" urine.
Serum resistance	This assay confirms the ability to avoid killing by serum.	The test was not performed with a heat-sensitive control, only heat-inactivated serum.
Invasion and adhesion to epithelial cells	The assay demonstrates the ability to adhere to and invade gastrointestinal and urinary tract epithelial cells. Invasion demonstrates the ability to avoid the immune system.	The assay is time-consuming, and an antibiotic is required to kill adherent bacteria to evaluate degree of invasion, which may be complicated by working with resistant isolates.
In vitro digestion	This assay limits the use of animals for animal models, and it is easy to perform in a laboratory setting. It is standardized and comparable between laboratories.	The assay does not include a full microbiota, the colonic stage is omitted, and the test is performed in set stages rather than with dynamic changes.
Colicin production	The test demonstrates the ability to produce unspecific colicins providing growth advantages to other *E. coli spp.*	The test does not distinguish between different colicins.

In both Paper 2 and Paper 3, the ability to adhere to and invade eukaryotic cells was investigated, in kidney cells and colorectal cells, respectively. The selected cells were thought to mimic the urinary epithelium and the colonic mucosal surface in these experiments. The HT-29 mtx cell line produces mucosa that would have been even more representative for this study. However, as this cell-line was impossible to obtain due to the Brexit and COVID-19 restrictions, an in-house available HT-29 cell-line was chosen. This cell line produces more mucus than e.g., Caco-2-cells, but does not develop a full mucosal layer, as HT-29 mtx does. In the phenotypical virulence experiments, a discrepancy between genotypic and phenotypic traits was observed. This could be due to up and down regulation of gene expressions or phenotypes in the experimental conditions provided.

To be able to distinguish between donors, recipient and transconjugants selective plates containing antibiotics were used. They contained cefotaxime and/or nalidixic acid. All included isolates were confirmed to grow on selective plates prior to the experiments of Paper 3. In addition, none of the isolates survived treatment of MEM Cell Medium containing nalidixic acid and gentamycin in Paper 3. In Paper 2, for unknown reasons, the controls for isolate 220 showed growth in the presence of nalidixic acid and gentamycin and were therefore excluded from the study.

Adherence and invasion were carried out in monoculture. However, in a colonization event of the gastrointestinal tract, the environment would already be packed with a high-diversity microbiota. Competition from residing microbiota is likely to influence the colonization event *in vivo.* For the urinary tract, that is considered a sterile environment with exception of the distal urethra, the colonization experiments are closer to that of reality. One limitation here is that the experiments were carried out in nutrient rich cell medium. The urine in a healthy individual is in general scarce on nutrients available for the bacteria, as demonstrated by growth curves performed in urine from healthy volunteers.

The *in vitro* digestion model used in this thesis is a static model that represents a simplified simulation of digestion. It has been developed by INFOGEST (an international network of excellence on the fate of food in the gastrointestinal tract) and aims to be a cost-effective, comparable, and simple to perform. While a simplified model is easy to set up and provides few variables and increase comparability it does not account for the highly advanced process of digestion and is probably the reason comprehensive models are both hard and expensive to establish. The current model has been documented to be physiologically comparable to *in vivo* porcine digestion of skim milk powder (Egger *et al*., 2017). In this work, the role of the microbiota was not considered, as the digestion procedure was performed in monocultures. However, setting up a model with a microbiota that has the same content for comparison and reproducibility over several replicates may be difficult to achieve. It has also been documented by others that the survival of enteropathogens during digestion vary when inoculated on different foods, and thus the protection effect of food particles has not been accounted for (Waterman and Small, 1998). This may be due to heterogenous pH in the food bolus in the gastric step, which in turn means that the type of food may be a variable to account for when assessing survival. Due to these experiments being performed in liquid broth,

the gastric step was limited to 40 min. These results may not be applicable to all consumers, for example individuals who are immunocompromised, elderly or children. Future perspectives would include conditions more accurate to the real-life conditions.

5.4.1.4 Virulence associated gene analysis

WGS data from the 31 isolates included in paper 3 were analyzed for virulence associated genes using a custom database previously used for characterizing environmental isolates (Finton *et al*., 2020). The database used contained 1191 genes/gene variants or genetic markers and has been developed by Lindstedt at Faculty of chemistry, biotechnology, and food science, NMBU. Sequence matches with 95% nucleotide identity or more with 60% or more query coverage were included in the results. The isolates included in Paper 3 were compared to *E. coli* K12.

5.4.2 General discussion

Food is one of the most important interfaces from a One Health perspective, linking both humans, animals, plants, and environments. In addition to connecting industry, consumers, and decision makers, it is given that food receives a lot of attention in the AMR context as well. The risk of exposure and spread may be high for some of the food-pathogen-resistance combinations, but exposure and projections may be uncertain. Correlations between antibiotic usage and resistance in food production have been shown in several countries in Europe (Chantziaras *et al*., 2014, Bergšpica *et al*., 2020). For poultry meat, synchronized jurisdictions and interventions have been shown to reduce the frequency of resistance determinants (Maron *et al*., 2013), thus limiting the potential of poultry as both a reservoir and an important vector for AMR (Mak *et al*., 2022). Several suggestions have been made on how to lower antibiotic usage in the food animal sector, including global regulations restricting antibiotic usage, limiting meat-intake, imposing higher fees on veterinary antibiotics, and putting antibiotic usage under veterinary control. It has been estimated that if these measures were implicated globally the usage of antibiotics in the food production sector would be reduced by 80% (Van Boeckel *et al*., 2017). An example of such measures carried out in the EU is the antibiotic categorization for veterinarians of which antibiotics to avoid (A), restrict (B), use with caution (C) and to exercise prudence (D) when using (EMA, 2019). Especially the importance of regulations in low- and middle- income countries has been highlighted regarding the potential of limiting antibiotic use (Van Boeckel *et al*., 2019). Synchronized global efforts are the essence for securing safe foods free from resistance determinants, and an important part of this is surveillance, in addition to gathering more knowledge about the various food-pathogen-resistance combinations.

The aim of this thesis was to shed light on the fate of ESC-resistant *E. coli* from poultry in Norway. This was achieved by first investigating one of the theories for why this resistance surfaced in Norwegian poultry. Second, investigating how the bacteria survived in an *in vitro* digestion model. And third, investigation of phenotypical traits relevant for virulence-potential, and evaluation of genes from WGS data. In the following sections, results are discussed in a broader context.

Classification of resistance in NORM-VET is based on epidemiological cut-off values (ECOFF), and follows the requirements set in the Commission-implementing decision of 12th November 2013 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria (2013/652/EU). Selective methods are used for detection of *E. coli* resistant to extended-spectrum cephalosporins amongst others. Selective screening has been implemented to detect resistance even in low-prevalence sources, and to ensure a representative sampling. The finding of ESC-resistant *E. coli* in the Norwegian poultry production chain as part of the NORM-VET surveillance was surprising as cephalosporins are not prescribed for poultry in Norway (Statens Legemiddelverk, 2022). This finding raised questions regarding the origin of these resistance traits, and several theories have been investigated by others (Mo *et al*., 2017). One theory that had not been investigated prior to the work of this thesis was that heavy metals in the poultry feed could contribute to this persistence of AMR in the absence of antibiotics. Heavy metals have previously been shown to increase spread of AMR (Zhang *et al*., 2018), and

poultry receive higher amounts of Zn and Cu in their feed than what they biologically require. When performing conjugation assays with various subinhibitory concentrations of Zn and Cu, a concentration-dependent downregulation in conjugation frequencies was observed. This contrasted with the hypothesis of the study, as subinhibitory concentrations of heavy metals have been shown to induce the bacterial SOS response, and in turn increase the spread of AMR determinants (Baharoglu *et al*., 2010). Zn and Cu are toxic to the bacterial (and eucaryotic) cell in larger doses but are still essential for normal cell functions. To investigate the effects of Zn and Cu on conjugation, the MICs for the included isolates were measured and compared to levels of Zn and Cu in the poultry intestines. This was done to ensure representative concentration of metals for the conjugation experiments. In cecum samples, the concentrations were higher than the observed MIC. Bacteria in the fecal flora apparently tolerate these increased levels of metals, which may be explained by biofilm formation, or a heterogenous distribution of metals in the gut. Bacteria behave differently in biofilm than as planktonic cells, and it is known from other species that Cu and Zn enhance biofilm formation and increases resistance to high concentrations of metals (Marguerettaz *et al*., 2014, Cobine *et al*., 2013, Navarrete and De La Fuente, 2014). In addition, Cu has been shown to induce a so-called viable nonculturable state in Gram-negative bacteria (Aurass *et al*., 2011). The effects of Zn and Cu on conjugation of ESC-resistant *E. coli* in biofilm is still unknown and may differ from the results of conjugation between planktonic cells. Even though a downregulation of conjugation in the presence of Zn and Cu was observed in this study, the sum of effects the heavy metals may have on the bacteria, may be in the resistance driving favor. One study showed that Zn influenced the growth of *E. coli,* but had little effect on established *E. coli* biofilm (Danilova *et al*., 2020).

Previous studies have shown that the donor isolates harbor an addiction-system (Mo *et al*., 2016). This is a toxin-antitoxin system, which means that once a bacterium has obtained the plasmid, it is not possible for the bacteria to eliminate it without inducing cell-death. In the VAG analysis of Paper 3, a large variety of toxin-antitoxin genes was discovered. Even though these genes were detected, their expression and their relative contribution in conjugation have not been explored further. In Paper 1 it was confirmed that the resistance plasmid of isolate 1292 remained stable after conjugation, and thus confirmed ability to persist through 300 generations. This suggests that the resistance plasmid can persist in *E. coli* over time, possibly years, complicating possible future infections, and increasing risk of spread to other bacteria.

The next question that was investigated was related to ingestion of resistant bacteria. When present on retail chicken meat ESC-resistant *E. coli* may end up in the gastrointestinal tract of consumers in case of compromised kitchen hygiene. To evaluate the degree of survival of *E. coli* during this step the harmonized static *in vitro* digestion model was set up. Usage of this model for bacteriological evaluations has been limited to one study on *Listeria monocytogenes* by Pettersen *et al* in 2019 prior to the work of this thesis (Pettersen *et al*., 2019). The model was considered suitable to evaluate bacterial survival through digestion. Future studies should aim to include inoculation on food, in addition to including other bacteria to resemble

the interactions of the microbiota. However, good solutions for such evaluations may be challenging to develop due to the complexity of human digestion.

In the *in vitro* digestion model, all isolates survived and were investigated for change in conjugation frequencies and cell association after digestion. Gastric acid is an early defence of the gastrointestinal immune system, and most microorganisms that reside in the fecal microbiota have some degree of acid resistance. Acid resistance has been shown to be induced in stationary phase or under starvation conditions (Small *et al*., 1994). The survival in the digestion assay was not surprising as *E. coli* is documented to be acid resistant (Gorden and Small, 1993). However, it has been documented that the ability of pathogens to resist low pH corresponds to their oral infective dose, meaning that even if few ESC-resistant *E. coli* are taken in through food, they are likely to end up in the residing microbiota (Lin *et al*., 1996). Acid-sensitive bacteria have been shown to survive low pH when inoculated on certain foods, and inoculation on food may change the outcome if this model were applied on acid-sensitive bacteria (Waterman Scott and Small, 1998).

After digestion the isolates demonstrated a generally decreasing ability to conjugate, compared to the non-digested control. This may be due to limited nutrients in the simulated digestive fluids, or other stressors influencing the expression of conjugation related genes, but this need to be further investigated. It may also be that the gastrointestinal environment provides a non-favorable environment for *E. coli* to conjugate in, and thus the resistance determinants of ESC-resistant *E. coli* may play a smaller role in spread of AMR than first expected.

An important part of establishment in new niches is the ability to adhere to and invade host tissues. For Paper 2, 18 of the isolates were included to study adhesion and invasion to kidney cells. The same 18 isolates, together with an additional 11 isolates, were included in study of adhesion and invasion to intestinal cells for Paper 3. When investigating adhesion and invasion to kidney cells, three isolates (3678, 1295 and 4991) displayed the highest levels of adhesion, while all the isolates showed low levels of invasion. After performing the same experiment with intestinal cells for Paper 3, the results showed a general display of low levels of invasion. However, a very high increase in invasion was observed for isolates 220, 5656 and 7037 after digestion (Figure 3, Paper 3). For isolate 7037 especially one of the replicates displayed an extremely high invasion-rate after digestion, which contributed to increase the mean and spread of data displayed in Paper 3. In addition, this isolate was the only isolate in the sample population that held the *gimB*-genomic island associated with invasion (Pokharel *et al*., 2020). Isolate 7037 is interesting as it was categorized as a UPEC strain in the VAG-analysis in Paper 3. A filamentous morphology was noted in isolate 7037 on the cefotaxime-containing plates, which may be explained by the fact that UPEC isolates, develop subpopulations, and adapt quickly to new environments (Gawel and Seed, 2011). The level of adhesion to intestinal cells displayed a larger variety within isolates compared to invasion. Except for isolate 771, that displayed a high increase in adhesion after digestion, no real increase was observed for the rest of the isolates after digestion. These results may indicate that the ability to colonize a tissue depends on the type of tissue and may also depend on previous events and entrance

routes, such as being exposed to the stress involved in digestion to be able to cause damage. These theories will need further investigations to be confirmed.

With the more frequent use of WGS tools, and ability to investigate detailed information in the *E. coli* genome, the scientific community has developed a new understanding of the bacterial genomic dynamics. It is also important to note that results determining different pathovars of *E. coli* based on virulence genes are highly dependent on the database used. As the main focus of this thesis is the phenotypical trait, only a brief discussion of the WGS is included. Virulence- and resistance associated genes were investigated by searching several databases (Res-finder, virulence-finder, and a customized database). The virulence potential of the investigated isolates (n = 31) was considered low, with exception of three isolates that stood out in the results from the VAG analysis in Paper 3 (isolates no. 7037, 220 and 3680). Even though virulence genes were investigated in Paper 2, a more detailed VAG analysis was performed in Paper 3. This analysis revealed isolate 7037 and 220 as UPEC isolates, and 3680 as a hybrid pathogen containing genes associated with IPEC, STEC and ETEC isolates. It is still important to emphasize that these isolates need to access and establish themselves in the urinary- or gastrointestinal tract to be able to cause disease. It is also important to note that the presence of such genes does not necessarily correspond with the expression of the given genes. This may be one of the reasons why phenotypic and genotypic traits did not correspond for all isolates in Paper 2. Another reason may be explained by epigenetics and regulation of expression of a given phenotype in a given environment, for example as the expression of the AmpC phenotype in the presence of cephalosporins. To provide further knowledge of expression to further build on the data in this thesis, RNA-sequencing or proteomics may have provided further information to fully paint the picture of regulation of these genes.

The number of doctoral consultations regarding UTIs have increased by 32,7 % in Norway from the year 2000 to 2015 (Haugom *et al*., 2021). According to the national surveillance system for resistant microbes the prevalence of resistance in isolates collected from UTIs have remained relatively stable the last ten years, with a small decrease in 2021 (NORM/NORM-VET, 2022). These low numbers get accredited to effective infection control measures and favorable usage patterns by both physicians and veterinarians in Norway. In 2021, the number of *E. coli* isolates registered from the urinary tract were 1335, where 3,1 % of them were considered ESBLs (definition include isolates resistant to ampicillin, cefalexin, cefotaxime and/or ceftazidime) (NORM/NORM-VET, 2022). Interestingly, the highest peak of registered UTIs with resistant microbes coincides with the highest peak of reported resistant *E. coli* found on poultry meat, all in 2014 (NORM/NORM-VET, 2022). Most women that have experienced a UTI are likely to experience relapsing events, and may risk becoming chronically colonized (Kim *et al*., 2018). Based on the data presented in this thesis the potential for ESC-resistant *E. coli* from Norwegian poultry to become uropathogens are considered unlikely. This is in accordance with other studies that demonstrate a low risk of poultry being a reservoir for AMR pathogens but may not be applicable for all countries (Day *et al*., 2019, Dorado-García *et al*., 2017, Mughini-Gras *et al*., 2019). In some countries, food products, and especially chicken meat, have been highlighted as a source of AMR pathogens (Díaz-

Jiménez *et al*., 2020, Vieira *et al*., 2011, Lazarus *et al*., 2014). It is therefore likely to believe that the potential risk of representing a reservoir for AMR is dependent on the national or regional AMR burden. Today, the prevalence of ESC-resistant *E. coli* isolates from poultry meat is low, as they have not been detected in Norway since 2018. This contributes to the risk of colonization being low. In Paper 2 it is concluded that even though single isolates may display some uropathogenic potential, it is not the case for the majority of the isolates investigated. As pointed out earlier, it is also a prerequisite that normal kitchen hygiene is not upheld leading to the bacteria being ingested. They then must find their way to the urinary tract to cause infection, possibly by surviving digestion, colonizing the gut and/or the urinary tract, where their resistance characteristics may complicate treatment. This is the case for consumers, but it is also worth mentioning that workers in the poultry production chain may risk exposure and colonization of these isolates as the exposure is higher (Dohmen *et al*., 2017, Van Gompel *et al*., 2020).

When evaluating the phenotypic and genotypic traits of ESC-resistant *E. coli* it is interesting to note that they appear to be extremely diverse despite being collected from the same source with the same selective methods and harboring the same resistance gene ($\textit{bla}_{\text{CMY-2}}$). In the study population of 141 isolates, 19 different STs were observed, with temporal variations. This means that the potential human health hazard varies with the dominating strain. Correlation between phylogroup and pathotype has been described by others; where phylogroup B2 and D have been linked to UPEC and other ExPEC isolates, respectively (Hutton *et al*., 2018). In addition, phylogroup D has been shown to more easily develop resistance to third-generation cephalosporins (Yu *et al*., 2021). The most prevalent phylogroup in the study population was phylogroup D. In Paper 2 the genotypic and phenotypic characteristics related to uropathogenicity were compared (Figure 3, Paper 2). Interestingly, the results contained discrepancies, meaning that the presence of genes detected did not always correspond to the results from the phenotypical tests. This may be due to variation of expression of genes, e.g., the gene coding for a given trait is present in the genome, but not expressed under the given conditions for the experiment, or that the expression of a phenotype is regulated by other genes in the presence of the gene searched for.

