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Beyond Colistin Withdrawal: tracking colistin-resistant Enterobacterales within One Health context from local to global drivers

Marisa Ribeiro-Almeida

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BEYOND COLISTIN WITHDRAWAL: TRACKING COLISTIN-RESISTANT ENTEROBACTERALES WITHIN ONE HEALTH CONTEXT FROM LOCAL TO GLOBAL DRIVERS

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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CHAVE MÓVEL

Marisa Isabel Ribeiro de Almeida

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To my family,

my greatest source of strength.

Becoming isn't about arriving somewhere or achieving a certain aim,

it's about the journey itself.

Michelle Obama, 2018

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ABSTRACT

Antimicrobial resistance (AMR) is a major global health threat, compromising critically important antimicrobials. Among these, colistin is a last-resort treatment for infections caused by multidrug-resistant (MDR) Gram-negative bacteria. However, decades of use of this antibiotic in livestock production have exerted strong selective pressure, promoting the emergence and dissemination of mobile colistin resistance (*mcr*) genes in food-producing animals. Restrictions on the veterinary use of colistin aimed to curb this dynamic; nevertheless, the long-term effectiveness of these measures is unclear. In particular, the drivers, reservoirs, and transmission pathways that may sustain colistin resistance post-withdrawal remain poorly understood. This thesis aimed to investigate the persistence and dissemination of colistin-resistant bacteria, particularly those carrying *mcr* genes, beyond colistin withdrawal, and the contribution of multiple local and broader environmental drivers - including antimicrobial and metal co-selection, environmental reservoirs, and transmission routes - to their maintenance and spread across agri-food and environmental settings within a One Health framework. Sampling was conducted in Portugal and encompassed 15 poultry and rabbit farms, 55 dog commercial pet food samples containing animal by-products and a population of wild birds (gulls). Colistin-resistant *Escherichia coli* and *Klebsiella pneumoniae* were characterized by culturomics and whole genome sequencing in order to identify colistin resistance mechanisms, along with associated mobile genetic elements and co-resistance determinants. The main results show that although colistin withdrawal markedly reduced *mcr*-mediated resistance the response to the colistin ban was species-specific. In poultry farms, colistin-resistant *K. pneumoniae* persisted via chromosomal mutations, and MDR - including genes associated with decreased copper susceptibility - remained frequent and often linked to IncF plasmids. No differences in MDR or colistin-resistant *K. pneumoniae* were observed between poultry fed organic or inorganic trace mineral/copper diets, highlighting the greater influence of farm environment and management practices. In rabbit farms, MDR *mcr*-positive *E. coli* persisted in association with IncHI2 plasmids carrying both antimicrobial resistance genes and determinants of decreased copper susceptibility. These findings underscore the role of copper and other antimicrobials as co-selection pressures, sustaining colistin resistance. The detection of genetically similar isolates in rabbit faeces and environmental samples indicates environmental reservoirs, contributing to recurrent contamination and reintroduction of *mcr* genes. Beyond farms, *mcr*-carrying MDR *E. coli* were detected in dog raw meat-based diets harbouring highly transferable IncX4 plasmids. Gulls carried *mcr-1*-positive MDR *E. coli* strains with IncI2 and IncHI2 plasmids, which also harboured genes conferring decreased susceptibility to metals. These findings emphasize the role of food chain and wildlife as reservoirs and vectors of AMR. Comparative genomic analyses revealed

overlaps between animal and human-lineages (e.g., *E. coli* ST1011, ST1196, ST10, B2-ST131-H22, ST297-ExPEC, ST162; *K. pneumoniae* ST15, ST392), alongside IncHI2, IncF and IncX4 plasmids resembling global counterparts. These results indicate the circulation of clinically relevant MDR lineages carrying *mcr*-1, with evidence of interspecies and interregional dissemination potential. In conclusion, colistin resistance exhibits distinct adaptive responses, with *mcr* genes disappearing faster than chromosomal mutations once colistin selective pressure ceases. Its persistence results from environmental contamination, farm-level cross-contamination, co-selection by other antimicrobials, and interspecies transmission. The detection of clinically relevant lineages and plasmids shared among animals, humans, food, and wildlife highlights the interconnectedness of local events with global AMR dissemination. Effective mitigation requires integrative One Health interventions, including stricter global regulation of antibiotic and metal use in food animal production, reinforced biosecurity across animal production systems and food chain, strengthened infection control and antimicrobial stewardship in both human and veterinary medicine, improved waste and water management, and enhanced cross-sectoral surveillance, particularly within the environmental sector. By linking farms, food chains, animal by-products, and wildlife, this work emphasizes the complex ecology of AMR and the urgent need for coordinated global actions to preserve colistin efficacy.

Keywords: Antimicrobial resistance, colistin, *mcr* genes, co-selection, food-animal production, One Health

RESUMO

A resistência aos antimicrobianos (AMR) constitui uma das principais ameaças globais à saúde pública. A colistina figura entre os antimicrobianos de último recurso para o tratamento de infeções causadas por bactérias Gram-negativo multirresistentes (MDR). No entanto, décadas de uso deste antibiótico na produção pecuária geraram uma enorme pressão seletiva em prol da emergência e disseminação de genes móveis de resistência à colistina (*mcr*) em animais de produção. As restrições impostas ao uso veterinário da colistina visaram travar esta dinâmica, mas os resultados a longo prazo destas medidas permanecem pouco claros, nomeadamente quanto aos fatores e vias de transmissão que sustentam a resistência à colistina, assim como o papel de outros setores, enquanto reservatórios ou vetores de bactérias portadoras de *mcr*. Esta tese teve como objetivo investigar a persistência e disseminação de bactérias resistentes à colistina, particularmente das portadoras de genes *mcr*, após a retirada da colistina, bem como a contribuição de múltiplos fatores, incluindo co-seleção por antimicrobianos e metais, reservatórios ambientais e vias de transmissão para a manutenção e disseminação destas bactérias em contextos agroalimentares e ambientais, no âmbito da abordagem One Health. A amostragem decorreu em Portugal, abrangendo 15 explorações de aves de capoeira e de coelhos, 55 amostras de ração de cão com subprodutos animais, e uma população de aves silvestres (gaivotas). As estirpes de *E. coli* e *K. pneumoniae* resistentes à colistina foram caraterizadas por culturómica e sequenciação genómica total, com o objetivo de identificar os mecanismos de resistência à colistina, juntamente com os elementos genéticos móveis associados e determinantes de co-resistência. Os principais resultados demonstraram que, embora a retirada da colistina tenha reduzido acentuadamente a resistência mediada por *mcr*, a resposta à proibição da colistina foi específica de acordo com a espécie. Nas explorações avícolas, *K. pneumoniae* resistente à colistina persistiu através de mutações cromossómicas, e a MDR - incluindo genes associados à diminuição da suscetibilidade ao cobre - manteve-se frequente e associada a plasmídeos IncF. Não foram observadas diferenças na ocorrência de *K. pneumoniae* MDR ou resistentes à colistina entre aves alimentadas com dietas contendo minerais/cobre na forma orgânica ou inorgânica, sugerindo maior influência do ambiente e das práticas de maneio. Nas explorações cunícolas, *E. coli* MDR *mcr*-positiva persistiram em associação com plasmídeos IncHI2 que albergavam tanto genes de resistência antimicrobiana como determinantes associados à menor suscetibilidade ao cobre. Estes resultados sublinham o papel do cobre e de outros antimicrobianos como agentes de co-seleção, levando à persistência da resistência à colistina. A deteção de isolados geneticamente semelhantes em fezes de coelhos e amostras ambientais indica a existência de reservatórios ambientais, contribuindo para a contaminação recorrente e reintrodução de genes *mcr*. Fora do contexto