Even if concluding that the pathogenic potential is dependent on the given strain colonizing, an infection is not only dependent on the single pathogen. It is a complex dynamic between the host, its current colonizing microbiota, available nutrients, and other immunologically related factors (Richter *et al*., 2018, Tenaillon *et al*., 2010, Srikanth and McCormick, 2008). An example of this is demonstrated by the fact that *E. coli* lacks the enzymes to degrade mucin-associated polysaccharides, and are therefore dependent on the mucinolytic activity of other commensal gastrointestinal anaerobes (Conway and Cohen, 2015). When interfering with the glycoproteins in the mucuslayer, *E. coli* get provided with ligands to facilitate adhesion and biofilm formation *in vivo* (Sicard *et al*., 2017). Iron availability is one of the main limiting factors for UPEC survival in human urine. In the extensive VAG analysis performed for Paper 3 several genes related to iron-metabolism were observed. Genes related to iron acquisition have been suggested as potential drug-targets to treat AMR UTIs (Sargun *et al*., 2021).

In the event of colonization of an ExPEC, a time-lag between exposure and infection/disease development exist. This further complicates tracing a possible infection route and identifying origin and the risks, and/or a relation to contaminated foods. Sarowska *et al.* pointed out that what appeared to be most difficult was to examine the transition from the asymptomatic colonization, to where the bacteria cause symptoms outside of the gastrointestinal tract (Sarowska *et al*., 2019). If proving the transfer route of ESC-resistant bacteria from animals to humans is challenging (Muloi *et al*., 2018), this may constitute an indirect risk to public health by increasing the gene pool from which pathogenic bacteria can pick up AMR-genes. Overall, events leading to the occurrence of AMR-genes and the transfer and spread of these seem very much multifactorial.

This thesis set out to investigate some of the knowledge gaps and health hazards of ESC-resistant *E. coli* in the Norwegian poultry production chain. The knowledge obtained through the work of this thesis may be of use for countries with a similar resistance-situation as Norway, with low use of antibiotics and a low AMR burden, and it can be concluded that Norwegian poultry meat still is considered safe to consume. In any regards, harmonized surveillance on a global level is of the essence to detect and control if AMR-isolates arise in the poultry production chain, as it cannot be ruled out as both a source of AMR and a vector of such.

5.5 Main Conclusions

- It is unlikely that Zn and Cu in poultry feed contribute to the spread of ESC-resistance plasmids in *E. coli* from poultry as the presence of heavy metals reduce the conjugation frequencies in a concentration dependent manner.

- ESC-resistant *E. coli* from poultry survive *in vitro* digestion, and the digestion process alters the characteristics of single isolates.

- ESC-resistant *E. coli* from poultry displayed both increase and decrease of conjugation frequencies after digestion, in addition to changes in ability to adhere to and invade gastrointestinal cells.

- Norwegian ESC-resistant *E. coli* in general is unlikely to become uropathogens.

- Individual strains may be of greater concern if colonizing the gastrointestinal tract than the general population, as these contain genes associated with ExPEC isolates.

5.6 Future perspectives

The first and most important aspect to highlight is the importance of continued surveillance to early detect if ESC- resistant E. coli isolates will reappear in the Norwegian poultry production. Further, information regarding which other factors might influence conjugation and the spread of AMR in poultry is needed. Studies investigating the effects of Zn and Cu on conjugation in biofilm, in addition to conjugation on various surfaces to simulate e.g., the food processing environment, or conjugation to other recipients would be interesting to see. The use of the INFOGEST model with inoculation of bacteria on food stuff would also provide further insights to the protective effects of exposure of AMR bacteria in a meal setting. A more complicated model for conjugation in microbiota in an intestinal environment could further simulate the events that occur in the human gastrointestinal tract. Such an improvement of the model would be a good alternative to animal- or clinical studies. Another aspect that has not been considered in the experiments performed in this thesis is what happens in immunocompromised, the elderly or children, and if they possess characteristics that might make them more- or less susceptible to colonization of- or exposure to ESC-resistant E. coli. Other measures for educating and encouraging consumers on how to treat their food and maintain good kitchen hygiene is necessary, and investigation of the poultry slaughter process with emphasis on how to make it a cleaner procedure would be beneficial.

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7 Scientific Papers 1-3

Paper 1

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Zinc and Copper Reduce Conjugative Transfer of Resistance Plasmids from Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli*

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The present work addresses the effect of excess levels of $ZnCl_2$ and $CuSO_4$ in the growth medium on the conjugative transfer of plasmids carrying the antibiotic resistance gene bla_{CMY-2} from extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*. Norwegian poultry are not treated prophylactically with antibiotics, but still, ESBL-producing *E. coli* are found in the chicken populations. Chickens receive higher amounts of Zn and Cu than their biological need, and several metals have been shown to act as drivers of antimicrobial resistance. In the present study, ESBL-producing *E. coli* strains collected from retail chicken meat were mated in broth containing various concentrations of $ZnCl_2$ and $CuSO_4$. Manual counting of transconjugants showed that $ZnCl_2$ and $CuSO_4$ reduced the conjugation frequency between *E. coli* strains in a concentration-dependent manner. Quantitative real-time PCR analyses showed that the presence of $ZnCl_2$ and $CuSO_4$ in the growth media reduced expression of the conjugation genes *traB* and *nikB*. By propagating monocultures over several generations, it was found that the bla_{CMY-2} plasmids remained stable in the recipient strains. Together the results show that exposure of ESBL-producing *E. coli* to Zn and Cu reduce horizontal transfer of the bla_{CMY-2} resistance plasmid by reducing expression of genes involved in conjugation in the plasmid donor strain.

Keywords: conjugation, CMY-2, antimicrobial resistance, poultry, zinc, copper

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Introduction

Spread of antimicrobial resistance (AMR) has become a significant threat against human and animal health.[1] Parts of this resistance has its origin within the agriculture sector and dissemination of resistant bacteria from the food production chains may be one out of several routes by which consumers can be exposed to AMR bacteria.[2–6] In 2012, the NORM-VET monitoring program for AMR in the veterinary and food production sectors detected cephalosporin-resistant *Escherichia coli* in 43% of the Norwegian broiler flocks.[7] In addition, 32% of *E. coli* from retail chicken meat were categorized as cephalosporin resistant, but even higher numbers were reported from Denmark and Sweden.[8] The NORM-VET findings were surprising as the use of antimicrobial agents in the poultry production in Norway is limited, and among the lowest in Europe.[7,9] In Norway, conjugative extended-spectrum beta-lactamase (ESBL)-encoding plasmids have frequently been discovered in bacteria isolated from broiler chicken, implicating that these animals are a potential reservoir for cephalosporin-resistant *E. coli.*[10]

Third and fourth generation cephalosporins have been defined as critically important antimicrobials by the World Health Organization.[11] However, ESBL-producing bacteria have been isolated from a variety of animal species in different European countries, which could represent a major threat to public health.[12–16] In 2015, the Norwegian Scientific Committee for Food and Environment concluded that "The probability of transfer of ESBL/AmpC-producing Enterobacteriaceae, quinolone-resistant *E. coli*, and their respective corresponding genes from live poultry and poultry meat is considered as non-negligible."[17] Exposure to ESBL-producing Enterobacteriaceae may result in consumers becoming carriers of resistant bacteria, if these bacteria establish themselves as part of the human gut microbiota.[3,18–20] In situations where these bacteria cause disease, or spread their resistance genes to other pathogenic bacteria, their resistance characteristics may lead to treatment failure and increased mortality.[21,22]

Conjugation allows bacteria to spread genetic information across diverse bacterial phyla by the use of mobile genetic elements.[23] Inter- and intraspecies dissemination of resistance plasmids is the main mechanism of horizontal gene

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transfer of AMR between bacteria and is mediated by the type IV secretion system (T4SS).[24] The plasmid-located *tra* operon encodes the genes important for transport of the plasmid from the donor to the recipient cell. The TraB protein exhibit ATPase activity thought to provide energy for the assembly of the T4SS machinery and is known to play a major role in the conjugative transfer of plasmid DNA.[25,26] Transfer of plasmid DNA is initiated and terminated at the origin of transfer, oriT. *NikB* encodes a relaxase, responsible for site- and strand-specific cleaving and rejoining of oriT at the nick site of the plasmid.[27] Hansen *et al.*[28] showed that horizontal transfer of plasmids is more important than clonal dissemination for transmission of bla_{CMY-2} mediated cephalosporin resistance between animals and humans.

Mo *et al.*[29] described two bla_{CMY-2} encoding plasmids (pNVI1292/IncK and pNVI2798/IncI1), which were found in *E. coli* strains isolated from retail chicken meat in Norway. They further showed that the plasmids could spread to a variety of Enterobacteriaceae species by conjugation. These plasmids encode two plasmid stability systems, namely relBE/stbDE and pndAC, which presumably facilitate dissemination and stability of the bla_{CMY-2} encoding plasmids. However, the importance of this stability system is not well studied.

Metals like Zn and Cu have antimicrobial effects; the bacterial toxicity of Zn may be due to the chemical affinity for thiol groups of biomolecules and Cu toxicity is based on production of hyperoxide radicals and interactions with cell membranes.[30] Bacteria acquire resistance genes against antimicrobials and metals with antibacterial properties on mobile genetic elements.[31] When two or more resistance genes are present on the same genetic element, or the same mechanism provides resistance against several substances, it may result in co-selection of genes conferring metal and antibiotic resistance. By these mechanisms, selection for resistance to zinc (Zn), copper (Cu), and other potentially toxic metals may act as drivers for spread of AMR.[32,33] However, data on the required concentration and time exposure for this effect to occur is still lacking.[34]

Zn and Cu are important elements in the cellular metabolism; they allow many critical enzymes to function properly and are also essential for wound healing, protein synthesis, and maintaining the strength of the skin, blood vessels, and various tissues in the organism.[35] Copper and zinc are routinely used as additives in animal feed in livestock farming, however, when animals receive feed containing larger amounts of Zn and Cu than what they biologically require the excess of metals are thereby released into the environment.[36] Zn and Cu are therefore commonly found in soil, water, plants, and in manure from various farm animals, including chicken.[37] The occurrence of Zn and Cu may have beneficial fertilizing properties, as these are important trace elements for plants, but may also be of environmental concern when present in large quantities, affecting groundwater, surface water, and aquatic animals.[38–41]

We hypothesize that excess levels of Zn and Cu promote transfer of resistance plasmids from ESBL-producing *E. coli*. The aim of our study was to assess the effect of Zn and Cu on conjugation of bla_{CMY-2} carrying plasmids from ESBL-producing *E. coli* collected from retail chicken meat.

Materials and Methods

Bacterial strains and growth media

Two *E. coli* strains isolated from retail chicken meat were used as plasmid donors in the conjugation experiments; *E. coli* 2012-01-1292 (pNVI1292/IncK) and *E. coli* 2012-01-2798 (pNVI2798/IncI1), hereafter named *E. coli* 1292 (IncK) and *E. coli* 2798 (IncI1), respectively. The strains were collected through NORM-VET in 2012, and harbored the bla_{CMY-2} gene,[42] the most common plasmid-mediated AmpC-beta-lactamase in *E. coli.*[43] Both strains have recently been whole genome sequenced (Mo *et al.*).[42] The *E. coli* DH5α strain, resistant to nalidixic acid (Nal^R), was used as recipient. Both donors ferment lactose, while the recipient does not. The bacteria were cultured in Luria-Bertani (LB) broth (Sigma-Aldrich, Germany) or Brain Heart Infusion broth (Sigma-Aldrich) throughout the whole study unless otherwise stated. $ZnCl_2$ (Sigma-Aldrich) and $CuSO_4$ (Merck, Germany) were used as sources of Zn and Cu throughout the whole study.

In vivo *and experimental concentrations of Zn and Cu*

The NORM-VET collects AMR-bacteria from the cecum of chickens. To determine the *in vivo* concentrations of Zn and Cu in chicken cecum, ten 25-day-old chickens were collected from a commercial chicken farm in Norway. The chickens were euthanized, cecum-content collected, and analyzed for Zn and Cu content at Eurofins Food and Feed Testing Norway (Moss, Norway).

Experimental concentrations for $ZnCl_2$ and $CuSO_4$ to be used in the conjugation assays were chosen according to the two following criteria: (1) without exceeding the respective minimum inhibitory concentrations (MICs) and (2) reflect the range of concentrations found *in vivo*.

Minimum inhibitory concentrations

MICs for $ZnCl_2$ and $CuSO_4$ were determined for all strains by serial dilutions (Supplementary Table S1) in LB-broth.[44] The concentrations tested ranged from 0.03 μg/mL to 3 mg/mL for $ZnCl_2$ and 0.01 μg/mL to 1 mg/mL for $CuSO_4$.

Bacterial growth of donor and recipient strains

Overnight cultures of each strain were diluted 1:1,000 in fresh LB-broth containing the selected experimental concentrations of $ZnCl_2$ and $CuSO_4$. A control without supplemented metals was included in each experiment. A volume of 200 μL of each sample was transferred to a 96-well microtiter plate (Greiner; Sigma-Aldrich) and incubated at 37°C in Tecan platereader. The optical density (OD_{600}) was measured in the cultures every 10 min for 24 hr. Each experiment was performed in three biological replicates, with three replicates of each sample.

Conjugation study

Conjugation experiments were conducted in LB-broth according to Sunde and Sorum,[45] with minor modifications. Briefly, the donor and recipient strains were grown overnight in LB-broth at 37°C and subsequently diluted to an OD_{600} equivalent to a McFarland standard no. 1 (~3E+8

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bacteria/mL).[46] A volume of 500 μL of the recipient strain culture and 10 μL of the donor strain culture was mixed in 4 mL LB-broth containing the selected experimental concentrations of $ZnCl_2$ or $CuSO_4$, respectively, including a control without supplements. All cultures were incubated for 4 hr at 37°C. LB-broth supplemented with Zn or Cu were prepared 1 day before the experiment and incubated at 37°C overnight under agitation to prevent precipitation of the added metal. Dilutions of each mating culture were plated on Mueller-Hinton agar plates (Sigma-Aldrich) supplemented with 20 mg/L nalidixic acid and/or 0.5 mg/L cefotaxime, and incubated for 24 and 48 hr at 37°C. To quantify the conjugation and to test for toxic effects of the metals on the donor and recipient strains individually, we plated the mating cultures on donor-, recipient-, and transconjugant selective plates. The conjugation frequency was determined by manual counting of colony forming units (CFU) and dividing the number of transconjugants with the number of recipients.

Representative colonies from each transconjugant-selective plate was plated on bromothymol lactose blue agar (Sigma-Aldrich) to distinguish transconjugants from spontaneously mutated donors. In addition to different abilities to ferment lactose, the transconjugants and mutated donors are distinguishable by colony morphology. PCR analysis of bacterial colonies was conducted to confirm that the transconjugants harbored the $bla_{CMY\text{-}2}$ gene.[47]

Plasmid stability

The transconjugants from the conjugation assay (DH5α with pNVI1292/IncK or pNVI2798/IncI1) were propagated by serial transfers as previously described.[48,49] with minor modifications. Briefly, 10 μL of stationary phase culture was transferred into 990 μL of fresh LB-broth supplemented with 0.05 mg/mL $ZnCl_2$ or 0.01 mg/mL $CuSO_4$ every 12 hr for 5 days, corresponding to ∼300 generations. Cultures in LB-broth without metal supplements were used as controls. The bacteria were grown at 37°C under agitation (180 rpm). To confirm the presence or absence of plasmids pNVI1292/IncK and pNVI2798/IncI1, serial diluted samples from each transfer were plated on Mueller-Hinton agar plates with or without antibiotics (20 mg/mL nalidixic acid and 0.5 mg/mL cefotaxime). Plates without antibiotics were incubated at 37°C for 24 hr and plates containing antibiotics were incubated for 48 hr. The number of CFU was counted manually. The presence of the $bla_{CMY\text{-}2}$ plasmid was confirmed by colony PCR.[47]

Sample preparation, RNA isolation, and quantitative PCR

The transcriptional analysis of genes involved in conjugation, *nikB* and *traB*, was performed in *E. coli* 1292 (IncK). An overnight culture was inoculated in fresh LB-broth with $ZnCl_2$ or $CuSO_4$. Bacteria cultured in plain LB media were used for comparison. The samples were incubated for 4 hr at 37°C. Total RNA was isolated from harvested *E. coli* using PureLink RNA Mini Kit (Life technologies, Carlsbad, CA) according to the manufacturer's protocol. On-column PureLink DNAse (Life technologies) treatment was performed according to the protocol. An amount of 100 ng of total RNA was used to synthesize cDNA using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, California, CA) according to the manufacturer's protocol. Primers were designed by Primer Express software, and the sequences are listed in Table 1. A standard curve using serial dilution of DNA from *E. coli* 1292 was made to calculate PCR efficiency for each primer pair. Real time reactions were performed using Power SYBR Green PCR Master Mix (Life Technologies) and real time amplification was carried out using Step One Real Time PCR system (Applied Biosystems). The data were collected and analyzed by normalization against the endogenous control gene *rpoA* using StepOne Software v2.3.

Statistical analysis

All experiments were performed as at least three independent experiments, with three technical replicates. As data were not normally distributed, we used a nonparametric regression through the quantile regression technique in Stata (Stata MP/16 for Windows), to evaluate the effect of $ZnCl_2$ and $CuSO_4$ levels on the experiments. We adjusted for the impact of strain and biological replicate. Results were

Table 1. Primers Used in This Study for Quantitative Polymerase Chain Reaction for Evaluation of Expression of Genes Involved in Conjugation

Gene	Primer sequences: Forward (5′-3′)	Primer sequences: Reverse (5′-3′)	Slope[a]	%Eff[b]	Gene description[c]
nikB	CGCCTGATAATGGCTGCTTT	CGCTGTTTTGCGCACAATA	–3.44	95.05	Conjugal transfer relaxase protein NikB
rpoA	GGCACAATCGATCCTGAAGAG	TTCCAGTTGTTCAGCCAGAATG	–3.37	97.85	DNA-directed RNA polymerase, alpha subunit
traB	GGCAAAAACCGCGAACAT	TCCAGGGAAGGACGTGTTG	–3.4	96.75	Type IV secretion/ conjugal transfer ATPase, VirB4 family

[a]The slope was calculated from the regression line in the standard curve.
[b]The efficiency was calculated using the slope of the regression line in the standard curve.
[c]According to UniProt Database.