produtivo, *E. coli* MDR portadoras de genes *mcr* foram detetadas em rações cruas para cães, albergando plasmídeos IncX4 altamente transferíveis. As gaivotas transportavam estirpes de *E. coli* MDR portadoras de genes *mcr-1* em plasmídeos IncI2 e IncHI2, que co-transportavam genes associados à menor suscetibilidade a metais. Estes resultados sublinham o papel da cadeia alimentar e da fauna selvagem como reservatórios e vetores de AMR. As análises genómicas comparativas revelaram sobreposições entre linhagens animais e humanas (por exemplo, *E. coli* ST1011, ST1196, ST10, B2-ST131-H22, ST297-ExPEC, ST162; *K. pneumoniae* ST15, ST392), e elevada similaridade de plasmídeos IncHI2, IncF e IncX4 com os seus homólogos globais. Estes resultados indicam a circulação de linhagens MDR clinicamente relevantes portadoras de *mcr*-1, com evidências de potencial de disseminação interespécies e inter-regional. Em conclusão, a resistência à colistina revela respostas adaptativas distintas, com os genes *mcr* a desaparecerem mais rapidamente do que as mutações cromossómicas após a remoção da pressão seletiva por colistina. A sua persistência resulta de uma complexa interação entre contaminação ambiental, contaminação cruzada nas explorações, co-seleção por outros antimicrobianos e transmissão interespécies. A deteção de linhagens e plasmídeos clinicamente relevantes partilhados entre animais, humanos, alimentos e vida selvagem evidencia a interligação entre eventos locais e a disseminação global da AMR. A mitigação eficaz requer intervenções integradas “One Health”, incluindo regulamentação global do uso de antimicrobianos e metais mais rigorosa na produção animal, o reforço de medidas de biossegurança nos sistemas de produção animal e ao longo da cadeia alimentar, melhoria da gestão de resíduos e águas, e o reforço da vigilância intersectorial, particularmente no compartimento ambiental. Ao estabelecer ligações entre explorações, cadeias alimentares, subprodutos animais e vida selvagem, este trabalho evidencia a complexidade ecológica da AMR e a necessidade urgente de ações globais coordenadas para preservar a eficácia da colistina.

Palavras-chave: resistência antimicrobiana, colistina, genes *mcr*, co-seleção, produção animal, One Health.

CONTENTS

LIST OF FIGURES

CHAPTER 1 – Introduction

CHAPTER 3 – Results and Discussion

From farm to fork: Persistence of clinically relevant multidrug-resistant and copper-tolerant K*lebsiella pneumoniae* long after colistin withdrawal in poultry production

Persistence of *mcr-1*-carrying *E. coli* in rabbit meat production: Challenges beyond long-term colistin withdrawal

Raw meat-based diet for pets: a neglected source of human exposure to *Salmonella* and pathogenic *Escherichia coli* clones carrying *mcr*, Portugal, September 2019 to January 2020

High diversity of pathogenic *Escherichia coli* clones carrying *mcr-1* among gulls underlines the need for strategies at the environment–livestock–human interface

LIST OF TABLES

High diversity of pathogenic *Escherichia coli* clones carrying *mcr-1* among gulls underlines the need for strategies at the environment–livestock–human interface

LIST OF ABREVIATIONS

AMR – Antimicrobial resistance

ARB – Antibiotic-resistant bacteria

ARGs – Antibiotic resistance genes

BPPL – Bacterial Priority Pathogens List

COVID-19 – Coronavirus disease 2019

DNA – Deoxyribonucleic acid

EC – European Commission

EFSA – European Food Safety Authority

EMA – European Medicines Agency

EU – European Union

FAO – Food and Agriculture Organization

GLASS – Global Antimicrobial Resistance and Use Surveillance System

GNB – Gram-negative bacteria

HGT – Horizontal gene transfer

HICs – High-income countries

HPCIA – Highest Priority Critically Important Antimicrobial

IM – Inner membrane

L-Ara4N – 4-amino-4-deoxy-L-arabinose

LMICs – Lower-middle-income countries

LPS – Lipopolysaccharide

MCR – Mobile colistin resistance

MDR – Multidrug-resistant

MGEs – Mobile Genetic Elements

MIC – Minimum inhibitory concentration

NDARO – National Database of Antibiotic Resistant Organisms

OM – Outer membrane

PCU – Population Correction unit

PEtN – Phosphoethanolamine

PhoPQ – PhoP-PhoQ two component systems

PmrAB – PmrA-PmrB two component systems

ROS – Reactive oxygen species

TCSs – Two-component systems

USA – United States of America

WGS – Whole genome sequence

WHO – World Health Organization

WOAH – World Organization for Animal Health

XDR – Extremely drug-resistant

LIST OF PUBLICATIONS

In compliance with the *Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto*, the author of this thesis hereby declare that she participated in the execution of the experimental work, analysis and interpretation of the data leading to the results presented and writing of the respective manuscripts. The manuscripts provided in this thesis were not previously included in other theses and correspond to their integral versions.

Under the scope of this thesis, the following manuscripts were published:

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CHAPTER 1 - Introduction

1. Antimicrobial resistance: a One Health challenge

1.1. Resistance to antimicrobials as a global problem

Antimicrobial resistance (AMR) is recognized as one of the top ten global public health threats, with profound implications for human, animal, and environmental health. This biological phenomenon occurs when microorganisms, particularly bacteria, acquire genetic determinants of resistance through horizontal gene transfer (HGT) or chromosomal mutations. When these confer clinically relevant resistance levels, infections become harder to treat, increasing the risk of disease spread, severe illness, and mortality (Murray et al., 2022; UNEP, 2023; Tang et al., 2023).

The following sections examine the impact of AMR across human, animal and environmental sectors, highlighting the interconnected nature of this global challenge.

1.1.1. Human health

The introduction of antimicrobials in human health, particularly antibiotics for the treatment of bacterial infections, was one of the greatest medical advances, significantly reducing mortality, morbidity and increasing life expectancy (Tang *et al*., 2023; WHO, 2022). The period from 1940s to the 1960s, often referred as the “Golden Age” of antibiotic discovery, saw the development of over 150 new antibiotics. However, in the decades that followed, there was a progressive decline in antibiotic research and innovation, despite the increasing need for novel antimicrobials to combat life-threatening infections. This decline is largely attributed to the rise of AMR, that undermines the effectiveness of antibiotic treatments, leading to increased disease burden, deaths, and substantial healthcare costs worldwide (Murray *et al*., 2022; UNEP, 2023; Salam *et al*., 2023; Hutchings *et al*., 2019).

Over recent decades, the global burden of AMR has grown alarmingly. In 2019, AMR was associated with 4.95 million human deaths globally, including 1.27 million deaths directly attributable to AMR infections (Murray *et al*., 2022). Each year in the European Union (EU), antibiotic-resistant bacteria (ARB) cause over 670.000 human infections, resulting in approximately 33.000 deaths - a stark reminder of the growing threat posed by AMR (ECDC, 2024; Murray *et al*., 2022; Tang *et al*., 2023; UNEP, 2023). Without immediate and effective preventive measures, projections indicate that AMR could cause up to 10 million deaths annually by 2050, potentially becoming the leading cause of death worldwide, with Asia and Africa facing the highest estimated burdens (Tang *et al*., 2023; O’Neill, 2014; Murray *et al*.,

2022). Lower-middle-income countries (LMICs) are particularly vulnerable due to limited access to clean water, poor sanitation and hygiene, inadequate infection control and restricted availability of quality-assured antimicrobials, vaccines and diagnostics, key drivers that contribute to AMR (IACG, 2019).

The global burden of AMR is strongly associated with the overuse and misuse of antimicrobials, practices that increased selective pressure and accelerate the development of bacterial resistance mechanisms (WHO, 2023). In human medicine, penicillins, cephalosporins, macrolides, fluoroquinolones, and tetracyclines remain the most used antibiotic classes globally (ECDC/EFSA/EMA, 2024; Klein et al., 2024). Recent studies have reported a 16.3% increase in human antibiotic consumption between 2016 and 2023, with the steepest increases observed in LMICs, where access to essential antibiotics is limited and inappropriate use is frequent (Klein *et al*., 2024). In contrast, high-income countries (HICs), including those in the EU, show lower overall antibiotic consumption. These countries experienced a temporary decline in antibiotic use during the COVID-19 (*Coronavirus disease 2019*) pandemic, followed by a return to pre-pandemic levels in 2022–2023 (EEA, 2024). These global trends have reignited concerns about the future trajectory of AMR, with projections indicating that, without effective international global intervention, global antibiotic consumption could increase by over 50% by 2030 (EEA, 2024; Klein *et al*., 2024).

In light of all these predictions, the World Health Organization (WHO) has classified AMR as one of the most critical global public health challenges (WHO, 2001), and a central concern within United Nations 2030 Sustainable Development Goals (WHO, 2021).

1.1.2. Animal health

Beyond its impact on human health, AMR poses serious challenges to animal health and global food supply. Antimicrobials have been widely used in veterinary practices, both in companion animals and especially in livestock production, contributing to animal health, increased productivity and economic efficiency (McEwen & Collignon, 2018; Durso *et al*., 2014; FAO, 2018). The livestock sector accounts for the majority of global veterinary antimicrobial consumption, whereas companion animals, despite lower consumption levels, present growing concerns about AMR due to the use of shared drug classes and close human-animal contact (Caneschi et al., 2023).

In food-producing animals, antimicrobials have been used since 1950s, mostly via oral routes (feed, water, or milk replacers), enabling cost-effective treatment of large groups. These drugs are commonly used not only for therapeutic purposes but also for prophylaxis (in healthy

animals that are not clinically ill but considered to be at risk of infection) and metaphylaxis (in a group of animals, after the diagnosis of disease in part of the group) (WHO, 2017; Tang *et al*., 2023; McEwen & Collignon, 2018). Such practices improved animal health, growth rates, and productivity (Caneshi *et al*., 2023), supporting the intensification of livestock systems to meet increasing demand for animal-derived foods, especially in LMICs (Komarek *et al*., 2022). Additionally, sub-therapeutic doses of antimicrobials have been used for growth promotion, enhancing animal growth and production efficiency (Caneschi *et al*., 2023; Cromwell *et al*., 2002; Gustafson & Bowen, 1997). However, the overuse and misuse of antibiotics, such as their administration at sub-therapeutic doses, exert significant selective pressure, driving the emergence and spread of resistant and multidrug-resistant (MDR) bacteria (resistant to three or more antimicrobial classes) (Magiorakos *et al*., 2012). These ARB and their antimicrobial resistance genes (ARGs) can spread across ecosystems and reach humans through multiple transmission pathways (Ho *et al*., 2025; Salam *et al*., 2023; Aslam *et al*., 2021; UNEP, 2023; Caneschi *et al*., 2023; Pires *et al*., 2022). In response, the EU banned antimicrobial growth promoters in 2006, followed by similar measures in the United States of America (USA) (2017) and China (2020) (European Union, 2003; EC, 2005; FDA, 2013; Maron *et al*., 2013). Nevertheless, this practice persists in some countries, especially LMICs, where regulatory frameworks are weaker (Caneschi *et al*., 2023; Palma *et al*., 2020).

In 2017, food-animal production accounted for approximately 73% of global antimicrobial consumption, with projections indicating continued increases in Asia, Africa, Oceania, and South America (Van Boeckel *et al*., 2019; Ho *et al*., 2025; Mulchandani *et al*., 2023). More recent reports from the World Organisation for Animal Health (WOAH) show some global progress: a 13% reduction in animal antimicrobial use from 2017 to 2019, and a further 5% decrease from 2020 to 2022, with a total of 70.648 tonnes of antimicrobial agents used in animals in 2022 (WOAH, 2024; WOAH, 2025). Tetracyclines remain the most used class, followed by penicillins, sulfonamides, macrolides, and cephalosporins - with tetracyclines and penicillins together accounting for nearly half of global use in 2022 (WOAH, 2025). Despite these reductions, regional disparities remain. Between 2017 and 2022, antimicrobial use declined in Europe (−23%), Africa (−20%), the Americas (−4%), and Asia-Pacific (−2%), but increased by 43% in the Middle East (WOAH, 2025). The top five consumers - China, Brazil, India, USA, and Australia - accounted for 58% of global veterinary antimicrobial use (Ho *et al*., 2025).

In 2023, about 25% of countries still reported using antimicrobials for growth promotion, with the highest prevalence in the Americas and Asia-Pacific (WOAH, 2025; Van Boeckel *et al*., 2019). This may explain why AMR rates tend to be higher in LMICs, despite lower per capita

antibiotic use (WHO, 2022). Of particular concern is the continued use in some countries (e.g. Pakistan, Bangladesh, Nigeria) of critically important antimicrobials for human health - such as colistin, and enrofloxacin - for growth promotion, increasing the risk of cross-species ARB and ARGs transfer (WOAH, 2025; Tang *et al*., 2023). Multiple reports confirmed a strong link between antimicrobial use and AMR, suggesting that reducing usage in both humans and animals can lower AMR prevalence (ECDC/EFSA/EMA, 2024). However, the continued use of colistin and third-generation cephalosporins in both sectors remains a major concern due to their potential role in AMR transmission between animals and humans (Tang *et al*., 2023).