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reported as coefficients with corresponding *p*-values when compared to the control. One-way ANOVA was used in the comparison of differences between samples with Zn and Cu and the control samples in the plasmid stability experiment and the analysis of gene expression. The level of statistical significance was set to $p < 0.05$.

Results

Selected Zn and Cu concentrations

The poultry feed contained 100 mg/kg Zn and 15 mg/kg Cu, and the results from the poultry cecum-content ranged from 8.52 to 83.5 mg/kg Cu and 71.9 to 225 mg/kg Zn. MIC data for *E. coli* 1292 (IncK) and *E. coli* 2798 (IncI1) is shown in Table 2. Thus, the experimental concentrations were selected as follows: 0.05, 0.125, and 0.2 mg/mL for $ZnCl_2$, and 0.01, 0.255 and 0.5 mg/mL for $CuSO_4$. This is equivalent to 0.024, 0.06, and 0.096 mg/mL elemental Zn and 0.0039, 0.101, and 0.199 mg/mL elemental Cu.

Bacterial growth

Growth curves for the donor and recipient strains are shown in Fig. 1. The lowest concentration of $ZnCl_2$ did not appear to have any effect on planktonic growth, while the two highest concentrations displayed a delayed growth rate (0.125 and 0.2 mg/mL). None of the concentrations of $CuSO_4$ appear to have any effect on the planktonic growth of the strains. The two donor strains *E. coli* 1292 (IncK) and *E. coli* 2798 (IncI1) showed higher growth rates compared to the recipient *E. coli* DH5α strain. Growth rates are provided in the supplementary material (Supplementary Table S2).

Conjugation study

The conjugation frequency was first determined in a pilot study where samples were taken after 4 and 24 hr of mating. No difference in the conjugation frequency was observed between the two time points (data not shown). Therefore, 4 hr was chosen for further conjugation and transcriptional studies. The mating cultures were plated on donor-, recipient-, and transconjugant selective plates to calculate the conjugation frequency (Table 3) and to evaluate any inhibiting effect of the metals in the mating cultures. Neither the donors nor the recipient showed any reduction in CFU compared to the control (data not shown).

As shown in Fig. 2 the effect of increasing levels of $ZnCl_2$ and $CuSO_4$ on conjugation were clear. These findings were supported by the nonparametric regression analysis, where strong effects of Zn and Cu levels were found ($p < 0.001$). There was no effect of the donor strains on the number of recipients in the conjugation experiments in $ZnCl_2$ ($p = 0.31$) and $CuSO_4$ ($p = 0.55$), which confirms that the recipients are not outcompeted by the donors (Supplementary Fig. S1). No statistical effect of replicate could be observed in the data. Our results show that conjugation of the IncK plasmid was reduced by more than 98% at all concentrations of Zn tested compared to the control. This was also observed for the two highest concentrations of $CuSO_4$ while 0.01 mg/mL $CuSO_4$ gave a 90% reduction of conjugation for the IncK plasmid. At the two highest concentrations of Zn and Cu there was a more than 90% reduced conjugation of the IncI1 plasmid, while the lowest concentrations of Zn and Cu gave

Table 2. Determined Minimum Inhibitory Concentrations of $ZnCl_2$ and $CuSO_4$ for Donor and Recipient Strains

Metal/ strain	E. coli *1292 (IncK), mg/mL*	E. coli *2798 (IncI1), mg/mL*	E. coli *DH5α, mg/mL*
$ZnCl_2$	0.4	0.4	0.3
$CuSO_4$	0.9	1	0.75

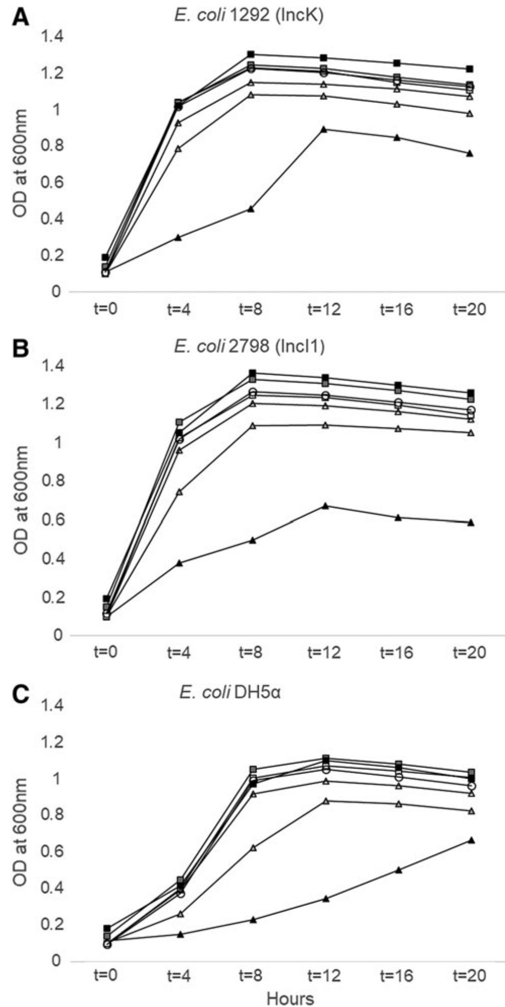

FIG. 1. Growth curves. **(A)** *Escherichia coli* 1292 (IncK), **(B)** *E. coli* 2798 (IncI1), **(C)** *E. coli* DH5α (△) 0.05 mg/mL $ZnCl_2$, (▲ grey) 0.125 mg/mL $ZnCl_2$, (▲) 0.2 mg/mL $ZnCl_2$, (□) 0.01 mg/mL $CuSO_4$, (■ grey) 0.255 mg/mL $CuSO_4$, (■) 0.5 mg/mL $CuSO_4$, (○) Control. The data are based on three biological replicates with three technical replicates each.

Table 3. Conjugation Frequencies in Response to Different Concentrations of $ZnCl_2$ and $CuSO_4$

Strain	*Additive*	*mg/mL*	*Conjugation frequency*[a]	*SD+/–*
E. coli 2798 (IncI1)	$CuSO_4$	0.01	1.26E–04	1.03E–04
		0.255	3.95E–06	9.12E–06
		0.5	NTD	0.00E+00
	$ZnCl_2$	0.05	9.31E–05	6.00E–05
		0.125	1.69E–05	6.85E–06
		0.2	NTD	0.00E+00
	Control	0	2.04E–04	1.31E–04
E. coli 1292 (IncK)	$CuSO_4$	0.01	3.37E–05	4.57E–05
		0.255	2.37E–06	2.28E–06
		0.5	NTD	0.00E+00
	$ZnCl_2$	0.05	1.04E–06	1.35E–06
		0.125	NTD	0.00E+00
		0.2	NTD	0.00E+00
	Control	0	9.21E–05	5.31E–05

[a]Conjugation frequencies were calculated as the mean number of transconjugants divided by the mean number of recipients from all replicates, for each combination.

NTD, no transfer detected (no colonies detected) on transconjugant selective plate.

a reduction of 58% and 41%, respectively. A representative selection of colonies was picked for further confirmation and all of them were confirmed PCR-positive for the bla_{CMY-2} gene. Furthermore, all tested transconjugants gave a negative result on the bromomethyl lactose agar, confirming the recipient phenotype.

Plasmid stability

Both plasmids carry genes encoding stability systems (*relBE/stbDE* and *pndAC*). We wanted to investigate whether these stability systems promote plasmid maintenance, and if Zn and Cu had any impact on the stability of the plasmids. By propagating the transconjugants in monocultures for ~300 generations and calculate CFU after plating on transconjugant selective plates, we could show that both plasmids were maintained within the transconjugants (Supplementary Fig. S2). Furthermore, the results showed that there was no difference in plasmid stability between the IncK- and the IncI1 plasmid and that subinhibitory concentrations of $ZnCl_2$ and $CuSO_4$ did not have any influence on the maintenance of the plasmids ($p < 0.05$).

Effect of Zn and Cu on expression of conjugative-related genes

Qualitative PCR analysis showed that genes involved in transfer of the IncK plasmid had a significantly reduced expression following exposure to 0.05 mg/mL $ZnCl_2$ or 0.01 mg/mL $CuSO_4$ compared to the control ($p < 0.05$) (Fig. 3). Specifically, the expression of *traB* was reduced by 87% and 92% in the samples with $ZnCl_2$ and $CuSO_4$ compared to the control without $ZnCl_2$ or $CuSO_4$. The expression of *nikB* was reduced by 97% and 96% in response to $ZnCl_2$ and $CuSO_4$ compared to the control.

Discussion

Conjugation is a complex mechanism that allows bacteria to spread genes encoding beneficial traits that will increase bacterial survival. A previous study has shown that the two plasmids included in this study are inter- and intra-species transferable at different conditions, indicating that they may contribute to the maintenance of antibiotic-resistant genes in the environment.[29] In contrast to earlier studies, which show

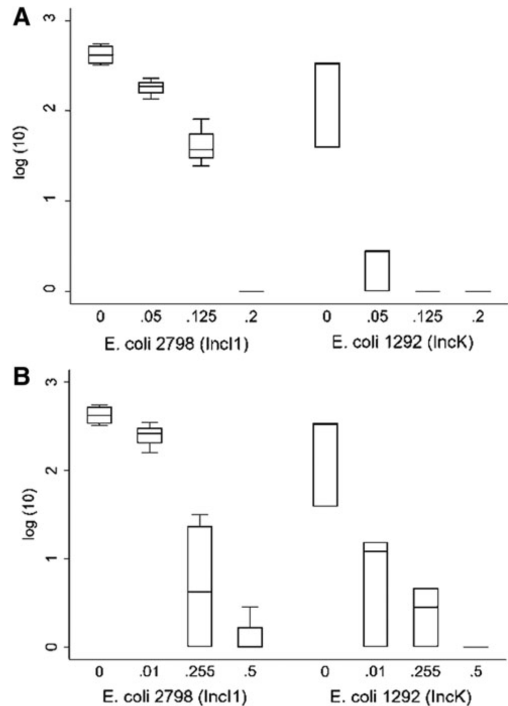

FIG. 2. Distribution of transconjugants demonstrating a dose-dependent reduction of transconjugants in the presence of $ZnCl_2$ **(A)** and $CuSO_4$ **(B)**. Concentrations are provided in mg/mL for $ZnCl_2$ and $CuSO_4$. The *horizontal line* within the *box* represents the median. *Boxes* represent 50% of the data and the whiskers, highest and lowest values, while *dots* represent outliers. The data are based on four biological replicates with three technical replicates each.

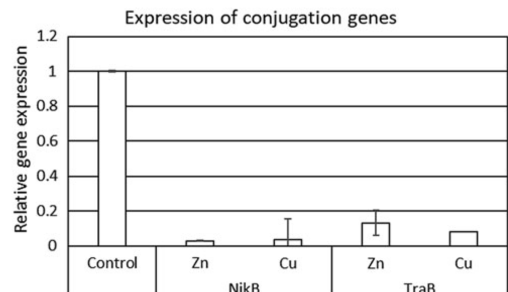

FIG. 3. Expression of genes involved in conjugation in *Escherichia coli* 1292 (IncK) in response to $ZnCl_2$ and $CuSO_4$. The data are presented as mean values ± SD ($n = 6$).

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that Zn and Cu are associated with increased conjugation,[50–53] we found that Zn and Cu reduced the conjugation frequency between *E. coli* strains in a concentration-dependent manner. There was also no difference in the number of colonies on the donor- and recipient-selective plates from the mating cultures, which confirms that Zn and Cu does not affect growth of donor- or recipient strains. Toxic effects of the metals can therefore not explain the observed reduction in conjugation frequencies. A pilot study was performed at both 4 and 24 hr of mating (data not shown). No difference in conjugation rate was observed between the two time points, which is consistent with a previous report by Mo *et al.*[29] Altogether, this justifies our choice of using the 4 hr time point in the mating experiments. The growth curves showed that the recipient strain grew slightly slower than the donor strain, however, this was compensated for by using larger amounts of recipient cells in the conjugation experiments.

The plasmids used in this study did not contain any known Zn or Cu resistance genes,[42] which rules out co-selective mechanisms. Our results are consistent with a recent study, which showed that metal stress (Zn and Cu included) decreased plasmid transfer frequencies to bacterial communities independent of metal-resistance.[54] Suzuki *et al.*[55] also showed a reduction in horizontal transfer of the tetracycline resistance gene *tet*(M) in response to Zn and Cu exposure. Reduced plasmid transfer in response to metal stress could be a consequence of changes in metabolic status, decrease in plasmid replication, activation of the SOS-response, or a combination of different mechanisms.[56–58] This study also showed that both the IncK and IncI1 plasmids remained stable in their host throughout several generations, independent of presence of Zn or Cu.

The molecular mechanisms of heavy metals on conjugative transfer of resistance genes, with exception of co-selective mechanisms, have rarely been investigated.[59] To understand the effect of Zn and Cu on conjugation in our study, we performed a real-time transcriptional analysis that showed a reduction in expression of conjugation-associated genes in response to subinhibitory concentrations of Zn and Cu. However, our findings contrast the report by Zhang *et al.*,[52] who found that different concentrations of heavy metals increased conjugation and upregulated the expression of genes involved in conjugation of plasmids. Strain background, concentrations of the metals, and experimental conditions might explain the contradictory results.

Conjugative transfer of plasmids are controlled by a wide range of genes.[60] A failure to form a functional relaxosome, mediated by the NikB protein, can result in an incapability of the plasmid to mobilize.[61] Our results from the transcriptional analysis could therefore indicate that the reduced expression of *nikB* in response to Zn and Cu could lead to a dysfunctional relaxosome, and the incapability of the plasmid to transfer from the donor to the recipient strain. The expression of the *traB* gene involved in plasmid transfer was also significantly reduced, which indicates that metals disturb the function of the conjugation machinery.

Our goal was to use concentrations of Zn and Cu that mimic the conditions found in the chicken intestines as closely as possible. We focused on the effect of heavy metals on the plasmid-encoded genes by using an experimental setup that excluded the effect of co-selection. However, we cannot exclude that Zn and Cu interfere with expression of chromosomal genes or genes located in the recipient strain. The effect of Zn and Cu on the SOS-response may be of future interest, in addition to investigate the combined effect of Zn and Cu on conjugation. The expression of other conjugational genes needs to be studied to learn more about how metals interfere with conjugation at a transcriptional level. It would also be beneficial to study horizontal gene transfer in more complex models than the ones used in the present study; preferable models that to a larger degree resembles the ''real life'' conditions in the chicken intestinal environment. Nevertheless, our results indicate altogether that Zn and Cu interfere with genes involved in conjugation, and thereby decrease the frequency of conjugational transfer of plasmids between *E. coli*.

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Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Figure S1
Supplementary Figure S2

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Paper 2

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RESEARCH ARTICLE Open Access

Population structure and uropathogenic potential of extended-spectrum cephalosporin-resistant *Escherichia coli* from retail chicken meat

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Abstract

Background: Food-producing animals and their products are considered a source for human acquisition of antimicrobial resistant (AMR) bacteria, and poultry are suggested to be a reservoir for *Escherichia coli* resistant to extended-spectrum cephalosporins (ESC), a group of antimicrobials used to treat community-onset urinary tract infections in humans. However, the zoonotic potential of ESC-resistant *E. coli* from poultry and their role as extraintestinal pathogens, including uropathogens, have been debated. The aim of this study was to characterize ESC-resistant *E. coli* isolated from domestically produced retail chicken meat regarding their population genetic structure, the presence of virulence-associated geno- and phenotypes as well as their carriage of antimicrobial resistance genes, in order to evaluate their uropathogenic potential.

Results: A collection of 141 ESC-resistant *E. coli* isolates from retail chicken in the Norwegian monitoring program for antimicrobial resistance in bacteria from food, feed and animals (NORM-VET) in 2012, 2014 and 2016 (*n* = 141) were whole genome sequenced and analyzed. The 141 isolates, all containing the beta-lactamase encoding gene bla_{CMY-2}, were genetically diverse, grouping into 19 different sequence types (STs), and temporal variations in the distribution of STs were observed. Generally, a limited number of virulence-associated genes were identified in the isolates. Eighteen isolates were selected for further analysis of uropathogen-associated virulence traits including expression of type 1 fimbriae, motility, ability to form biofilm, serum resistance, adhesion- and invasion of eukaryotic cells and colicin production. These isolates demonstrated a high diversity in virulence-associated phenotypes suggesting that the uropathogenicity of ESC-resistant *E. coli* from chicken meat is correspondingly highly variable. For some isolates, there was a discrepancy between the presence of virulence-associated genes and corresponding expected phenotype, suggesting that mutations or regulatory mechanisms could influence their pathogenic potential.

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Conclusion: Our results indicate that the ESC-resistant *E. coli* from chicken meat have a low uropathogenic potential to humans, which is important knowledge for improvement of future risk assessments of AMR in the food chains.

Keywords: Antimicrobial resistance, AMR, Poultry, Foodborne, Norway, Phenotype, *E. coli*, Virulence, Urinary tract infection, UTI

Background

Escherichia coli is a highly diverse species that includes commensals, pathogens, and opportunistic pathogens. *E. coli* that cause infections outside the intestinal tract are commonly referred to as extraintestinal pathogenic *E. coli* (ExPEC). ExPEC is usually phenotypically indistinguishable from gut-colonizing commensal *E. coli.* Based on their virulence traits, they are often divided into subgroups such as uropathogenic *E. coli* (UPEC), isolates causing septicemia, neonatal meningitis-causing *E. coli* (NMEC), and avian pathogenic *E. coli* (APEC) [1, 2]. As ExPEC are mainly considered opportunistic pathogens, it has been challenging to define a set of virulence factors for this group of bacteria. Terms like "fitness factors", "colonizing factors", and "virulence-associated traits" have been suggested as being more accurate for describing specific traits that distinguish ExPEC from other *E. coli* [2–4]. Johnson et al. proposed a list of ExPEC virulence-associated traits which included various adhesins, toxins, nutritional characteristics, protectins, and miscellaneous traits [3]. The common denominator for all these traits is provision of competitive advantages and survival outside the intestinal tract, with potential to cause disease in various other tissues [2–4]. Several reservoirs for ExPEC have been described, for example the intestinal tract of humans, companion animals, and food-producing animals [5]. Different typing methods have been applied for epidemiological purposes and understanding of the transmission of ExPEC between different reservoirs and hosts, allowing for differentiation of *E. coli* into group levels. Multilocus sequence typing (MLST) groups *E. coli* into various sequence types (STs), and some STs are known to possess a higher pathogenic potential than others [6].