1.1.3. Environment

The environment plays a central role in the ecology and persistence of AMR, acting as both a receptor and reservoir of ARB and ARGs. Environmental compartments - soil, water, air, and wildlife - are not isolated systems, they form an interconnected network that continuously interacts with human and animal sectors through natural and anthropogenic processes (Greig et al., 2015). The continuous discharge of resistant microorganisms and resistance genes into soils and aquatic environments through anthropogenic activities, such as agricultural runoff, aquaculture effluents, hospital wastewater, and manure application, promote conditions that favour AMR development, either through spontaneous mutation or through HGT events (Gomes, 2024; UNEP, 2023). This convergence brings together resistant bacteria naturally present in the environment, harbouring intrinsic resistance determinants as part of their ecological adaptation, with ARB and ARGs originating from human and animal sources (Vikesland *et al*., 2017). This bi-directional exchange between environmental and human/animal bacteria represents a major route for the emergence of new resistance traits, particularly in aquatic ecosystems and soils enriched with organic waste. The higher resistance traits enhance the persistence and dissemination of these bacteria across environmental matrices (soil, water, air) and colonization of wild animals and plants (Singer et al., 2016).

Plants, given their high interaction with soils and water, serve as living bridges connecting contaminated environmental matrices with the food chain. In plant agriculture, the use of antimicrobials - although less widespread than in human or veterinary medicine – played an important role in managing bacterial diseases of high-value crops (McManus et al., 2002). Antibiotics such as streptomycin and oxytetracycline, introduced in the 1950-1960s to control diseases in high-value crops, became widely adopted in orchards and vegetable production, particularly in the USA and Asia (Sundin & Wang, 2018; Miller *et al*., 2022). However, excessive use of antibiotics in plants has resulted in the emergence of resistant bacteria and

transferable resistance genes that persist in soil and plant-associated microbiomes, facilitating HGT to human and animal pathogens through environmental or food chain pathways (FAO, 2018; UNEP, 2023). Due to their ecological impact, the EU banned antibiotics in plant production under Regulation (EC) No 1107/2009, yet global data on antimicrobial use in crop production remain scarce (FAO, 2018; Haynes, 2020).

Healthy soils and water bodies host vast and complex microbial ecosystem that are fundamental to planetary health and overall ecosystem stability. However, the release and development of ARB into the environment through anthropogenic activities can disrupt these microbial communities (Gomes, 2024; UNEP, 2023). Such disturbances reduce microbial diversity, weakening ecosystem resilience and disrupting the natural balance of these microbiomes (Gomes, 2024). Wildlife is also affected, since when animals are exposed to ARB in their habitats, their natural microbiota may be altered, increasing disease susceptibility and potentially impacting population dynamics, especially protected species (Radhouani *et al*., 2014; Vezeau & Kahn, 2024). Consequently, the environmental compartment acts as a dynamic interface where ARB and ARGs can circulate and ultimately re-enter human and animal populations, through multiple pathways with a local and/or global impact, in a natural bi-directional flow between human, animals and environment (Palma *et al*., 2020).

In addition to the dissemination of resistant microorganisms, the presence of antimicrobial residues in the environment plays a crucial role in maintaining and intensifying AMR. Antimicrobial compounds excreted unmetabolized by humans and animals or derived from improper disposal of pharmaceutical and agricultural waste, enter wastewater and natural habitats where they persist at sub-inhibitory concentrations. Even at low levels, these residues exert selective pressure that favours the growth of resistant subpopulations and stimulates genetic exchange among bacteria (Singer et al., 2016; UNEP, 2023).

Ultimately, the environment not only receives and sustains resistant bacteria but also amplifies and redistributes them, connecting environmental, animal, and human microbiomes, and reinforcing AMR as a global ecological and public health challenge. This complex and multifaceted issue requires a One health approach, with coordinated action and surveillance efforts at national and international levels, as it has implications for human, animal, plant, and ecosystem health (WHO, 2017; ECDC/EFSA/EMA, 2024; IACG, 2019).

1.2. Enterobacterales as priority AMR pathogens

A substantial proportion of global deaths attributable to AMR can be traced to a limited number of bacterial pathogens. In 2019, six key bacteria - *E. coli, K. pneumoniae, S. aureus, S. pneumoniae, A. baumannii,* and *P. aeruginosa* - were directly responsible for approximately 929,000 (73%) of the 1.27 million deaths directly attributed to AMR globally. Among these, *E. coli* accounted for the highest number of deaths, followed closely by *K. pneumoniae*, underscoring their critical impact on global health (Murray *et al*., 2022).

The *E. coli* and *K. pneumoniae*, both members of the order Enterobacterales, are among the most clinically significant MDR pathogens. These organisms exhibit resistance to multiple antibiotic classes, including last-resort drugs such as carbapenems and colistin, complicating treatment of nosocomial and community-acquired infections (Oyenuga *et al*., 2024).

Reflecting the severity of these threats, the WHO updated its Bacterial Priority Pathogens List (BPPL) in 2024. Enterobacterales resistant to third-generation cephalosporins and carbapenems, including *E. coli* and *K. pneumoniae,* were placed in the “critical group”, a designation reserved for pathogens that pose a high threat to public health due to their resistance patterns, high mortality and morbidity, limited treatment options, and rising prevalence (WHO, 2024a). Given their global impact, MDR, and prioritization by both clinical and public health authorities, *E. coli* and *K. pneumoniae* represent two of the most urgent targets for AMR research, surveillance, and intervention.

1.3. Emergence and dissemination of AMR: mechanisms and pathways

Although AMR is a natural evolutionary phenomenon driven by HGT and genetic mutations in microorganisms, anthropogenic activities have markedly accelerated its emergence and dissemination, especially the overuse and misuse of antimicrobials (Tang *et al*., 2023; Caneschi *et al*., 2023; Holmes *et al*., 2016). Bacteria can exhibit intrinsic, acquired, and adaptive forms of resistance (**Figure 1**) (Salam *et al*., 2023; Darby *et al*., 2023). Intrinsic resistance refers to the natural resistance found in certain bacterial species, associated with the absence of a drug target, reduced target affinity or reduced membrane permeability (EFSA, 2021; Ho *et al*., 2025). Acquired resistance arises either through chromosomal mutations, passed vertically across bacterial generations or via HGT (EFSA, 2021). HGT is particularly significant and involves the acquisition of ARGs from other bacteria predominantly through mobile genetic elements (MGEs) (Salam *et al*., 2023; Darby *et al*., 2023; von Wintersdorff *et al*., 2016). Adaptive resistance, in contrast, involves reversible phenotypic