Urinary tract infection (UTI) is one of the most common bacterial infections encountered in the human population worldwide, and comes with great societal costs [7]. A UTI starts with bacteria, such as UPEC, colonizing the distal parts of the urethra, thereby ascending into the bladder, adhering to the surface of the bladder, followed by biofilm formation, and then invasion and replication within the hosts cells [8–10]. Both structural and secretory features are involved in UPECs ability to cause UTI [8]. Structural virulence-associated traits including adhesins, fimbriae, flagella, and other surface components are involved in colonization of the mucosal surfaces in the urinary tract, while secreted components, such as toxins and enzymes, are responsible for cell-damage [11]. UTIs can vary from a mild bacteriuria to severe urosepsis, and antimicrobials are often needed for curing the infection [12].

Antimicrobial resistance (AMR) is one of the largest threats against global public health in our time [13, 14]. Use of antimicrobials is regarded as the most important driver for development and dissemination of AMR, although the exact and relative amounts distributed to and between human and veterinary medicine vary considerably from country to country [15–17]. On a global basis, the use and overuse of antimicrobials in food-producing animals is extensive, and the co-occurrence of AMR, including extended-spectrum cephalosporin (ESC)-resistant *E. coli,* in the food chains is considerable [15, 17, 18]. The Norwegian monitoring program for antimicrobial resistance in bacteria from food, feed, and animals (NORM/NORM-VET) have for several years documented that Norway is among the European countries with the lowest levels of antimicrobial use and corresponding low levels of AMR [13, 14, 19, 20]. NORM-VET is governed by the legislation ensuring harmonized AMR monitoring within the EU, and poultry are sampled every other year [21]. Results document that the Norwegian broiler production has a low level of antimicrobial use with only one to seven flocks treated yearly between 2013 and 2017 [22–27]. ESC-resistant *E. coli* have nevertheless been detected in healthy broilers and retail chicken meat using selective methods since their first observed appearance in 2006, and with significant reduction since 2012 [28, 29].

Food-producing animals and their products are considered a possible source for human acquisition of AMR *E. coli* [30–32]. ESC-resistant *E. coli* isolates are of particular interest, as extended-spectrum cephalosporins are listed as critically important antimicrobials by the World Health Organization [33]. Genetic comparisons of *E. coli* isolates from poultry and clinical UPEC isolates have been observed to have a high degree of similarity [34], and *E. coli* isolates from meat have also been shown to cause UTI in murine models [35]. In a review from 2015, Lazarus et al. considered poultry to be the most likely source of human acquisition of ESC-resistant

ExPEC from food-producing animals [36], and consumption of chicken meat could thus be a possible route of ExPEC transmission, including UPECs [35–38]. In a report from 2015 the Norwegian Scientific Committee for Food and Environment (VKM) also concluded that poultry and poultry products are regarded as the most important reservoirs of ESBL/AmpC-producing *Enterobacteriaceae*, quinolone resistant *E.coli* (QREC), and their corresponding resistance determinants [39]. However, lack of data has made it difficult to reach any firm conclusions regarding the probability of AMR transmission from food to humans.

The aim of this study was to characterize ESC-resistant *E. coli* isolated from domestically produced retail chicken meat regarding their population genetic structure, the presence of virulence-associated geno- and phenotypes as well as their carriage of AMR genes, in order to evaluate their uropathogenic potential. To ensure a highly relevant collection of bacterial isolates, all ESC-resistant *E. coli* isolates collected from retail chicken meat through NORM-VET in 2012, 2014, and 2016 were included in the initial screening and description. Representative isolates were selected for further phenotypic virulence characterization.

Results

Characterization of population structure

All the 141 ESC-resistant isolates were whole genome sequenced and the isolates were grouped into 19 different sequence types (STs) based on 7-gene MLST. A core genome (cg) MLST including 2360 genes clustered each ST separately (Fig. 1). There was an annual variation in the presence of STs; ST38 was the most common ST in both 2012 ($n = 57$, 86%) and 2014 ($n = 16$, 28%) but was not detected in 2016. A total of 241 allele differences were present among the 73 ST38 isolates, and none of the isolates displayed identical cgMLST profiles. ST1158 was also common among isolates collected in 2014 ($n = 15$, 26%). However, this ST was not present in either 2012 or 2016. Fifty allele differences were detected among isolates belonging to ST1158, and two isolates shared an identical cgMLST profile. In 2016, ST2040 emerged as a new ST, and was also the dominant ST that year ($n = 11$, 65%). A total of 32 allele differences were observed between the isolates belonging to ST2040, with two isolates displaying identical cgMLST profiles. The highest diversity of STs was observed in 2014, with 12 different STs being represented among the isolates, followed by seven different STs in 2012, and five in 2016. Two STs were present in all three years, namely ST10 ($n = 3 + 2 + 1$) and ST1594 ($n = 1 + 1 + 1$). In the cgMLST analysis, 39 and 44 allele differences were detected among ST10 and ST1594 isolates, respectively. None of the ST10 nor the ST1594 isolates shared identical cgMLST patterns (Fig. 1). An overview of the results from the ST-profiling is presented in Fig. 2.

Virulence-associated geno- and phenotypes

In order to study the virulence potential of the 141 ESC isolates we used Virulence Finder and vfdb_core databases to detect virulence genes. Based on the authors' knowledge and descriptions in the databases specific UPEC-associated virulence genes were identified. A complete overview of all virulence genes identified in each of the 141 isolates investigated is found in the Supplementary material (Table S1). In general, a limited number of genes encoding UPEC-associated virulence factors were present among the isolates. Genes encoding the UPEC-associated toxins hemolysin (*hly*) or cytotoxic necrotizing factor 1 (*cnf1*) were not identified in any of the isolates. The most common UPEC-associated virulence genes detected among the *E. coli* isolates encoded proteins involved in iron uptake, synthesis of type 1 fimbriae, serum survival, and capsule formation (Table S1). Furthermore, incomplete operons encoding certain traits, such as P-fimbriae (*papA-K*) and type 1 fimbriae (*fimA-I*) were identified in several isolates.

To evaluate the expression of detected genes and virulence-potential, a selection of isolates was chosen for further phenotypical testing. These isolates were selected based on the following criteria: 1) representatives from each phylogroup (A, B1, B2, or D) and different sequence types, 2) isolates with the most and the fewest virulence genes, 3) representatives from the most prevalent sequence types, 4) at least one representative from common ExPEC STs. An overview of these 18 isolates and their UPEC- and AMR-associated genes are summarized in Table 1. A summary of the results from the in vitro phenotypic tests related to the presence of selected virulence-associated genes is presented in Fig. 3. In total, there was limited consistency between the occurrence of genes and the corresponding traits detected in the in vitro phenotypic assays.

Expression of the type 1 fimbriae was assessed by a yeast agglutination test which tests the ability of type 1 fimbriated bacteria to bind to mannose receptors on the surface of yeast cells. Seven out of 18 isolates did not agglutinate the yeast cells. Among the remaining 11 positive isolates, six harbored the complete *fim A- I* operon, while at least one of the genes in the *fim* operon was not detected in the other five. Among the seven isolates that were not able to agglutinate yeast cells, a complete *fim* operon was identified in two isolates (Fig. 3).

To be able to migrate from the gastrointestinal tract to other tissues, resistance to killing by human serum provides a huge advantage for bacterial survival. None of the isolates were classified as sensitive in the serum-resistance assay. Most of the isolates exhibited

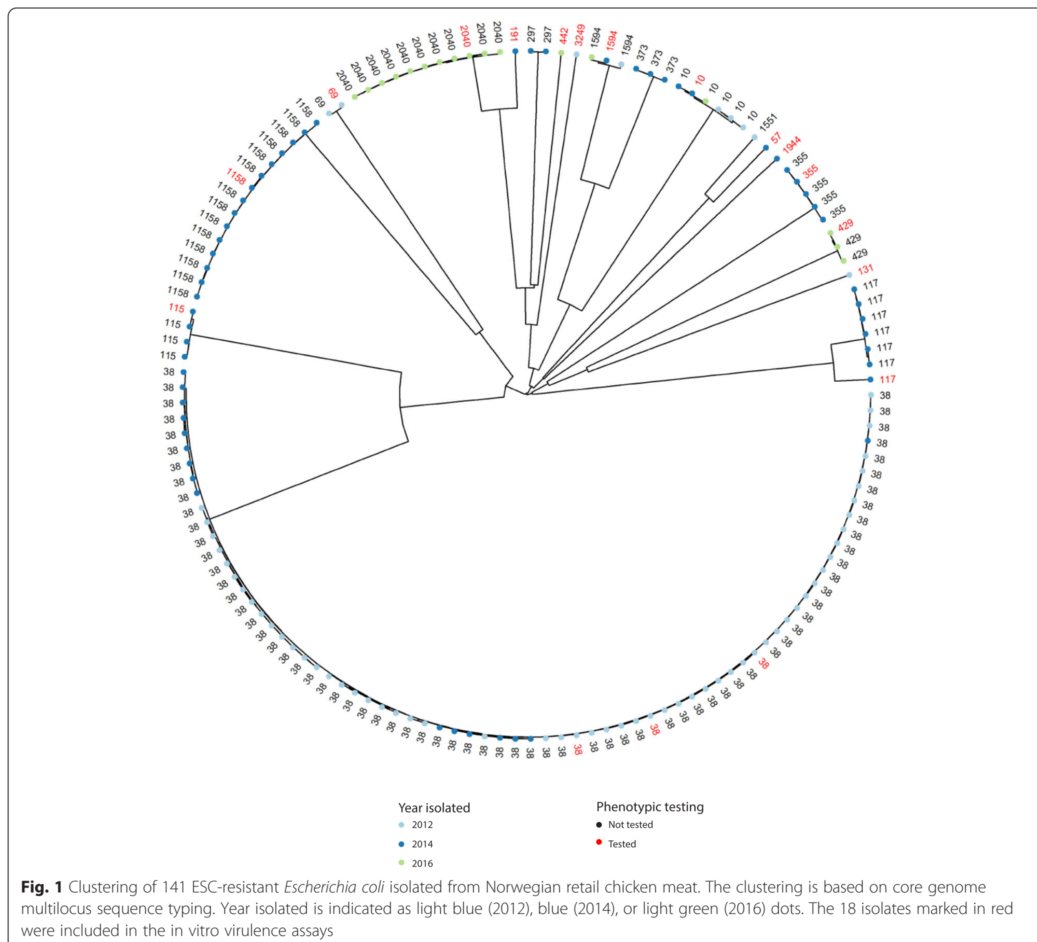

Fig. 1 Clustering of 141 ESC-resistant *Escherichia coli* isolated from Norwegian retail chicken meat. The clustering is based on core genome multilocus sequence typing. Year isolated is indicated as light blue (2012), blue (2014), or light green (2016) dots. The 18 isolates marked in red were included in the in vitro virulence assays

intermediate serum resistance ($n = 10$), whereas the remaining isolates were resistant to inactivation by human serum. Five of the resistant isolates and six of the isolates that exhibited intermediate resistance harbored the *iss* gene.

Production of colicins can provide competitive advantages by inhibiting growth of other coliform bacteria, ensuring the isolate to prevail. Thirteen isolates produced colicins. The most prevalent colicin-encoding gene detected among the *E. coli* isolates was *cma,* encoding Colicin M. The *cma* gene was present in eight of the colicin-producing isolates. In two of these isolates, *cma* was found in combination with *celB* (Colicin E2) and *cba* (Colicin B), in two isolates in combination with only *celB* or in combination with *mchB*, *mchC* and *mchF* (Microcin H47), respectively. One of the colicin-producing isolates carried only the *celB* gene, while *celB* was present in combination with *mchB* and *mchC* in another isolate. There was also one isolate that only carried the *mcmA* gene (Microcin M/Colicin V). Finally, there were two colicin-producing isolates which did not carry any of the investigated colicin-encoding genes. In four of the five isolates without colicin production, genes associated with colicin production were identified (Fig. 3).

We wanted to characterize the potential of our isolates to colonize a host. Thus, the ability to adhere to and invade Vero cells were studied. The isolates showed a high capability of bacterial adhesion to eukaryotic cells, but low capability of cell invasion. Data for cell adhesion and invasion for isolate 2016–22-220 (ST429) is not included in the results as concentrations of antibiotics used to kill adherent bacteria were not effective for this isolate,

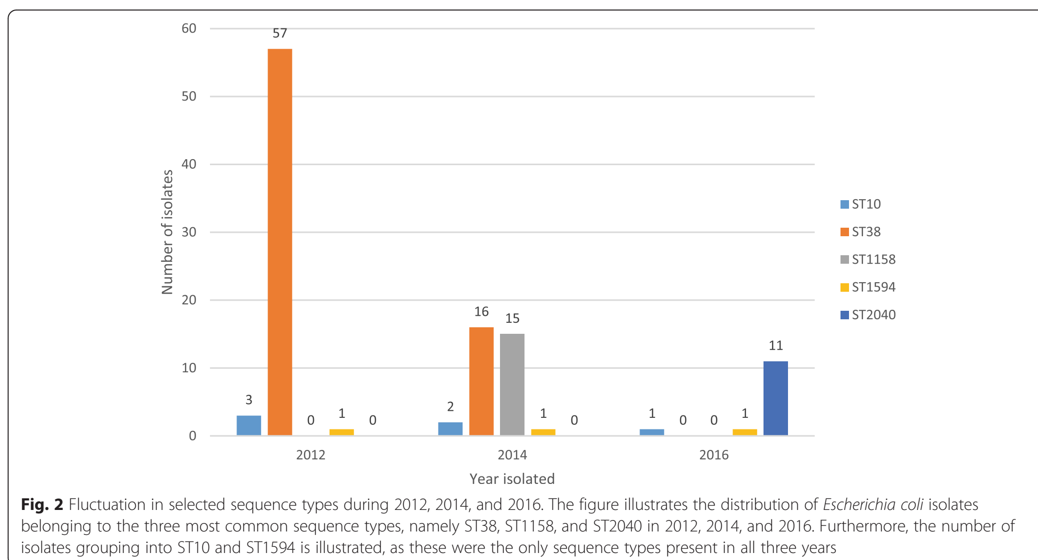

Fig. 2 Fluctuation in selected sequence types during 2012, 2014, and 2016. The figure illustrates the distribution of *Escherichia coli* isolates belonging to the three most common sequence types, namely ST38, ST1158, and ST2040 in 2012, 2014, and 2016. Furthermore, the number of isolates grouping into ST10 and ST1594 is illustrated, as these were the only sequence types present in all three years

despite exceeding the predicted MIC values. Several genes may be involved in the process of cell adhesion. Genes investigated in this study include the *sfaX* gene, the *pap* operon and the *fim* operon. In only one of the isolates, 2014-01-3678 (ST117), both the *sfaX* gene and a complete *pap* operon were identified. However, an incomplete *fim* operon was also identified in the same isolate. This isolate displayed a high degree of cell adhesion (> 400 CFU/ml). The degree of cell adhesion among the eight isolates with a complete *fim* operon was observed to be very variable. Although isolate 2014-01-4991 (ST57) carried incomplete *fim*- and *pap* operons, and the *sfaX* gene was absent it showed a high degree of cell adhesion.

Motility was evaluated as the ability of the bacteria to move through semi-liquid LB agar. None of the isolates were able to move in 0.7% agar. Eight of the isolates were non-motile in 0.2% agar, but only isolate 2014-01-7011 (ST1944) was non-motile in 0.03% agar. The ability to form biofilm varied among the isolates, between temperatures and culture incubation times. Biofilm production was defined by OD three times as high as the control. Isolate 2012–01-707 (ST38) produced the strongest biofilm at 37 °C, while isolate 2016-22-1061 (ST2040) was the strongest biofilm producer at 20 °C. Nine of the isolates displayed poor biofilm formation at both 37 °C and 20 °C. All isolates were able to grow in human urine (Fig. 4). Isolate 2016-22-1061 (ST2040) exhibited the most rapid growth of the 18 isolates, given the experimental conditions provided, both in urine and in LB. Growth rates are provided in the supplementary material (Table S2).

Acquired AMR genes

The presence of $bla_{\mathrm{CMY\text{-}2}}$ in all isolates was confirmed by the WGS data, and some of the isolates carried additional resistance genes. These included ampicillin-resistance gene bla_{TEM1B} ($n = 13$), sulfonamide-resistance genes *sul1* ($n = 5$) and *sul2* ($n = 11$), streptomycin- and spectinomycin-resistance gene *aadA1* ($n = 6$), aminoglycoside-resistance genes *aac (3)*-VIa ($n = 3$) and *aph* ($n = 2$), tetracycline-resistance genes *tet*(A) ($n = 3$) and *tet*(B) ($n = 2$), and trimethoprim-resistance genes *dfrA1* ($n = 2$) and *dfrA5* ($n = 1$). In general, isolates with the same ST had highly similar AMR genes. An overview of acquired AMR genes and their association with ST in all included isolates is presented in the Supplementary material (Fig. S1 and Table S1).

Discussion

We have characterized by WGS all ESC-resistant *E. coli* isolated from retail chicken in Norway in 2012, 2014, and 2016 obtained through the NORM-VET monitoring program. This enabled in-depth characterization of the population dynamics among ESC-resistant isolates over a prolonged time period. As mentioned earlier NORM-VET is governed by the legislation for surveillance of AMR, and the sampling was therefore performed

Table 1 Overview of genetic characteristics, including virulence and AMR genes, for 18 extended-spectrum cephalosporin-resistant *Escherichia coli* isolates included in the phenotypic characterization

ID	Year	ST	Phylo-group	Serotype	AMR genes	UPEC associated virulence genes
2012-01-1292	2012	38	D	O7:H18	bla_{CMY-2}	*fimA-I, iucA-D, iutA, chuA, chuS-Y, entA-F, entS, fepA-D, fepG, iroN, kpsM, iss, cma,*
2012-01-1295	2012	38	D	O7:H18	bla_{CMY-2}	*fimA-I, iucA-D, iutA, chuA, chuS-Y, entA-F, entS, fepA-D, fepG, iroN, kpsM, iss, iha, cma,*
2012-01-2798	2012	3249	A	O8:H9	bla_{CMY-2}, *sul1*	*fimC-I, entA, entC, entE-F, entS, fepC-D, fepG, iss, astA, celB, cma*
2012-01-3586	2012	131	B2	O25:H4	bla_{CMY-2}	*fimB-I, iucA-D, iutA, chuA, chuS-Y, entA-C, entE-F, entS, fepA-D, fepG, iroN, tsh, kpsM, iss, fyuA, iha, usp, celB, mchB, mchC, tsh*
2012–01-707	2012	38	D	O7:H18	bla_{CMY-2}, *sul2*	*papB, papI, fimA-I, iucB-D, iutA, chuA, chuS-Y, entA-F, entS, fepA-D, fepG, iroN, kpsM, iss, iha, cma*
2012–01-771	2012	69	D	O17/O44, O17/O77:H18	bla_{CMY-2}, *sul2, aadA, dfrA*	*fimB-D, fimF-I, iucA-D, iutA, chuS, chuU-Y, entA-C, entE-F, entS, fepA-D, fepG, iss, astA, cma*
2014-01-1336	2014	1594	A	O21:H4	bla_{CMY-2}, bla_{TEM1}	*fimA-I, iucA-D, iutA, entA-C, entE-F, entS, fepA-D, fepG, kpsM, astA, celB*
2014-01-3678	2014	117	D	O24:H4	bla_{CMY-2}, *sul1, aadA*	*papB-papK, sfaX, fimB-I, iucB-D, iutA, chuA, chuS-Y, entA-C, entE-F, entS, fepA-D, fepG, iroN, pic, vat, ireA, fyuA, mcmA*
2014-01-3680	2014	1158	D	O17/O44, O17/O77:H34	bla_{CMY-2}	*fimA-C, fimE-I, iucA-D, iutA, chuA, chuS-Y, entA-C, entE-F, entS, fepA-D, fepG, kpsM, iss, iha*
2014-01-4267	2014	191	A	O150:H20	bla_{CMY-2}	*fimA-I, entA-C, entE-F, entS, fepA-D, fepG, iha, mchB, mchC, mchF*
2014-01-4991	2014	57	D	ONT:H18	bla_{CMY-2}	*fimA-C, fimE-I, chuA, chuS-Y, entA-C, entE-F, entS, fepA-D, fepG*
2014-01-5104	2014	115	D	O102:H6	bla_{CMY-2}	*fimA-I, iucB-D, iutA, chuV-Y, entA-F, entS, fepA-D, fepG, kpsM, iss, astA, cba, celB, cma*
2014-01-5656	2014	10	A	O125ab:H4	bla_{CMY-2}	*fimB-I, iucB-D, iutA, entA-C, entE-F, entS, fepA-D, fepG, iss, fyuA, astA, iha*
2014-01-7011	2014	1944	D	O38:H39	bla_{CMY-2}	*fimF-H, chuA, chuS-Y, entA-F, entS, fepA-D, fepG, iha, cma, mchB, mchC, mchF*
2014-01-7037	2014	355	B2	O2:O50/O2:H5	bla_{CMY-2}	*fimB-I, iucA-D, iutA, chuA, chuS-Y, entA-C, entE-F, entS, fepA-D, fepG, kpsM, iss, fyuA, astA, iha, usp, cba, celB, cma*
2016–22-220	2016	429	B2	O50/O2:H1	bla_{CMY-2}, *sul1, aadA, aac, tetA*	*fimA-I, iucB-D, iutA, chuA, chuS-Y, entA-F, entS, fepA-D, fepG, iroN, kpsM, iss, fyuA, usp, mchF*
2016–22-832	2016	442	B1	O91:H21	bla_{CMY-2}, *dfrA*	*fimB-I, iucB-D, iutA, entA-C, entE-F, entS, fepA-D, fepG, iroN, mchF*
2016-22-1061	2016	2040	A	O159:H20	bla_{CMY-2}	*fimA-I, iucB-D, iutA, entA-F, entS, fepA-D, fepG, iroN, tsh, cma*

following defined schemes designed to be representative for the entire population [21].

Since 2012 there has been a significant decrease in the occurrence of ESC-resistant *E. coli* in Norwegian retail chicken [40]. Newly published data showed that only 0.4% of samples were positive in 2018 [40]. Furthermore, semi-quantitative methods have revealed that in the vast majority of Norwegian retail chicken samples where ESC-resistant *E. coli* were detected, only very low levels of these bacteria were present (≤ 0.2 cfu/g) [19, 41]. This is consistent with recent trends reported from Denmark, Sweden, and the Netherlands, where a decrease in the number of ESC-resistant isolates has also been described over the last decade [42–44]. In 2014, the Norwegian poultry industry initiated an action plan against antimicrobial resistant bacteria in broiler production [22]. The sum of measures taken by the industry, both nationally and internationally, has likely contributed to improve the situation.

Although the occurrence of ESC-resistant *E. coli* in Norwegian retail chicken decreased during the five-year study period, the relatively large variation in STs indicates that there are annual fluctuations in the population of ESC-resistant *E. coli* STs. This underlines the complex epidemiology of ESC-resistant *E. coli*, which has also been highlighted by others [45]. Understanding the epidemiology is further complicated by the occurrence of both vertical and horizontal dissemination of ESC-

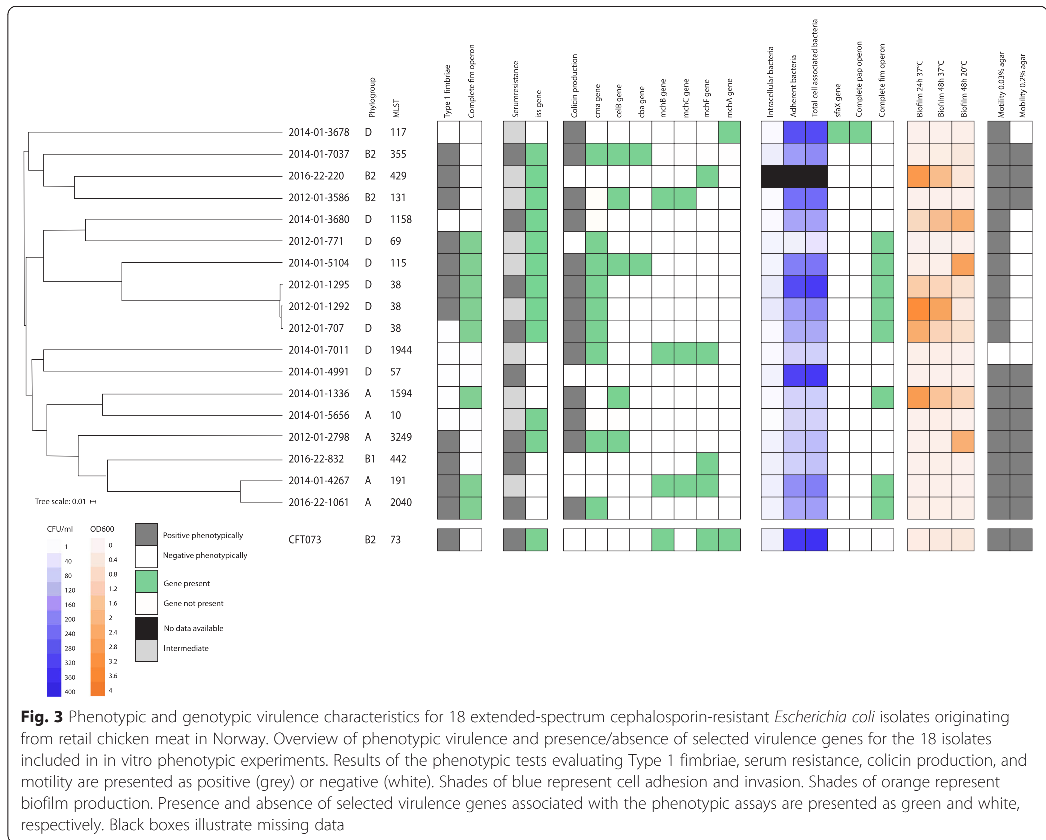

Fig. 3 Phenotypic and genotypic virulence characteristics for 18 extended-spectrum cephalosporin-resistant *Escherichia coli* isolates originating from retail chicken meat in Norway. Overview of phenotypic virulence and presence/absence of selected virulence genes for the 18 isolates included in in vitro phenotypic experiments. Results of the phenotypic tests evaluating Type 1 fimbriae, serum resistance, colicin production, and motility are presented as positive (grey) or negative (white). Shades of blue represent cell adhesion and invasion. Shades of orange represent biofilm production. Presence and absence of selected virulence genes associated with the phenotypic assays are presented as green and white, respectively. Black boxes illustrate missing data

resistance. The vast majority of ESC-resistant *E. coli* included in this study were known to carry $bla_{\text{CMY-2}}$ on self-transferable IncK plasmids [46]. However, in the emerging ST2040, $bla_{\text{CMY-2}}$ was present on a non-transferable IncI1 plasmid (unpublished data). We have not investigated if virulence genes were located on plasmids or a possible co-location of AMR- and virulence genes. In order to do so, it is necessary to perform long-read sequencing to enable reliable hybrid assemblies. This would enable us to consider the potential of dissemination and co-dissemination of AMR- and virulence determinants in the bacterial population.

Only two STs were present over all three years, namely ST10 and ST1594. However, only one or a limited number of isolates belonging to these STs were detected each year, indicating that they were not common in the ESC-resistant *E. coli* population. The three major STs in our material, ST38, ST1158, and ST2040, have all been previously described to occur in the broiler production in Europe [47–49]. This observation intrigues us to claim that certain successful ESC-resistant *E. coli* STs disseminate widely in the European broiler production, but further comparisons of sequence- and epidemiological data are warranted to confirm this hypothesis.

Five of the 19 *E. coli* STs identified among our isolates (ST131, ST117, ST38, ST10, and ST69) are included in the "top 20 ExPEC ST"-list published in a meta-analysis [50]. This included 217 studies that performed MLST or whole-genome sequencing to genotype *E. coli* recovered from extraintestinal infections or the gut. Our results from analyses of virulence associated genes and in vitro virulence assays revealed a large variation in the estimated virulence potential among the different STs including those that previously have been classified as ExPEC. The high diversity of virulence-associated traits suggests that the uropathogenic potential of ESC-resistant *E. coli* from poultry meat is isolate dependent and/or dependent on the sensitivity of the individual host. Based on data from the in vitro virulence assays, none of the isolates belonging to known ExPEC STs

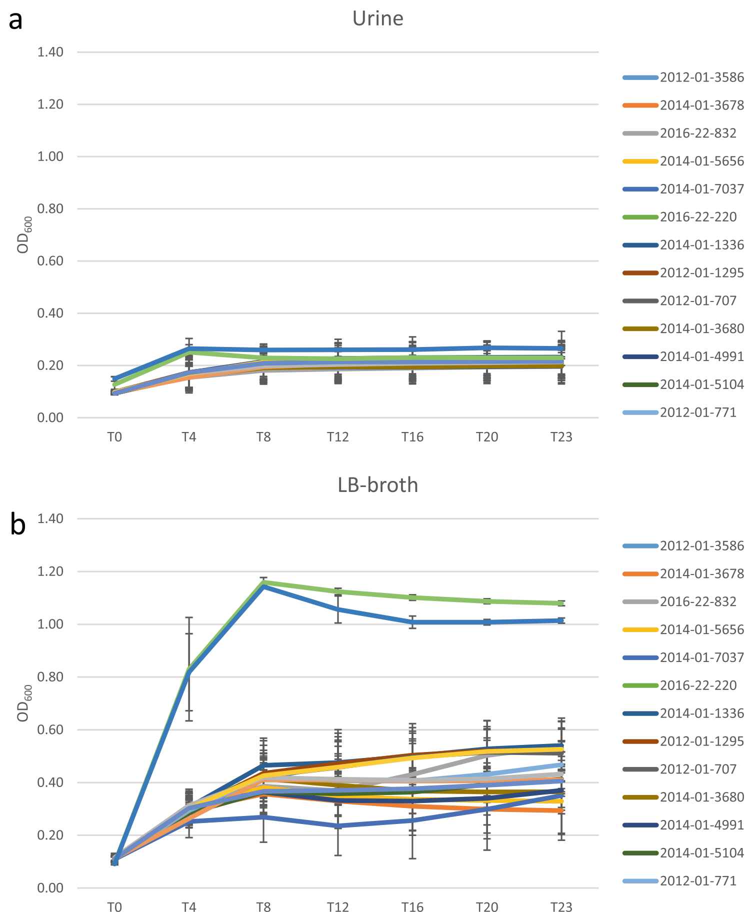

Fig. 4 Bacterial growth. The figure illustrates the growth of the 18 *E. coli* isolates included in the in vitro phenotypic studies in sterile filtered human urine (**a**) and LB broth (**b**). Growth in LB was included as a measure on optimal growth conditions and used as basis for comparison with growth in sterile filtered human urine. Growth curves were obtained from a Tecan plate reader in which the optical density was measured at 600 nm every ten minutes for 23 h. Time is given in hours on the x-axis, and the measured optical density on the y-axis. The experiment was performed in triplicate, and the standard deviations are indicated by whiskers

appeared to have higher uropathogenic potential than isolates belonging to the other STs. However, host related factors have not been considered in this work, and thus may affect the clinical outcome in an infection.

An important prerequisite for developing a UTI is the ability of uropathogenic *E. coli* to adhere to and invade the uroepithelium, and thus colonize the mucosal epithelial surfaces in the urinary tract. Expression of type 1 fimbriae which mediate bacterial adhesion to mannose-containing structures on the uroepithelium is particularly associated with UTIs [51], and this feature was found in half of our selected isolates. Notably, several of the isolates were able to form functional type 1 fimbriae despite lacking a complete *fim*-operon. None of the isolates showed a high degree of invasiveness in Vero cells under the experimental conditions used, and this could indicate that long-term survival in the uroepithelium would be limited.

We evaluated biofilm formation, colicin production and ability to grow in urine, all of which may influence the ability of isolates to colonize the human urinary tract [52, 53]. All isolates were able to grow in human urine, which may be a predictor of the colonization ability of

uropathogens [54]. This is consistent with growth of *E. coli* CFT073 performed in urine by others [55, 56].

One of the first steps of colonization is the establishment of biofilm, which provides protection from the shear forces of passing urine in the urinary tract. In addition, the forming of biofilm will also provide advantages in avoiding the immune system and giving increased protection from antimicrobials [57]. The ability of the isolates to form biofilm in Luria-Bertani (LB) broth without NaCl was tested at two different temperatures, and at two different time points. Biofilm formation is highly dependent on environmental conditions and the access to nutrients. Half of the tested isolates were able to form biofilm and most isolates of those that were positive formed more biofilm at 37 °C compared to 20 °C, possibly indicating ability to form biofilm in the human body. Previous reports have indicated that uropathogenic bacteria within biofilms or in biofilm-like communities may promote virulence under certain growth conditions, for example by creating intracellular pod-like bulges inside of the bladder epithelial cells [58, 59]. This is considered important especially for recurrent UTIs, where the intracellular bacterial communities (IBC) facilitate persistence in the urinary epithelium [58, 59]. Our study provided limited experimental conditions, where the ability to form IBCs was not studied. Further studies of the ability of ESC-resistant *E. coli* to form biofilm on human urinary tract epithelium are needed to evaluate their uropathogenic potential. The production of specific enzymes and toxins, for example colicins, can provide beneficial colonization conditions as it limits competition from other bacteria [52]. As most of our isolates produced colicins, we can assume that these isolates possess competitive advantages over other *E. coli* isolates in the intestines.

Serum resistance is a key virulence trait of isolates that cause urosepsis and all isolates tested were serum resistant. Urosepsis is a serious complication in UTIs that requires immediate medical care to avoid a possible life-threatening situation [60]. Furthermore, if an UTI is caused by an UPEC isolate resistant to clinically relevant antimicrobials, the treatment could be complicated, prolonged and costly [61, 62].

Isolate 2016-22-1061 (ST2040) stood out as the isolate which expressed the most UPEC-associated virulence factors (type 1-fimbriae, production of colicins, survival in human serum, and the fastest growth in urine). Interestingly, this isolate belongs to phylogroup A, which is rarely described to cause extraintestinal infections. Nevertheless, due to its estimated pathogenicity and recent emergence as described above, attention should be paid to this ST in future surveillance.

Isolate 2012-01-3586 belonged to ST131, serotype O25:H4 and phylogroup B2, which is known to be a notorious ExPEC ST and is considered a high-risk clone [63, 64]. Surprisingly, this isolate appeared have a lower virulence potential than expected; it produced colicin, expressed the type 1-fimbriae and adhered to eukaryotic cells, but it was among the isolates that expressed the lowest serum resistance. It did not invade Vero cells, and was a weaker biofilm producer compared to all isolates tested. Furthermore, none of the genes encoding toxins commonly produced by pathogenic ST131 isolates, namely *pic*, *vat*, *sat*, *hlyA/D*, *astA*, *cdtB*, and *cnf1* [65, 66] were present in the genome of this isolate. We detected the *fimH*38 allele in isolate 2012-01-3586, while the global high-risk ST131 clone has been associated with the *fimH*30 allele [63, 67]. Thus, it is possible that this isolate belongs to a sub-group with lower pathogenic potential compared with the previously described ST131 ExPEC clone.

The lack of direct correlation between the observed genotypes and phenotypes in our experiments complicates interpretation of the results. Many of the traits that we investigated have complex genetic backgrounds, and several genes may give rise to the same phenotype. For example; isolate 2014-01-4991 (ST57) carried incomplete *fim*- and *pap* operons, and the *sfaX* gene was absent but still showed a high degree of cell adhesion in the phenotypic testing. This genotype to phenotype divergence illustrates the importance of performing in vitro virulence tests in order to assess the possible pathogenic potential of isolates rather than relying solely on comparative genomics [68, 69]. Furthermore, the study of virulence associated traits was limited to only 18 isolates, but discrepancy observed between genotype and phenotype indicates that there could be differences in pathogenic potential within the same ST. For comparison of phenotypic characteristics of relevance for pathogenicity, we used *E. coli* CFT073 as a positive control strain in all our in vitro experiments. This strain was originally isolated from the blood of a woman with acute pyelonephritis and is regarded as an UPEC prototype [70]. However, none of the isolates that we investigated had an identical phenotypic profile to that of *E. coli* CFT073 (Fig. 3).