changes in response to antimicrobial exposure, often comprising transient alterations in gene expression that enable survival under antibiotic stress conditions (Salam *et al*., 2023; Tang *et al*., 2023). The antibiotic resistance mechanisms target four essential components in a bacterial cell: the cell wall, the cell membrane, protein synthesis and nucleic acid synthesis (Ho *et al*., 2025; Blair *et al*., 2015; Darby *et al*., 2023). The mechanisms underlying AMR include: i) decreased antibiotic permeability through the cell membrane (modifications of the membrane limit the antibiotic access to intracellular targets); ii) alteration of antibiotic target sites (reducing the binding affinity of the antimicrobial agent); iii) enzymatic degradation or modification of the antimicrobial compound (transfer of chemical groups to specific sites on the antimicrobial compound and neutralization of it); and iv) overexpression of efflux pumps (actively transport antimicrobial agents out of the bacteria, reducing the intracellular drug levels to sub-therapeutic concentrations) (Ho *et al*., 2025; Blair *et al*., 2015; Darby *et al*., 2023; Christaki *et al*., 2020). In addition, the ability of bacteria to form biofilms significantly contributes to increased resistance and long-term persistence in various environments. Biofilms, formed by structured microbial communities encased in a self-produced extracellular matrix, act as physical and chemical barriers that limit antibiotic penetration (Salam *et al*., 2023; Blair *et al*., 2015).

The rapid spread of antibiotic resistance is a critical global health issue, largely driven by HGT, which enables ARG dissemination across diverse bacterial species (von Wintersdorff *et al*., 2016). Unlike vertical transmission, HGT allows bacteria to acquire adaptive traits from their environment, including resistance to antimicrobials. Environmental bacteria act as reservoirs of ARGs, facilitating their transfer to human and animal pathogens in clinical, agricultural, and natural settings (Darby *et al*., 2023; UNEP, 2023; Palma *et al*., 2020). HGT occurs through four main mechanisms: transformation (uptake of free DNA), transduction (transfer of DNA by bacteriophages), extracellular vesicles (vesicles secreted by bacteria that transport DNA between cells), and conjugation (direct DNA transfer via pili) (**Figure 1**) (von Wintersdorff *et al*., 2016; Darby *et al*., 2023; Biller *et al*., 2025; Christaki *et al*., 2020). Conjugation is particularly concerning, as it is the most efficient and frequent mechanism and often involves plasmids carrying multiple ARGs, thus promoting MDR across environmental, animal, and human sectors (Blair *et al*., 2015; Salam *et al*., 2023; von Wintersdorff *et al*., 2016).

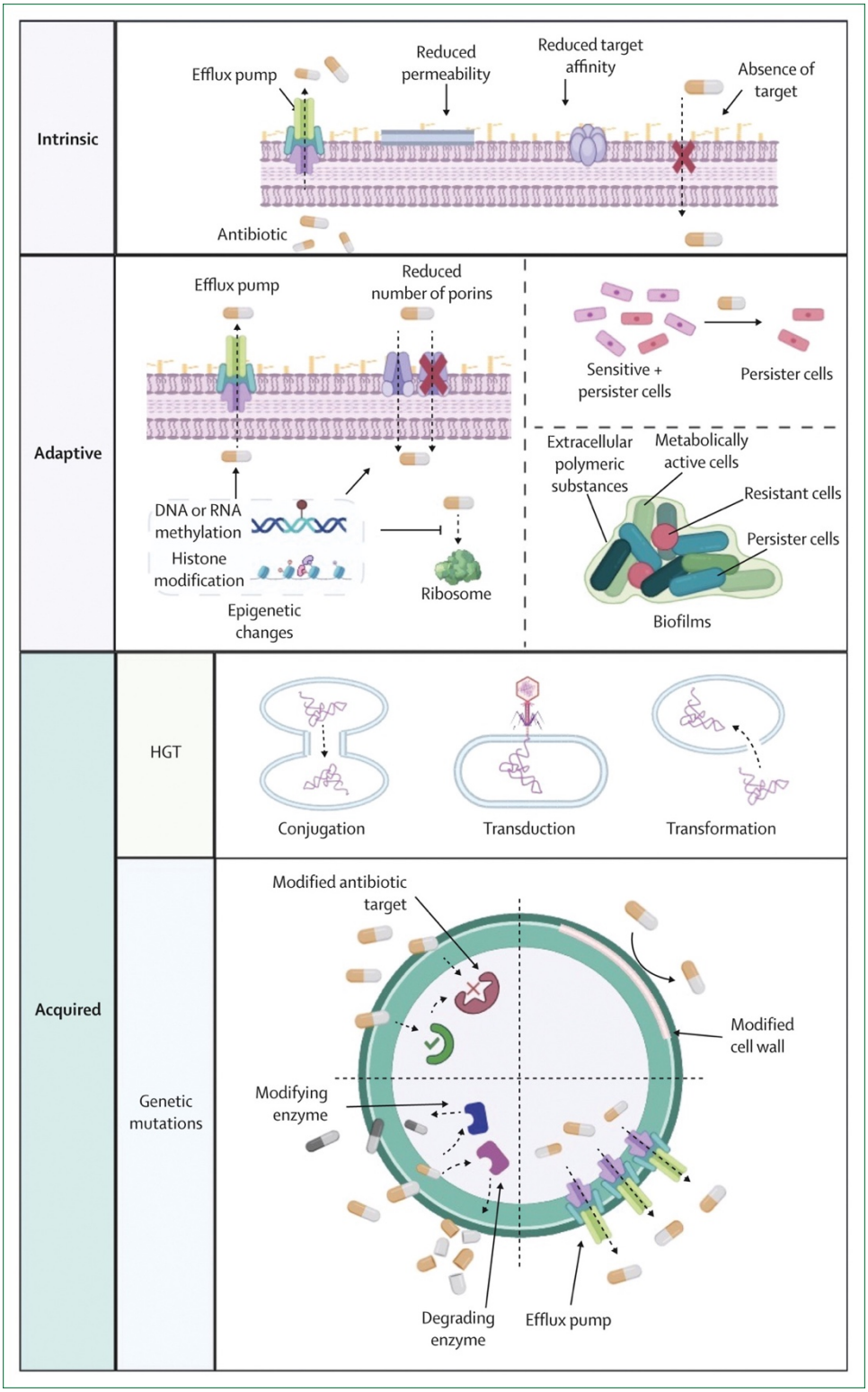

Figure 1. Antimicrobial resistance mechanisms (Ho *et al*., 2025).