The plasmid mediated AmpC beta-lactamase encoding gene $bla_{\text{CMY-2}}$ has played a major role in conferring ESC-resistance in the Norwegian *E. coli* isolates, while genetic linkages to genes encoding resistance to other antimicrobial classes have not been prominent [46, 71]. Previous studies of the phenotypical resistance patterns of these isolates have confirmed that the occurrence of co-resistance to other antimicrobials is limited [41, 71, 72]. In this study we focused on ESC-resistant *E. coli*. However, AMR and virulence genes are not necessarily linked, and it is possible that susceptible *E. coli* of broiler origin may have a different virulence potential than the ESC-resistant isolates investigated here. Several studies

have suggested that food products, especially chicken meat, are an important source of ESC-resistant ExPEC [31, 36]. On the other hand, other more recent studies have reported a limited contribution of chicken meat to the overall occurrence of ESC-resistant *E. coli* in humans [73–76]. By revisiting previously analyzed materials using WGS, de Been et al. failed to provide any evidence for recent clonal transmission of ESC-resistant *E. coli* strains from poultry to humans [77]. All these studies were performed in countries where the occurrence of ESC-resistant *E. coli* in poultry and/or chicken meat is higher than in Norway. The occurrence of ESC-resistance among clinical UPEC isolates in Norway is considered very low, only 3.4% in 2018 [40]. However, results from one Norwegian study indicated that clonal transfer of ESC-resistant *E. coli* from chicken meat to humans may occur, and that these bacteria could be a source of ESC-resistance plasmids that could be transferred to bacteria residing in the human gut microbiota [78]. However, only a limited number of isolates were included in the Norwegian study, and the presence of virulence factors known to be associated with UTIs was not in focus.

One strength of our study is that all ESC-resistant *E. coli* isolated from retail chicken in Norway in 2012, 2014, and 2016 were characterized in depth using cgMLST and that acquired AMR genes and virulence genes were investigated. As well as providing an overview of the population structure, these data also demonstrate the occurrence or absence of genes that encode virulence factors of possible relevance regarding pathogenic potential.

Conclusion

Our study showed a fluctuation in the ST composition of ESC-resistant *E. coli* isolated from retail chicken meat in 2012, 2014, and 2016. Five of the STs present have previously been associated with ExPEC. However, results from the in vitro virulence assays did not indicate that our isolates from these STs had a higher pathogenic potential than isolates from other STs. These observations suggest that the estimated pathogenic potential of ESC-resistant *E. coli* from poultry meat is highly dependent on the individual isolate. In conclusion, our results indicate that the uropathogenic potential of ESC-resistant *E. coli* from the Norwegian poultry reservoir is limited. It is reasonable to assume that the risk of being exposed to ESC-resistant *E. coli* with pathogenic potential through handling and consumption of chicken meat in Norway is low. However, we have also shown that the population structure of the ESC-resistant *E. coli* is dynamic and the genetic diversity of the fluctuating STs is considerable. It is therefore important to maintain monitoring programs and the implementation of preventive measures to hinder the emergence of AMR and potential pathogenic variants in the Norwegian broiler production.

Materials and methods

Bacterial isolates

All ESC-resistant *E. coli* isolated from domestically produced retail chicken meat in the NORM-VET program in 2012 ($n = 66$), 2014 ($n = 58$), and 2016 ($n = 17$) were included in the study (total $n = 141$). All isolates were known to carry the $bla_{\text{CMY-2}}$ gene encoding ESC resistance [19, 41, 72]. Year of isolation, phylogroup, serotype, and AMR profile of the 18 isolates selected for in vitro virulence characterization are described in Table 1. *E. coli* CFT073 [79], a known UPEC strain, was also analyzed for comparison in the in vitro experiments.

DNA extraction

Total genomic DNA was extracted using the DSP DNA Mini Kit (Qiagen, Hilden, Germany) and the QIAsymphony automated extractor (Qiagen), or manually either using the QIAmp DNA Mini kit or the Qiagen Blood and Tissue kit (Qiagen). DNA concentration was determined on a Qubit™ fluorometer (ThermoFischer Scientific, Waltham,) using the Qubit™ dsDNA BroadRange assay kit (ThermoFischer Scientific). DNA purity was measured on a Nanodrop 2000 spectrophotometer (ThermoFischer Scientific).

Whole genome sequencing

Samples were prepared with either the Nextera XT or Nextera Flex library preparation kit (Illumina, San Diego, CA, USA). Whole genome sequencing was performed on an Illumina HiSeq X ($n = 12$) or Illumina NextSeq 500 ($n = 107$), resulting in 150 bp paired-end reads, or on a HiSeq 2500 using rapid mode ($n = 9$), resulting in 250 bp paired-end reads. In addition, sequence data for 13 isolates (five from 2012 and eight from 2014) had been sequenced previously [46, 78], and raw reads were available for inclusion in the present study. An overview of which isolates were sequenced on the different platforms is provided in the Supplementary material (Table S1).

Bioinformatics

Initial quality control and assembly of samples was done using the Bifrost pipeline [80]. Briefly, quality control of the paired end reads was done using the FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the results were merged using MultiQC [81]. Further, PhiX was removed by BBDuk (https://sourceforge.net/projects/bbmap/), and sequences were trimmed with Trimmomatic [82]. Assembly was done using SPAdes [83], polished using Pilon [84], and the quality of the assemblies evaluated with QUAST [85].

Subsequently, the Bifrost pipeline for identification of specific genes such as MLST, AMR genes and virulence genes were used [86] In detail, the ARIBA (Antimicrobial Resistance Identification By Assembly) software [87] was used to determine multilocus sequence type (MLST) according to the Achtman scheme [88]. The presence of acquired resistance genes as well as virulence genes was determined using the ResFinder [89] and VirulenceFinder [90] and vfdb_core [91] databases, respectively. The analyses were performed in September 2019 and the updated databases were used. The *E. coli* serotypes were determined using SerotypeFinder [92].

Results from ARIBA were summarized using VAMP IR- Virulence, AMR, MLST and Plasmid analysis in R (https://github.com/hkaspersen/VAMPIR, commitid 54d687a) in R version 3.5.2 [93].

All isolates were subjected to cgMLST analysis, including 2360 genes, in order to investigate the genetic relationship between isolates. This was done using the cgMLST scheme available from Enterobase (https://enterobase.warwick.ac.uk/) in the chewBBACA suite [94]. The cgMLST tree was visualized using the ggtree package [95] in R version 3.5.2 [93]. Sequence data is available at European Nucleotide Archive (ENA). Accession numbers are given in Table 2.

In vitro virulence expression

Results from the genetic analysis and in vitro testing were summarized and visualized using iTOL (http://itol.embl.de) [96], and are presented in Fig. 3.

Expression of type 1 fimbriae The ability to express a D-mannose-binding phenotype, characteristic for functional Type 1 fimbriae, was assayed by the ability to agglutinate yeast cells (*Saccharomyces cerevisiae)* [97]. Each isolate was inoculated into LB broth and incubated over night at 37 °C. One ml of overnight culture was centrifuged (3000 x g, 5 min) and the pellet resuspended in 100 μl PBS. Ten μl of the bacterial suspension was mixed with 10 μl yeast cells (5 mg/ml, resuspended in PBS) with and without 1% D-mannose solution on a microscopy slide, and agglutination observed visually. Suspension containing 1% D-mannose was considered a negative control.

Table 2 Accession numbers for sequence data at European Nucleotide Archive (ENA)

Group	Read length	Setsize	N	Project accession
Nextseq	150	2	107	PRJEB40941
HiseqX	150	2	12	PRJEB40952
Hiseq2500	100	2	13	PRJEB40969
Hiseq2500rapid	250	4	9	PRJEB41003

Motility test One colony from a fresh blood agar plate of each isolate was perpendicularly inoculated into a tube containing 5 ml semi-solid LB agar, at concentrations of 0.03, 0.2, and 0.7% agar, and incubated for 24 h at 37 °C [98]. Motile bacteria appeared as a "cloud" of bacterial growth in the agar around the stab-line.

Biofilm production Biofilm production was evaluated as described by Stromberg et al., with minor modifications [99]. Briefly, overnight cultures in LB broth were diluted 1:200 in LB without NaCl, and 200 μl added to a 96-well microtiter plate (Greiner, Sigma-Aldrich, Germany). Wells containing uninoculated media were used as negative controls. The plates were incubated at 37 °C for 24 h and at 20 °C for 48 h. After incubation, the plates were washed three times with PBS to remove planktonic cells. Adhered bacteria were stained with 0.1% crystal violet for 15 min, followed by washing three times with PBS. Thereafter, 200 μl ethanol was added to each well and OD_{600} was measured using Infinite M200 plate reader (Tecan, Männedorf, Switzerland). Biofilm formation was considered when the OD_{600} was at least three times greater than that of the negative control [99].

Bacterial growth Overnight cultures of each isolate were diluted 1:1000 in fresh LB broth. Thereafter, 200 μl of each isolate was transferred to a 96-well microtiter plate (Greiner, Sigma-Aldrich, Germany) and incubated at 37 °C in Infinite M200 plate reader (Tecan). OD_{600} was measured every 10 min for 24 h. The experiment was repeated three times for each isolate.

In addition, bacterial growth was tested in sterile-filtered human urine (pH = 6.5) with the same protocol as for LB. Urine was collected from healthy female volunteers with no history of UTI or antibiotic use in the previous two months.

Serum resistance In order to investigate resistance to human serum, 250 μl of the overnight cultures were added to 750 μl 20% human serum (Sigma-Merck) (HS, diluted in PBS) or heat-inactivated serum (HIS, control for comparison). Serum was inactivated by incubating in a water bath at 56 °C for 60 min. The mixtures were incubated at room-temperature, and samples were taken every hour for three hours. Samples were serially diluted and plated on LB agar plates. The plates were incubated for 24 h at 37 °C and colonies counted. The colonies from HS samples were calculated as a percentage of the HIS samples. The results were categorized as follows: < 1% = serum sensitive, > 90% = serum resistant, and all other results were considered as intermediate [100].

Adhesion to and invasion of eukaryotic cells Adhesion to, and invasion of, cells was tested in Vero cells (Vero C1008, ECACC, Item number 85020206) grown at 37 °C [101]. The cells were grown to 80% confluence, and 200 μl of cells in fresh minimal essential medium (DMEM (GibcoTM 11,568,876)) with 10% Fetal Bovine Serum (GibcoTM 10,270,106) and 1 ml penicillin/streptomycin solution (GibcoTM, 15,140,122, containing 10,000 units/ml penicillin and 10,000 μg/ml streptomycin) added to 100 ml DMEM was transferred to a microtiter plate (Greiner, Sigma-Aldrich, Germany). This was done in duplicate, at a concentration of approximately $5*10^4$ cells/ml (counted in Countess (Thermo Scientific,), and the cells grown to confluence. Overnight cultures of bacteria were diluted 1:100 in fresh LB broth to $OD_{600} = 0.1$. One ml was centrifuged at 500 x g for 5 min and the pellet was resuspended in 500 μl fresh DMEM cell-medium without antibiotics. The bacterial suspension was diluted 1:100 in DMEM cell-medium without antibiotics and 50 μl was added to the confluent Vero cells with fresh cell medium, equivalent to approximately 30 bacteria per cell (MOI 30:1). The plates were centrifuged at 100 x g for 2 min to increase contact between bacteria and cells, and incubated for two hours at 37 °C.

To assess adhesion to cells, the cells were washed three times with PBS to remove non-adherent bacteria and lysed with 30 μl 1% Triton X for 10 min. The lysates were serially diluted in PBS and plated on LB agar. To assess bacterial invasion, 200 μl of fresh medium with antibiotics (0.1 mg/ml gentamicin and 20 mg/ml nalidixic acid) was added to the cells before incubation at 37 °C for two hours to kill adherent bacteria. The cells were lysed and plated as described for the adhesion assay.

Colicin production Colicin production was investigated as described previously [102]. Briefly, 100 μl overnight culture of *E. coli* DH5α was spread onto LB agar plates and left to dry for approximately 10 min at room temperature. One ml overnight culture of the respective isolates was centrifuged at 13000 x g for 10 min and the supernatants sterile filtered through a 0.22-μm Minisart® syringe filter (Sartorius Stedim Biotech GmbH, Germany). Ten μl of the filtrate was spot inoculated on the dried LB agar plates with *E. coli* DH5α and incubated at 37 °C for 24 h. Production of colicin was determined by the presence of an inhibition zone around the place of inoculation.

Statistical analysis All assays were performed in three parallels for each isolate, and each experiment was repeated three times. Standard deviations (SDs) for quantitative data were calculated (supplementary material).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-021-02160-y.

Additional file 1.
Additional file 2.

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Authors' contributions
All authors have contributed to the ideas and concepts, the study design and manuscript preparation. MLB and SSM performed the experiments and the genomics, wrote the main manuscript and prepared Figs. CS assisted the bioinformatics. ILW assisted laboratory experiments. MLB, SSM, ILW, CS, MS, YW reviewed the manuscript. All authors of the manuscript have read and agreed to its content and are accountable for all aspects of the accuracy and integrity of the manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Sequence data is available at European Nucleotide Archive (ENA). Accession numbers are given in Table 2.

Declarations

Ethics approval and consent to participate
Not applicable. Urine samples were provided by the authors themselves; no ethical parameters are required.

Consent for publication
Not applicable.

Competing interests
The authors have no competing interest to declare.

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In vitro digestion of ESC-resistant *Escherichia coli* from poultry meat and evaluation of human health risk

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Introduction: The spread of antimicrobial resistance (AMR) has become a threat against human and animal health. Third and fourth generation cephalosporins have been defined as critically important antimicrobials by The World Health Organization. Exposure to Extended spectrum cephalosporin-resistant *E. coli* may result in consumers becoming carriers if these bacteria colonize the human gut or their resistance genes spread to other bacteria in the gut microbiota. In the case that these resistant bacteria at later occasions cause disease, their resistance characteristics may lead to failure of treatment and increased mortality. We hypothesized that ESC-resistant *E. coli* from poultry can survive digestion and thereby cause infections and/or spread their respective resistance traits within the gastro-intestinal tract.

Methods: In this study, a selection of 31 ESC-resistant *E. coli* isolates from retail chicken meat was exposed to a static in vitro digestion model (INFOGEST). Their survival, alteration of colonizing characteristics in addition to conjugational abilities were investigated before and after digestion. Whole genome data from all isolates were screened through a custom-made virulence database of over 1100 genes for virulence- and colonizing factors.

Results and discussion: All isolates were able to survive digestion. Most of the isolates (24/31) were able to transfer their bla_{CMY2}-containing plasmid to *E. coli* DH5-á, with a general decline in conjugation frequency of digested isolates compared to non-digested. Overall, the isolates showed a higher degree of cell adhesion than cell invasion, with a slight increase after digestion compared non-digested, except for three isolates that displayed a major increase of invasion. These isolates also harbored genes facilitating invasion. In the virulence-associated gene analysis two isolates were categorized as UPEC, and one isolate was considered a hybrid pathogen. Altogether the pathogenic potential of these isolates is highly dependent on the individual isolate and its characteristics. Poultry meat may represent a reservoir and be a vehicle for dissemination of potential human pathogens and resistance determinants, and the ESC-resistance may complicate treatment in the case of an infection.

KEYWORDS

virulence, ExPEC, conjugation, INFOGEST, hybrid pathogen

Introduction

The diverse family of *Enterobacteriaceae* includes one of the most studied microbes, *Escherichia coli*. Within *E. coli*, we find commensals, opportunistic bacteria, and pathogens. Some of the pathogenic variants cause infections in the intestinal tract (intestinal pathogenic *E. coli*, IPEC), while extraintestinal pathogenic *E. coli* (ExPEC) can survive in other tissues of the host and are associated with neonatal meningitis (NMEC) and sepsis and urinary tract infections (UPEC) among others (Riley, 2014). Several virulence genes characterizing ExPEC have been described (Pitout and Laupland, 2008; Pitout, 2012; Mellata, 2013), which include

genes encoding for adhesins, invasins, toxins, and siderophores and genes related to iron metabolism (Dale and Woodford, 2015). The increasing occurrence of antimicrobial-resistant ExPEC isolates has led to prolonged hospital stays and higher mortality rates (Gastmeier et al., 2012). This rise in antimicrobial resistance (AMR) has a significant impact on human and animal health (Brinkac et al., 2017; Centers for Disease Control Prevention, 2019). Recently, hybrid pathogens have been explored (Lindstedt et al., 2018), demonstrating the plasticity of the *E. coli* pangenome (Mellata, 2013) and blurring the lines between human-made bacterial classifications. An increased understanding of the dynamic flow and transmission routes of antimicrobial-resistant bacteria (ARB) and antimicrobial resistance genes (ARG) between animal and human bacterial reservoirs and host interactions is important for developing and implementing targeted measures to further prevent AMR development (VKM, 2020).

Poultry has been described as one of the main reservoirs for extended-spectrum beta-lactamase (ESBL) producing bacteria, as well as *E. coli,* which is most closely linked to human ExPEC (Carattoli, 2008). The European Food Safety Agency (EFSA) concluded in 2011 that AMR *E. coli* isolates from humans and poultry are more frequently genetically related than antibiotic-susceptible isolates and that transmission of ESBL genes, plasmids, and clones from poultry to humans is most likely to occur through the food chain (EFSA, 2011; Manges and Johnson, 2012). In 2015, the report "Assessment of AMR in the food chains in Norway" concluded that the probabilities of human exposure to ESBL-producing *Enterobacteriaceae* and their corresponding genes, from live poultry and poultry meat, were considered non-negligible (VKM, 2015). Since then, extensive measures have been taken in the Norwegian poultry industry to limit previously discovered ARB and ARGs (Mo et al., 2014, 2021; Nortura, 2016), leading to the prevalence being substantially reduced in the last few years (NORM/NORM-VET, 2017, 2019). The European Center for Disease Prevention and Control (ECDC) published in their report from 2018/2019 that the proportion of presumptive ESBL/Ampicillinase C (AmpC) producing *E. coli* was low in the animal sector, as 14 countries reported a decrease in overall prevalence, while 11 countries reported an increase of ESBL/AmpC producing bacteria (European Food Safety Authority European Centre for Disease Prevention Control, 2021).