Although the mechanisms and dynamics of HGT under real-world conditions are not fully understood, it is well established that subinhibitory concentrations of antimicrobial compounds - including antibiotics, disinfectants and metals - commonly present in environments impacted by human activity, can enhance HGT, increase the frequency of gene transfer events and selection of ARB (Vikesland *et al*., 2017; von Wintersdorff *et al*., 2016). Beyond antibiotics, increasing attention is being paid to other antimicrobial compounds, such as biocides and metals, which are widely used in agri-food settings and may exert similar selective pressures (Rebelo *et al*., 2023; Baker-Austin *et al*., 2006). These substances, although functionally important, can contribute to the co-selection and dissemination of ARGs (**Figure 2**) (Pal *et al*., 2017; Davies & Wales, 2019; Rebelo *et al*., 2023). Co-selection may occur through i) Co-resistance: genes conferring resistance to antibiotics and to metals/biocides are located on the same MGE, so that exposure to one selecting for the other; ii) Cross-resistance: a single mechanism (e.g., efflux pumps) confers resistance to multiple compounds; iii) Co-regulation: shared regulatory pathways that simultaneously activate different resistance mechanisms (**Figure 2**). These processes are particularly relevant in high-stress environments, such as food production systems. Persistent exposure to low concentrations of these compounds may promote mutations and gene exchange, further enhancing ARG spread (Seiler & Berendonk, 2012; Rebelo *et al*., 2023). However, many of these interactions occur outside the scope of current surveillance systems, which tend to focus mainly on antibiotic use (WHO, 2017; Seiler & Berendonk, 2012).

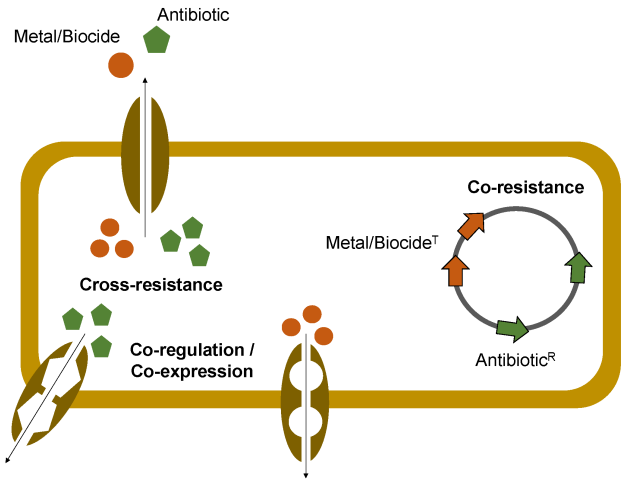

Figure 2. Mechanisms of metal/biocide and antibiotic co-selection: cross-resistance, co-resistance and co-regulation/co-expression (Rebelo *et al*., 2023).

A deeper, integrated understanding of these non-antibiotic selective pressures is essential. Identifying these "hidden" layers of selection within the food chain is crucial to fully comprehend the complex drivers of AMR. This requires a multidisciplinary approach, bridging microbiology, food safety, veterinary medicine, environmental science, and public health, within a One Health framework.

1.4. Interconnectedness of Antimicrobial Resistance

The transmission of ARB and ARGs between humans, animals and the environment occur through complex interactions and multiple routes, contributing to the accelerated spread of AMR (UNEP, 2023; Tang *et al*, 2023, Vikesland *et al*., 2017). The animal sector, particularly intensive food-animal production, plays a major role in AMR development, with the environment acting as a reservoir for AMR bacteria and genes, facilitating the spread of resistance across various ecosystems (Palma *et al*., 2020). Human activities are by no means exempt from responsibility, as practices across healthcare, industrial, and urban domains significantly contributed to the global burden of AMR, creating favourable conditions for the proliferation and dissemination of ARB and ARGs (UNEP, 2023; Vikesland *et al*., 2017).

In the human sector, hospitals and healthcare facilities are high-risk environments, acting as AMR hotspots due to frequent antibiotic usage and the presence of highly susceptible individuals (Salam *et al*., 2023; UNEP, 2023). Beyond clinical settings, the discharge of medical and industrial waste contributes to the accumulation of antimicrobial residues in the environment, fostering further AMR development (Salam *et al*., 2023; Vikesland *et al*., 2017). Global travel and migration further contribute to the spread of AMR, allowing ARB to cross borders and establish themselves in new environments. This poses significant risks in densely populated areas and in regions with limited healthcare infrastructure, such as LMICs, where AMR can proliferate unchecked, exacerbating the global health challenge (UNEP, 2023).

In intensive food-animal production, the need to meet the increasing demand for animal-derived food products has led to the widespread use of antimicrobials - especially antibiotics - for therapeutic, prophylactic, and, in some regions, growth-promoting purposes. This has imposed substantial selective pressure on microbial populations, driving the selection and proliferation of ARB (Van Boeckel *et al*., 2017; Tang *et al*., 2023, Palma *et al*., 2020). The use of specific antibiotic classes in food-animal production is strongly correlated with antibiotic resistance profiles observed in human pathogens (ECDC/EFSA/EMA, 2024; Marshall & Levy, 2011). This connection is underscored by specific cases of resistance, such as the use of fluoroquinolones and tetracyclines in livestock, which has been linked to resistant strains of *E. coli*, *Salmonella*, and *Campylobacter* species in both animals and humans (Rukambile, *et al*., 2019; Caneschi *et al*., 2023). The spread of ARB and ARGs from food-animal production to humans can occur through direct and indirect pathways. Direct transmission occurs through handling of livestock or consumption of contaminated animal products, such as undercooked meat or improperly processed dairy (Lekshmi *et al*., 2017; Ardakani *et al*., 2023). Manure used

as fertilizer and agricultural effluents are critical examples of indirect transmission pathways, leading to the introduction of resistance elements in agricultural soils, contaminate water sources, and spread across broader ecosystems (Greig *et al*., 2015; Zhang *et al*., 2022). Indirect transmission poses a boarder threat, with ARB and ARG excreted into the environment and disseminated through environmental routes including water, soil, and air, exacerbating the spread of AMR (UNEP, 2023).

The environment serves as a critical interface in the transmission and amplification of AMR. Wastewater treatment plants, which receive effluents from hospitals, households, industries and farms, often serve as junctions where bacteria from diverse sources mix and exchange genetic material via HGT (Salam *et al*., 2023; UNEP, 2023; Vikesland *et al*., 2017). If not adequately designed or maintained to remove antimicrobial residues and ARB, these facilities may become hubs for AMR amplification and dissemination. Aquatic environments are particularly vulnerable, with natural water bodies - rivers, lakes, and seas - frequently receiving agricultural effluents, aquaculture discharge, pharmaceutical waste, and wastewater, often containing persistent ARB and ARG that accelerate AMR spread across ecological compartments (Vezeau & Kahn, 2024; Murray *et al*., 2022; Proia *et al*., 2016; Ballash *et al*., 2024). These contaminated waters can carry ARB across vast distances, connecting communities, ecosystems, and continents (Bürgmann *et al*., 2018; Vikesland *et al*., 2017). Once present, ARB can contaminate drinking water supplies, and irrigation systems, or persist in soil, spreading to crops or becoming airborne through dust and aerosols (UNEP, 2023). This widespread dissemination poses serious risks to wildlife, animals, and humans, as ARB may enter food systems or human populations through direct contact, consumption of contaminated water, or agricultural products irrigated with such water (UNEP, 2023; Vikesland *et al*., 2017). Wildlife species, especially those at human-wildlife interface, are increasingly recognized as reservoirs and vectors of ARBs and ARGs, due to contact with contaminated water, soil, food sources, and domestic animals (Laborda *et al*., 2022; Vezeau & Kahn, 2024). The detection of ARB and ARG in remote or pristine environments, such as polar regions, deep-sea sediments, and protected wildlife reserves, demonstrates the global reach of AMR and highlights the complex interactions between environmental reservoirs and anthropogenic drivers (Hwengwere, 2022).

These environmental pathways enable the silent and pervasive spread of AMR, accelerating its global reach across diverse ecosystems and regions. Moreover, subinhibitory concentrations of antibiotics and other selective agents - such as disinfectants and metals - in polluted environments can promote HGT and selection of ARB (Berglund, 2015). Many ARGs found in clinically relevant pathogens have environmental origins, highlighting the ecosystem's

role in the evolution of AMR (Finley *et al*., 2013). Whether in soil or water, ARB and their ARG are constantly in motion, silently bridging the gaps between environment, human and animal populations (**Figure 3**).

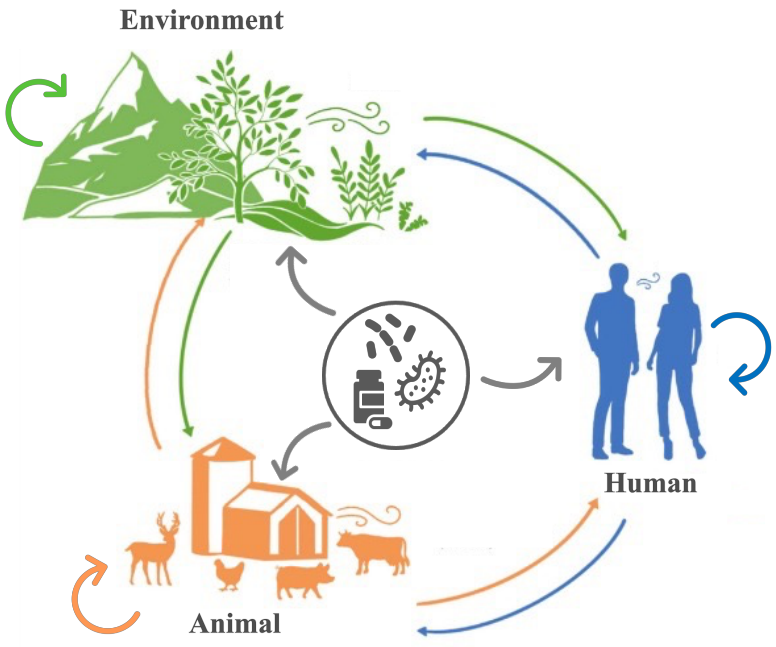

Figure 3. Interconnectness of antimicrobial resistance in human-animal-environment (adapted from Despotovic *et al.,* 2023).

Given the deeply intertwined nature of AMR across human, animal, and environmental domains, the implementation of One Health strategies is imperative (Tang *et al*., 2023, Palma *et al*., 2020). Tackling AMR in isolated compartments has proved to be insufficient, as AMR does not recognize sectoral boundaries. One Health offers a comprehensive, transdisciplinary framework that fosters collaboration across public health, veterinary medicine, agriculture, and environmental sciences (Tang *et al*., 2023; Aslam *et al*., 2021, Palma *et al*., 2020). As the spread of AMR accelerates through global trade, environmental contamination, and human movement, the adoption of robust One Health interventions stands as a critical pillar in safeguarding the efficacy of antibiotics - and other compounds with antimicrobial activity - and in protecting the health of current and future generations. Through coordinated surveillance, responsible antimicrobial stewardship, and integrated policymaking, One Health approaches can curb the AMR (Aslam *et al*., 2021; Palma *et al*., 2020; Vezeau, 2025).

2. Colistin resistance and the food-animal production link

2.1. Colistin and its relevance in human health

Colistin, also known as polymyxin E, belongs to the group of cyclic polycationic antimicrobial peptides called polymyxins, which comprise five chemically distinct compounds: polymyxin A, B, C, D, and E (Mondal *et al*., 2024). In clinical practice, only two polymyxins are used therapeutically - polymyxin B and colistin - with the latter playing a renewed role in the treatment of MDR infections (Gogry *et al*., 2021; Andrade *et al*., 2020).

Colistin was first discovered in 1947 in Japan by Y. Koyama, who isolated it from *Paenibacillus polymyxa* subsp. *colistinus*, a spore-forming Gram-positive soil bacteria (Hussein *et al*., 2021; Diani *et al*., 2024). Due to its remarkable efficacy against Gram-negative bacteria (GNB), it was initially considered a “miraculous” antimicrobial and was widely used in clinical settings from the 1950s to the 1970s. At that time, in was available as colistin sulfate for oral or topic administration, and as colistin methanesulfonate sodium for parenteral and inhalation use (El-Sayed Ahmed *et al*., 2020; Diani *et al*., 2024; Mondal *et al*., 2024). However, serious neurotoxic and nephrotoxic effects led to its decline in medical use, with its application largely restricted to treating cystic fibrosis and localized infections (e.g., eye and ear) caused by MDR GNB. It was gradually replaced by less toxic alternatives such as β-lactams, aminoglycosides, and quinolones (Elias *et al*., 2021; Hamel *et al*., 2021).

In recent years, the rapid rise of MDR and, in particular, extremely drug-resistant (XDR) GNB - notably carbapenem-resistant Enterobacterales - has driven the renewed use of colistin in human medicine. This resurgence is largely explained by colistin’s sustained activity against these pathogens; a feature partly preserved due to its historically limited use in clinical settings throughout the 20th century. In the absence of effective new antimicrobials, colistin has re-emerged in the end of 1990s as a critical therapeutic option for treating infections caused by MDR GNB (Gogry *et al*., 2021; Andrade *et al*., 2020).

2.2. Mechanism of action and spectrum of activity

Colistin is a cyclic polycationic heptapeptide with a hydrophobic acyl tail, whose structure enables it to act as a detergent and membrane destabilizer, leading to bacterial lysis across a broad spectrum of GNB (Diani *et al*., 2024; Elias *et al*., 2021). The positively charged amino groups in the colistin structure facilitate electrostatic interactions with negatively charged bacterial components, which are the key to its bactericidal activity (Andrade *et al*., 2020).

The exact mechanism of action of colistin is not fully elucidated. However, it is widely accepted that colistin exerts direct antibacterial activity against GNB. This activity is primarily attributed

to i) membrane lysis though self-promoted uptake mechanism, alongside several parallel mechanisms, including ii) the hydroxyl radical death pathway, iii) the inhibition of respiratory enzymes, and iv) the anti-endotoxin activity, which together exert a synergistic effect that enhances the overall antimicrobial activity of this antibiotic (**Figure 4**) (Diani *et al*., 2024; Mondal *et al*., 2024; El-Sayed Ahmed *et al*., 2020).