The most common AmpC beta-lactamase encoding gene in *E. coli* is the bla_{CMY-2}, which is predominantly located on plasmids (Alfei and Schito, 2022). It has been reported to occur in bacteria from both human infections and various animal and food sources with increasing prevalence (Pires et al., 2022). Some authors have assessed that ESBL-producing *Enterobacteriaceae* from the broiler production chain is a considerable public health risk due to both their virulence and resistance characteristics (Pitout and Laupland, 2008; Liebana et al., 2013; Vounba et al., 2019). At the poultry slaughterhouse, it is unavoidable that intestinal bacteria contaminate carcasses during the slaughtering process (Rouger et al., 2017; Rasschaert et al., 2020; Boubendir et al., 2021). Consequently, as chicken filets are often sold as fresh products, compromised kitchen hygiene habits may result in consumers becoming exposed to these bacteria (Bloomfield et al., 2017; Santos-Ferreira et al., 2021). However, even though the probability of exposure of consumers to ESC-resistant bacteria may be high depending on the prevalence levels in live animals, less is known about the consequences of such exposure (Buberg et al., 2021). The potentially long timespan from exposure to the development of infection makes infection routes hard to trace, and there is a need for more comprehensive genetic analysis of poultry isolates to unravel their pathogenic potential and further evaluate their role as a possible risk to human health (Leverstein-van Hall et al., 2011; Manges and Johnson, 2012; Berg et al., 2017).

To be able to determine the consequences of exposure to Extended Spectrum Cephalosporin (ESC)-resistant *E. coli* through food, further investigations of the fate of these isolates through the digestion process are needed. Their survival in humans through intake by the oral route has not yet been quantified and questions regarding survival, horizontal spread of resistance genes in the gastrointestinal tract, their interactions with the intestinal cells, and possible alterations of characteristics during the digestion process remain unanswered. Many different protocols for digestion models have been described making a comparison of studies between researchers challenging (Kong and Singh, 2010; Mulet-Cabero et al., 2019; Li et al., 2020; Mackie et al., 2020). However, in 2014, the INFOGEST network published a static *in vitro* digestion model that aimed to harmonize human-digestion conditions by being an easy and applicable model that could be compared between studies (Minekus et al., 2014; Brodkorb et al., 2019). Despite the limited use of this model for microbiological purposes, it is appropriate for the evaluation of the growth and survival of *Listeria monocytogenes* (Pettersen et al., 2019).

This study aimed to contribute to the understanding of the human health risk represented by ESC-resistant *E. coli* from the poultry food chain. We hypothesized that these bacteria would survive the human digestive process and have the potential to interact with the host and/or to transfer their resistance traits to other gastrointestinal bacteria. We addressed this hypothesis by using the above-mentioned *in vitro* digestion model for the evaluation of selected isolates' survival, conjugation abilities, and ability to adhere to and invade human colorectal cells. Furthermore, we assessed the presence of virulence factors characteristic of ExPEC through an in-depth analysis of whole genome sequence data.

Materials and methods

Isolates and selection

A total of 141 ESC-resistant *E. coli* was isolated from domestically produced retail chicken meat in the NORM-VET programs from 2012 to 2016 (NORM/NORM-VET, 2013, 2015, 2017). All these isolates were previously whole genome sequenced and known to carry the bla_{CMY-2} gene encoding ESC resistance and have previously been included in studies by Mo et al. (2014, 2016). A selection of 31 isolates was made from this collection by including isolates from all phylogroups and the most frequent sequence types, resulting in 11 isolates from 2012, 14 isolates from 2014, and six isolates from 2016. Eighteen of the isolates were previously partly characterized by Buberg et al. (2021). An overview of the included isolates is listed in Table 1.

TABLE 1 Isolates included in the study.

Whole ID	Year	ST	Phylogroup	Previously studied published
2012-01-3586	2012	131	B2	Mo et al., 2016; Buberg et al., 2021
2014-01-3678	2014	117	D	Mo et al., 2016; Buberg et al., 2021
2016-22-832	2016	442	B1	Buberg et al., 2021
2014-01-5656	2014	10	A	Mo et al., 2016; Buberg et al., 2021
2014-01-7037	2014	355	B2	Mo et al., 2016; Buberg et al., 2021
2016-22-220	2016	429	B2	Buberg et al., 2021
2014-01-1336	2014	1,594	A	Mo et al., 2016; Buberg et al., 2021
2012-01-1295	2012	38	D	Mo et al., 2016; Buberg et al., 2021
2012-01-707	2012	38	D	Mo et al., 2016; Buberg et al., 2021
2014-01-3680	2014	1,158	D	Mo et al., 2016; Buberg et al., 2021
2014-01-4991	2014	57	D	Mo et al., 2016; Buberg et al., 2021
2014-01-5104	2014	115	D	Mo et al., 2016; Buberg et al., 2021
2012-01-771	2012	69	D	Mo et al., 2016; Buberg et al., 2021
2014-01-7011	2014	1,944	D	Mo et al., 2016; Buberg et al., 2021
2014-01-4267	2014	191	A	Mo et al., 2016; Buberg et al., 2021
2012-01-1292	2012	38	D	Mo et al., 2016, 2017; Buberg et al., 2020, 2021
2012-01-2798	2012	3,249	A	Mo et al., 2016, 2017; Buberg et al., 2020, 2021
2016-22-1061	2016	2,040	A	Buberg et al., 2021
2012-01-1988	2012	38	D	Mo et al., 2016
2012-01-2350	2012	38	D	Mo et al., 2016
2012-01-1659	2012	10	A	Mo et al., 2016
2012-01-5334	2012	1,594	A	Mo et al., 2016
2012-01-5997	2012	10	A	Mo et al., 2016
2014-01-14	2014	38	D	Mo et al., 2016
2014-01-1676	2014	117	D	Mo et al., 2016
2014-01-2452	2014	117	D	Mo et al., 2016
2014-01-7149	2014	10	A	Mo et al., 2016
2014-01-2454	2014	38	D	Mo et al., 2016
2016-22-75	2016	1,594	A	Unpublished
2016-22-226	2016	10	A	Unpublished
2016-22-1059	2016	2,040	A	Unpublished

Isolation and species determination is described by Mo et al. (2016).
For clarification purposes and easy reading, the four last digits of the isolate ID are used when referred to in the text.

In vitro digestion model

Survival after digestion was evaluated by the static *in vitro* protocol developed by INFOGEST with minor modifications to adapt the protocol for studies of bacteria (Pettersen et al., 2019). Tests determining enzymatic activity for standardization were carried out according to the protocol before the experiment. In short, overnight cultures of all bacterial isolates in LB-broth were used in the *in vitro* digestion model. The "bacterial mixture" consisting of 0.5 ml of overnight culture in broth was added to a 5-ml Eppendorf tube (Eppendorf, Hamburg, Germany). Simulated salivary fluid (SSF) was added to obtain a 1:1 ratio. $CaCl_2(H_2O)_2$ was added to achieve a total concentration of 1.5 mM in SSF. As the bacterial mixture did not contain starch, amylase was omitted, and the mixture was incubated at 37°C for 2 min on a hematology mixer. For the gastric step, preheated simulated gastric fluid (SGF) was added to the bacterial mixture in a ratio of 1:1. The pH was adjusted to 3.0 by adding a pre-defined volume of HCl. $CaCl_2(H_2O)_2$ was added to a final concentration of 0.15 mM in SGF. Porcine pepsin (Sigma-Aldrich, batch no. SLCF7636) (2,000 U/ml), Rabbit Gastric extract (RGE15, Lipolytech) (60 U/mL), and water were mixed to achieve a 1× concentration of SGF, which was then added to the mixture before incubation at 37°C for 40 min (estimated passing time for liquid boluses). For the intestinal phase, preheated

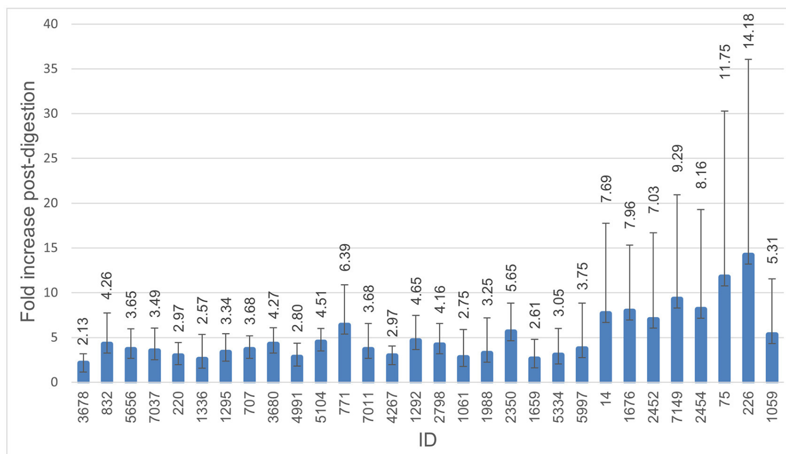

FIGURE 1
Survival through digestion. Histogram demonstrating fold-increase in CFU/ml after digestion compared to non-digested. The error bar represents the standard deviation. All isolates were able to survive digestion, and all were able to continue growth during the digestion process. Isolate 3,586 was excluded from this figure due to a failure of growth in the non-digested control.

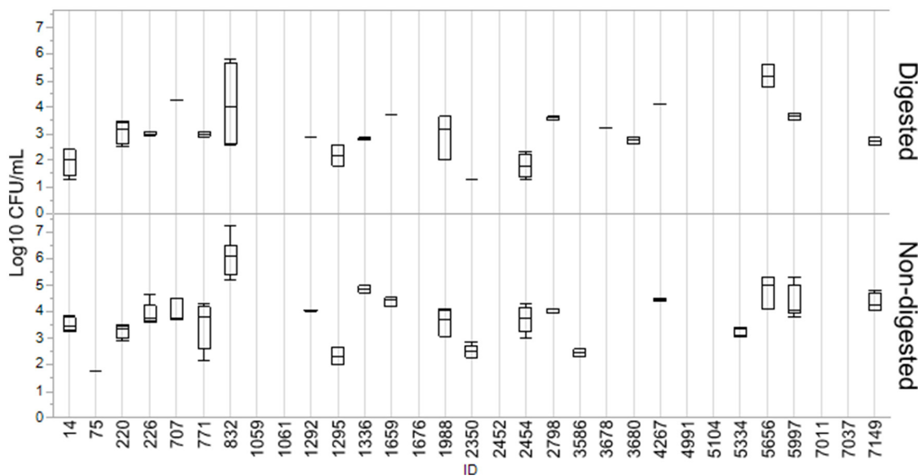

FIGURE 2
Log10 CFU/ml of transconjugants for digested and non-digested isolates. Boxes represent the quartiles and the median value, with included confidence intervals as the standard error of mean indicated by whiskers. Values are given in the number of Log10 CFU/ml. No whiskers are visible for isolates where the outer quartile is equal to the minimum or maximum value. For isolates where no transfer was detected, no box is present.

simulated intestinal fluid (SIF) was added to the bacterial mixture in a ratio of 1:1. The pH was adjusted to 7.0 by adding a predefined volume of NaOH. A final concentration of 10 mM bile was then added to the mixture. $CaCl_2(H_2O)_2$ was added to achieve a final concentration of 0.6 mM in SIF. Pancreatin (Sigma-Aldrich, batch no. SLCF4576) with a trypsin activity of 100 U/ml was then added to the mixture. Autoclaved ddH_2O was added to gain a 1× concentration of the SIF before samples were incubated at 37°C for 2 h on a hematology mixer. After incubation, the bacterial mixture was diluted and plated on selective agar plates (Müeller-Hinton agar containing 0.5 mg/L cefotaxime) and incubated for 24 h at 37°C. Colonies were counted manually and CFU/ml was determined after digestion. The number of CFU for the non-digested parallel was calculated by direct plating of overnight culture on selective agar plates. The experiments were carried out in triplicate.

TABLE 2 Ability to conjugate in liquid broth.

Conjugation: digestion	Total	Conjugation after digestion	NTD after digestion
Total	100% (31)	64.51% (20/31)	35.49% (11/31)
Conjugation non-digested	67.74% (21/31)	85.71% (18/21)	14.28% (3/21)
NTD non-digested	32.25% (10/31)	20.00% (2/10)	80.00% (8/10)

NTD, no transfer detected.
Binary distribution of the number of isolates able to successfully transfer bla_{CMY-2} to *E. coli* DH5-α after digestion compared to non-digested. If no confirmable transconjugants were present on the transconjugant-selective plates, it was considered NTD.

Conjugation assay

Conjugation experiments were performed in LB-broth according to Buberg et al. (2020) with minor modifications. In short, the donors (all 31 isolates individually) and recipient strain (*E. coli* DH5-α) were grown overnight in LB-broth at 37°C reaching OD600 equivalent to a McFarland standard no. 1 (*3E+8 bacteria/ml). A volume of 500 µL of the recipient strain culture and 10 µL of the donor strain culture (donor:recipient ratio = 1:50) were mixed in 4 mL LB-broth and incubated for 4 h at 37°C. Dilutions of each mating culture were plated on Müeller-Hinton agar plates (Sigma-Aldrich, Germany) supplemented with 20 mg/L nalidixic acid and/or 0.5 mg/L cefotaxime, and incubated for 24 and 48 h at 37°C. The mating mixture was then diluted and plated on the recipient- and transconjugant-selective plates containing nalidixic acid, or both nalidixic acid and cefotaxime, respectively. The number of transconjugants was reported for quantification and comparison between the donors. Conjugation frequency was calculated by dividing the number of transconjugants by the number of recipients in CFU/mL. For control, representative colonies from each transconjugant-selective plate were plated on bromothymol lactose blue agar (Sigma-Aldrich, Germany) to distinguish transconjugants from spontaneously mutated donors (i.e., mutated to nalidixic acid resistance). In addition, PCR analysis of transconjugants was conducted to confirm that they harbored the bla_{CMY-2} gene proving conjugation.

For conjugation after digestion, the same procedure was carried out immediately after the donors had gone through the *in vitro* digestion. The experiments were carried out in triplicate.

Adhesion and invasion assay

The ability to adhere to and invade eukaryotic cells was tested in HT-29 cells (RRID: CVCL_0320) grown at 37°C (Ammerman et al., 2008) between passages 10 and 25. The cells were grown to 80% confluence, and 200 µL of cells in fresh McCoy medium (Merck, USA) with 10% Fetal Bovine Serum (GibcoTM 10270106) (referred to as McCoy+) were transferred to a microtiter plate (Corning, Costar, Fischer Scientific, USA). Plates were incubated overnight for establishing cell attachment. Cell concentration for the experiments ranged between 3 and 6 E+06 cells/ml (>90% living cells) counted with Bio-Rad TC20. Overnight culture and digested culture (digested and non-digested) of bacteria were diluted in the ratio of 1:100 in fresh LB-broth. One mL was centrifuged at 2,000 rpm for 5 min and the bacterial pellet was resuspended in 500 µL fresh McCoy+ medium without antibiotics. The bacterial suspension was diluted in the ratio of 1:100 and 50 µL of bacterial suspension was added to each well (equivalent to MOI 30:1). Plates were centrifuged at 1,000 rpm for 2 min to increase contact between bacteria and cells, and incubated for 2 h at 37°C. To assess total cell association (number of adhering and invading bacteria), the cells were washed three times with PBS (1×) to remove non-adherent bacteria and lysed with 30 µL 1% Triton X for 10 min. The lysates were serially diluted in PBS (1×) and plated on selective agar as previously described. To assess bacterial invasion, 200 µL of fresh medium with antibiotics (0.1 mg/ml gentamicin and 20 mg/mL nalidixic acid) was added to the cells before incubation at 37°C for 2 h to kill adherent bacteria. The cells were washed, lysed, and plated as described previously. Adherence was calculated by subtracting numbers from the cell invasion from the total amount of cell-associated bacteria. Digested and non-digested were compared, and experiments were carried out in triplicate.

Detection of virulence genes

An extended virulence-associated gene analysis was carried out. The sequences were scanned (i.e., BLAST search) against a custom database previously used for characterizing environmental isolates (Finton et al., 2020) now expanded to include 1,191 genes/gene variants or genetic markers. The database contains genes related to both ExPEC and IPEC as well as loci suspected to contribute to virulence, e.g., the ETT2 locus. Only matches with 95% or more nucleotide identity with 60% or more query coverage were included in the results.

Results

In vitro digestion

All isolates were able to survive digestion in the INFOGEST *in vitro* digestion model. The numbers of the colony-forming units (CFU/mL) for digested and non-digested isolates are shown in Figure 1. The number of bacteria after digestion varied between isolates and was highly increased for some isolates and replicates. Mean CFU/mL for non-digested isolates was 4.91E+08, compared to 1.82E+09 for digested isolates. All isolates showed a 1-fold increase or higher in CFU/ml, with an average of 5.31-fold increase. More than a 4-fold increase was seen in 15 of the 31 isolates, with isolate 226 displaying the highest increase in the number of CFU/ml after digestion at a 14.18-fold increase.

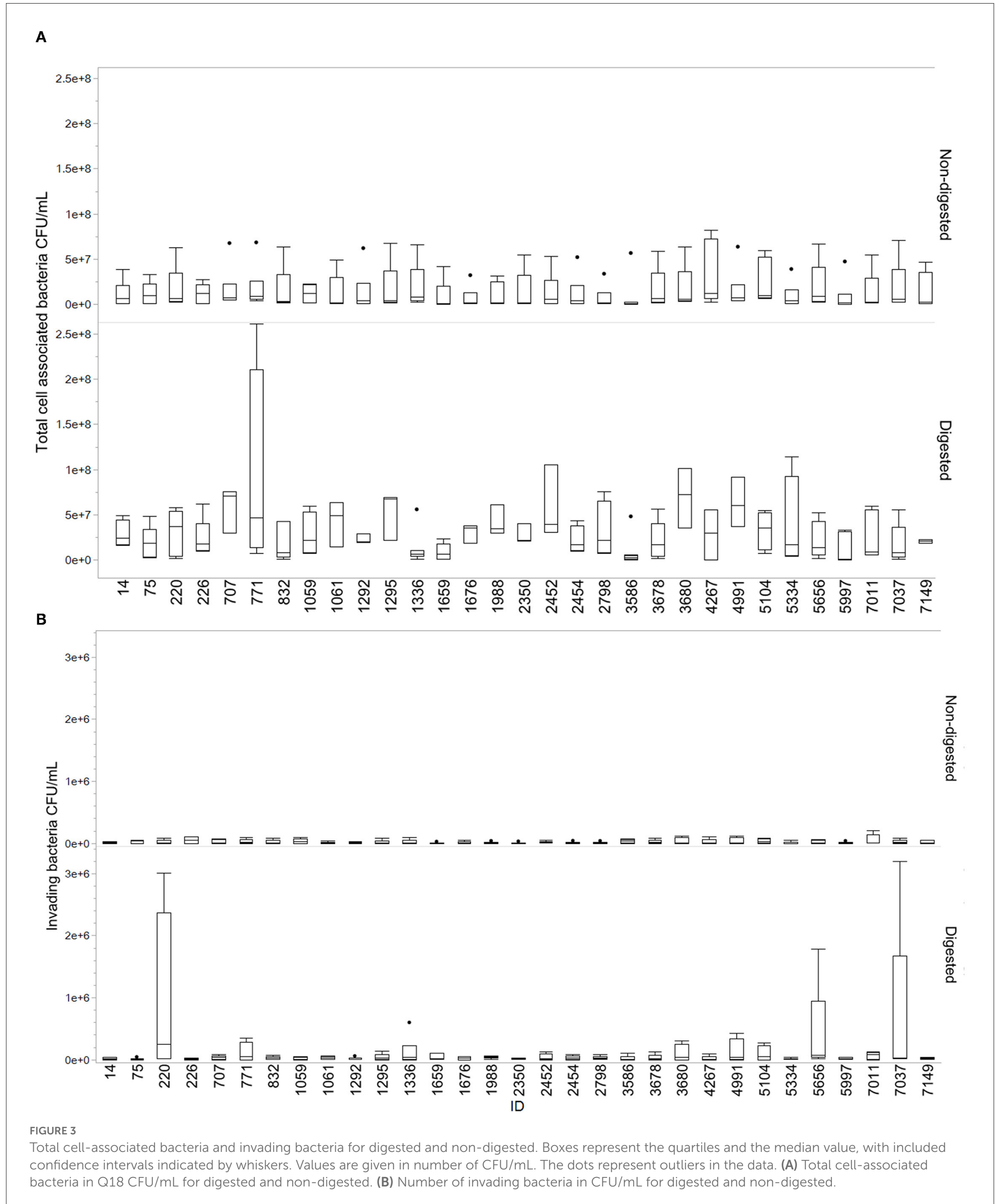

FIGURE 3
Total cell-associated bacteria and invading bacteria for digested and non-digested. Boxes represent the quartiles and the median value, with included confidence intervals indicated by whiskers. Values are given in number of CFU/mL. The dots represent outliers in the data. **(A)** Total cell-associated bacteria in Q18 CFU/mL for digested and non-digested. **(B)** Number of invading bacteria in CFU/mL for digested and non-digested.

Conjugation

Numbers of CFU/ml of transconjugants digested and non-digested for each donor are illustrated in Figure 2.

Table 2 gives an overview of the sample population and highlights the variation of conjugation frequencies before and after digestion. Conjugation frequencies are available in the Supplementary material.

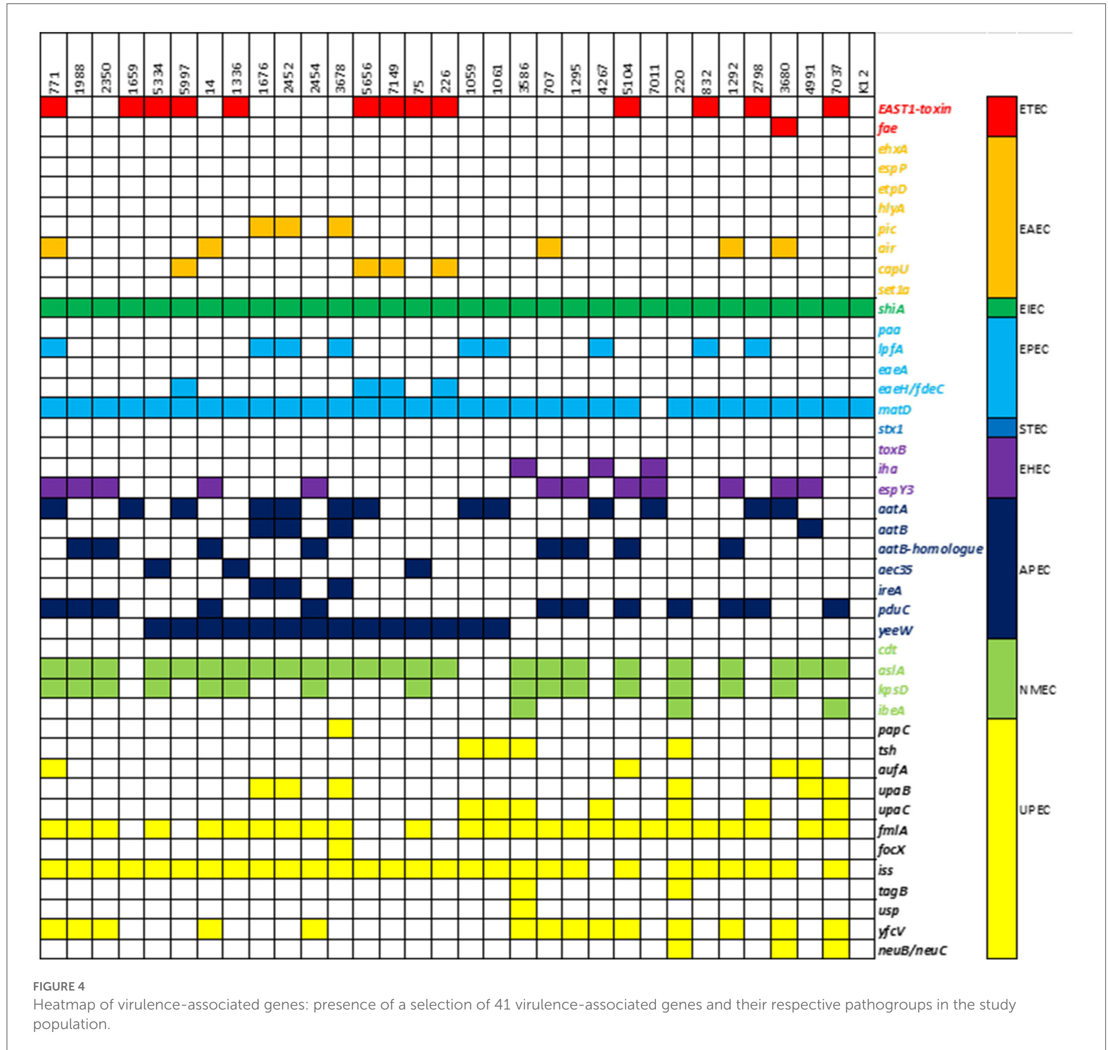

FIGURE 4
Heatmap of virulence-associated genes: presence of a selection of 41 virulence-associated genes and their respective pathogroups in the study population.

In general, most isolates that were able to transfer their $bla_{\text{CMY}-2}$ gene displayed a decrease in conjugation frequencies after digestion. Out of the 31 isolates, 24 were able to transfer $bla_{\text{CMY}-2}$ before or after digestion, and 19 consequently before and after. Eight isolates (7037, 4991, 5104, 2452, 1059, 1061, 1676, and 7011) were not able to conjugate at all. Conjugation frequency (the number of CFU/mL of transconjugants divided by the number of CFU/mL recipients) for each digested and non-digested isolate is reported in Supplementary Table S1. The mean conjugation frequency for non-digested was 2.04E−03, compared to digested which was 3.60E−04. Isolates 3687 and 3680 were only able to conjugate after being exposed to digestion. While isolates 0075, 3586 and 5334 were only able to conjugate before being digested, and not after. Figures are made in JMP Pro 16.0.0.

Adhesion and invasion assay

Overall, the isolates displayed a greater ability to cell adhesion compared to cell invasion. Most isolates showed an increase in total cell association after digestion compared to non-digested isolates. Results from this assay are demonstrated in Figure 3. The mean of cell adhesion for non-digested was 1.55E+07 compared to digested at 3.36E+07. For invasion, the mean for non-digested was 2.10E+04 and digested was 1.03E+05. Some variability was observed between the different replicates. Isolates 1336, 1659, 3586, and 4267 were the only isolates for which a decrease in adhesion was observed after digestion. For invasion, especially isolates 220, 5656, and 7037 stood out with highly increased invasion numbers after digestion compared to non-digested. Figures are made in JMP Pro 16.0.0.

Virulence-associated gene analysis

The presence of virulence-associated genes was compared to a reference *E. coli* K12 strain. Out of 1,191 genes in the database, our isolates had 123–193 (mean 161) different genes, compared to 107 in *E. coli* K12. Fifty-one genes were found in all isolates, including the control strain, and were considered core genes of the *E. coli* genome. An overview of 41 relevant virulence genes is presented in Figure 4, while a more detailed overview is available in the Supplementary material. According to the UPEC definition by Spurbeck et al. (2012), two of the isolates (220 and 7037) were predicted to be human UPEC isolates by containing the genes *chuA, fyuA,* and *yfcV* while being negative for *vat.* These two isolates were also both positive for other well-documented ExPEC/UPEC-associated genes, e.g., *papC, upaC, ibeA, irp1, irp2,* and *kpsMII.* In addition, isolate 7037, as the only isolate in our collection, contained the *gimB*-genomic island, which is associated with the invasion process of the host cells, particularly in NMEC strains, but is also found in APEC strains (Ewers et al., 2007). Isolate 220 held the *tagB/tagC* (Pokharel et al., 2020), and three isolates (220, 3680, and 7037) were considered K1-isolates as they held *neuB/neuC,* which is related to ExPEC virulence. The ability to acquire iron is related to pathogenicity. The majority of isolates contained iron acquisition loci in which *chuA*, *sit,* and the aerobactin loci were prominent. None of the isolates contained the ExPEC-related toxin genes *pic, sat, vat, hlyA,* or *cnf.* Genes associated with IPEC strains were detected as isolate 3680 contained *ehaA* (adhesion) and *espY3* non-LEE effector gene associated with EHEC/EPEC and *fae* (F4 fimbriae) genes associated with ETEC (Larzábal et al., 2018), and can thus be considered a hybrid pathogen.

Discussion

This study aimed to assess potential health risks represented by ESC-resistant *E. coli* from poultry meat. We showed that the selected isolates were able to survive and multiply during gastrointestinal digestion *in vitro* and that they were able to adhere to and invade human colorectal cells after digestion. In addition, we demonstrated that the stress of being digested changed the conjugation frequency of the $bla_{\text{CMY}-2}$-containing plasmid harbored by these bacteria. The presence of virulence-associated genes was evaluated for further determination of the pathogenic potential of the selected isolates. In general, the isolates showed a large variety of gene content despite being of the same origin and carrying the same AmpC phenotype and $bla_{\text{CMY}-2}$ resistance gene. The impact on human health due to exposure to ESC-resistant *E. coli* from poultry meat therefore strongly depends on the individual bacterial isolate being involved.

It was an expected finding that all the investigated isolates were able to survive digestion and displayed an increase in CFU/ml after digestion (Figure 1), as the fecal-oral pathway for infection is common for *Enterobacteriaceae* (Tenaillon et al., 2010). The bacteria were not too hampered by the low pH in the gastric step and were able to replicate despite the limited nutrients available in the simulated gastrointestinal fluids throughout digestion. One study investigated the survival of acid-sensitive bacteria and showed that survival increased in the presence of solid foods (Waterman and Small, 1998). The survival of *E. coli* is therefore expected to increase further with more nutrients available in the form of a meal. If, for example, consumers get exposed to ESC-resistant *E. coli*, these bacteria might therefore reach, encounter, and subsequently interact with the resident microflora.

The current study assessed the conjugational abilities of the $bla_{\text{CMY}-2}$ gene in a liquid broth (Table 2). The overall tendency when analyzing the conjugation data was that the frequency of spread of $bla_{\text{CMY}-2}$ decreased after digestion (Figure 2). Several studies have suggested that stress may enhance the further spread of resistance or virulence plasmids by triggering the SOS response (Baharoglu et al., 2010; Pribis et al., 2019). As the process of digestion includes an extreme change in pH, in addition to digestive enzymes, it can be considered a stressful procedure for the bacteria. We hypothesized that this stress would increase the spread of resistance genes and increase the bacteria's ability to adhere to and invade gastrointestinal cell lines, making colonizing of the gut more likely. Some of the isolates did not transfer the $bla_{\text{CMY}-2}$ gene to the recipient *E. coli*, as demonstrated in Table 2. The reason for this conjugation failure was not further investigated. Another interesting observation in this study is the large variation in the isolates' ability to transfer the $bla_{\text{CMY}-2}$ gene with changing conditions, demonstrating that despite their similarities, they likely have a variable ability to adapt to the changing environment of the gastrointestinal tract. Replicon typing of the respective isolates has been previously performed by Mo et al. (2016). In brief, all isolates included in this study hold an IncK plasmid, with exception of 1061 and 2798 which hold only an IncI1 plasmid. Isolate 1336 holds both IncK and IncI1, while isolate 1295 has IncK, IncFII, and IncFIB. No correlation between conjugation and replicon type was seen in this study and thus has not been investigated further.

Adherence and invasion are important characteristics of pathogen–host interactions (Kalita et al., 2014; Desvaux et al., 2020). We observed a change in the ability of cell interaction after digestion compared to non-digested (Figure 3). The results varied between isolates, which may demonstrate that single isolates display a higher probability than others to establish themselves in the gastrointestinal tract. The act of colonizing the intestinal tract is not dependent on individual bacterial characteristics alone but is a complex dynamic involving host factors, the residing microbiota, the nutrients available, and qualities of the colonizing strain (Srikanth and McCormick, 2008; Tenaillon et al., 2010; Richter et al., 2018). Interestingly, isolate 7037 displayed an extremely high invasion rate in one of the replicates, which contributed to the increase in the mean and spread of data (Figure 3). This isolate holds the GimB operon, which is important for NMEC pathogenicity, in addition to *ibeA*, which is related to invasion (Ewers et al., 2007). Only two other isolates contained the *ibeA* gene, isolate 3586 and 220, the latter also displaying an increased cell invasion after digestion. When assessing the ability to adhere to and invade human colorectal cells, the current study found an overall increasing trend of cell association after the digestion procedure.

To our knowledge, the survival of ESC-resistant *E. coli* through an *in vitro* digestion model has not been previously studied. Our findings are in concurrence with a study using an *in situ* model that demonstrated the survival and colonizing abilities of an ESBL-resistant *E. coli* strain from poultry. However, this study only focused on the latter steps of digestion (cecum and colon) and was only performed for a single isolate (Smet et al., 2011). *In vivo* digestion

models are thought to resemble the most life-like conditions but raise ethical questions as they require living animals or human volunteers to carry them out. They are in addition time-consuming, expensive, and require specialized facilities. A large variety of *in vitro* digestion models have been established as good alternatives to *in vivo* models. The dynamic models are the ones that simulate the most accurate digestion; however, these are still expensive, and the comparison of results between different laboratories has proven difficult. Due to the recent harmonization of the INFOGEST static *in vitro* digestion model, it is now possible to standardize research regarding digestibility across laboratories. The current model has been adjusted with minor modifications to fit microbiological studies such as the survival of bacteria as used in this study. A limitation of this study is that a static *in vitro* assay does not exactly replicate the conditions in the gastrointestinal tract. However, this model has been documented to be physiologically comparable to *in vivo* porcine digestion of skim milk powder (Egger et al., 2017). In addition, dietary and genetic host factors that may affect the individual host-bacteria interactions in the gastrointestinal tract have not been considered in the current study.

Determination of different pathovars of *E. coli* based on the analysis of their virulence gene content is highly dependent on the database used, as there may be individual differences in which genes are included in the defining criteria. This study used a custom-made database containing over 1,191 genes to increase the coverage of the number of VAGs and compared the results to a common K12 *E. coli* strain. Based on the genotypic results, isolates 220 and 7037 should be classified as human UPEC. A previous analysis of a subgroup of the isolates included in this study concluded that the risk of developing UTI upon exposure to ESC-resistant *E. coli* from poultry was limited. Nevertheless, with this expanded knowledge by applying a wider VAGs search, the risk of causing disease appears to be higher than first anticipated (Buberg et al., 2021). Except for isolates 220 and 7037, there were very few virulence traits connected to known human pathogenic variants of *E. coli*. The strain 3680 contained some genes (e.g., *ehaA* and *espY3*) associated with both enterohemorrhagic/enteropathogenic *E. coli* (EHEC/EPEC) and Shigatoxin-producing *E. coli* (STEC) (Figure 4). However, the lack of specific toxins makes it questionable whether this isolate should be classified as an IPEC strain.

In summary, the current study aimed to evaluate the consequences of consumer exposure to ESC-resistant *E. coli* using a static *in vitro* digestion model and evaluated conjugation and cell adhesion and invasion for digested and non-digested isolates, in addition to performing an in-depth VAG analysis to evaluate ExPEC potential. We conclude that the pathogenic potential is highly dependent on the characteristics of the individual isolates. The isolates 7037, 220, and 3680 contained genes and characteristics classifying them as ExPEC. As they additionally encode and express ESC resistance, they may complicate treatment in the case they cause disease in a human host. This study demonstrates that poultry meat may, although to a limited extent, represent a reservoir and be a vehicle for the dissemination of potential human pathogens and plasmid-borne resistance determinants such as $bla_{\text{CMY}-2}$.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MB planned, carried out the experiment, and interpretated and analyzed the results. MB wrote the manuscript with support from YW, BL, and IW. BL performed the VGA analysis. YW and IW supervised the project. All authors provided critical feedback and helped shape the research, analysis, and manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1050143/full#supplementary-material

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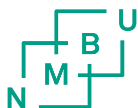

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