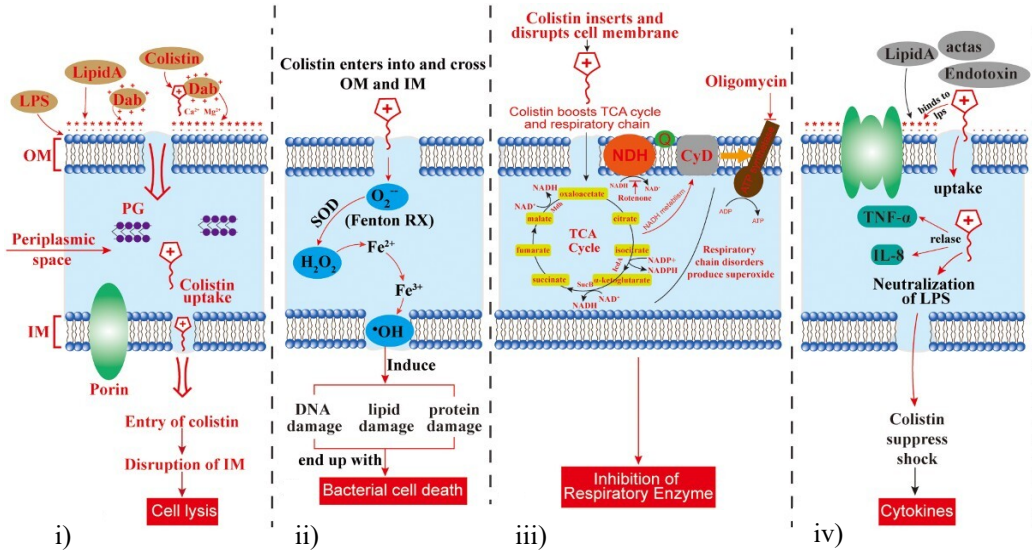

Figure 4. Colistin mechanisms of action. i) membrane lysis though self-promoted uptake mechanism; ii) hydroxyl radical death pathway; iii) inhibition of respiratory enzymes; iv) anti-endotoxin activity (Adapted from El-Sayed Ahmed *et al*., 2020).

i) Membrane lysis and self-promoted uptake mechanism

The primary and best-characterized mechanism of colistin is membrane lysis, which causes rapid membrane disruption, cytoplasmic leakage, and cell death. Colistin binds strongly via electrostatic interactions to lipid A, a key component of LPS (lipopolysaccharide) present in the outer membrane (OM) of GNB, displacing stabilizing divalent cations (Mg^{2+} and Ca^{2+}) and disrupting membrane integrity (Hamel *et al*., 2021; Diani *et al*., 2024; El-Sayed Ahmed *et al*., 2020). Its hydrophobic acyl tail further inserts into the lipid bilayer, promoting pore formation and leakage of cellular contents (Andrade *et al*., 2020; Seethalakshmi *et al*., 2023; Mondal *et al*., 2024). This destabilization increases OM permeability, facilitating colistin's entry into the periplasmic space and interaction with the inner membrane (IM), where it induces further disruption through: a) vesicle-vesicle contact leading to membrane fusion and osmotic imbalance, and b) targeting lipid A during its biosynthesis in the IM, compromising membrane stability (Elias *et al*., 2021; Mondal *et al*., 2024).

ii) Hydroxyl radical death pathway

Colistin induces oxidative stress by triggering the production of reactive oxygen species (ROS), that damage cellular components, disrupt metabolism, and enhance membrane destabilization, contributing to bacterial cell death (El-Sayed Ahmed *et al*., 2020; Elias *et al*., 2021).

iii) Inhibition of respiratory enzymes

Colistin also inhibits essential bacterial respiratory enzymes, present in the IM of GNB, impairing respiratory function, and affecting bacterial viability (Mondal *et al*., 2024; Elias *et al*., 2021).

iv) Anti-endotoxin activity

Colistin binds to lipid A, neutralizing LPS and attenuating endotoxin-induced immune activation, thereby reducing inflammation and the risk of septic shock in severe GNB infections (El-Sayed Ahmed *et al*., 2020; Diani *et al*., 2024; Mondal *et al*., 2024; Elias *et al*., 2021).

Colistin is highly effective against a broad range of GNB, including members of the *Enterobacteriaceae* family (*Escherichia coli, Klebsiella* spp.*, Enterobacter* spp.*, Salmonella* spp.*, and Shigella* spp.), as well as non-fermentative GNB such as *Pseudomonas aeruginosa, Acinetobacter baumannii*, and *Stenotrophomonas maltophilia* (Elias *et al*., 2021). However, it is ineffective against Gram-positive bacteria, anaerobes, and mycoplasmas (Diani *et al*., 2024). Certain GNB are intrinsically resistant to colistin, including species from the genera *Proteus*, *Providencia*, *Serratia*, *Edwardsiella, Brucella*, *Neisseria*, *Legionella*, *Chromobacterium*, as well *Morganella morganii*, *Pseudomonas mallei*, *Burkholderia cepacia*, *Helicobacter pylori*, *Stenotrophomonas mallei, Vibrio cholerae*, some *Aeromonas* species and anaerobic Gram-negative cocci (Gogry *et al*., 2021; Andrade *et al*., 2020; El-Sayed Ahmed *et al*., 2020; EUCAST, 2023). The intrinsic resistance to colistin of some of these GNB arises from structural or metabolic characteristics, such as OM modifications or colistin-inactivating enzymes (Gogry *et al*, 2021; El-Sayed Ahmed *et al*., 2020).

2.3. Chromosomally mediated colistin resistance mechanisms

Despite colistin's potent bactericidal activity against GNB, its clinical efficacy is increasingly threatened by the emergence of resistant strains (Elias *et al*., 2021; Diani *et al*., 2024; Mondal *et al*., 2024). Colistin resistance often arises through acquired or adaptive resistance, mechanisms involving modifications to its target sites, which reduce binding affinity and impair antimicrobial activity. Chromosomal mutations affecting membrane structures are considered the majors drivers of colistin resistance (Mondal *et al*., 2024; Gogry *et al*, 2021; El-Sayed Ahmed *et al*., 2020). The primary mechanism involves modifications of lipid A in the LPS, resulting in a decreased electrostatic attraction between colistin and the bacterial surface. This is achieved through the addition of phosphoethanolamine (PEtN) and/or 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A, which reduce its net anionic charge, thereby diminishing colistin binding (**Figure 5**) (Elias *et al*., 2021; Diani *et al*., 2024; Sharma *et al*., 2022). Among these, L-Ara4N addition appears to confer a higher level of resistance compared to PEtN (Olaitan *et al*., 2014; Gogry *et al*., 2021).

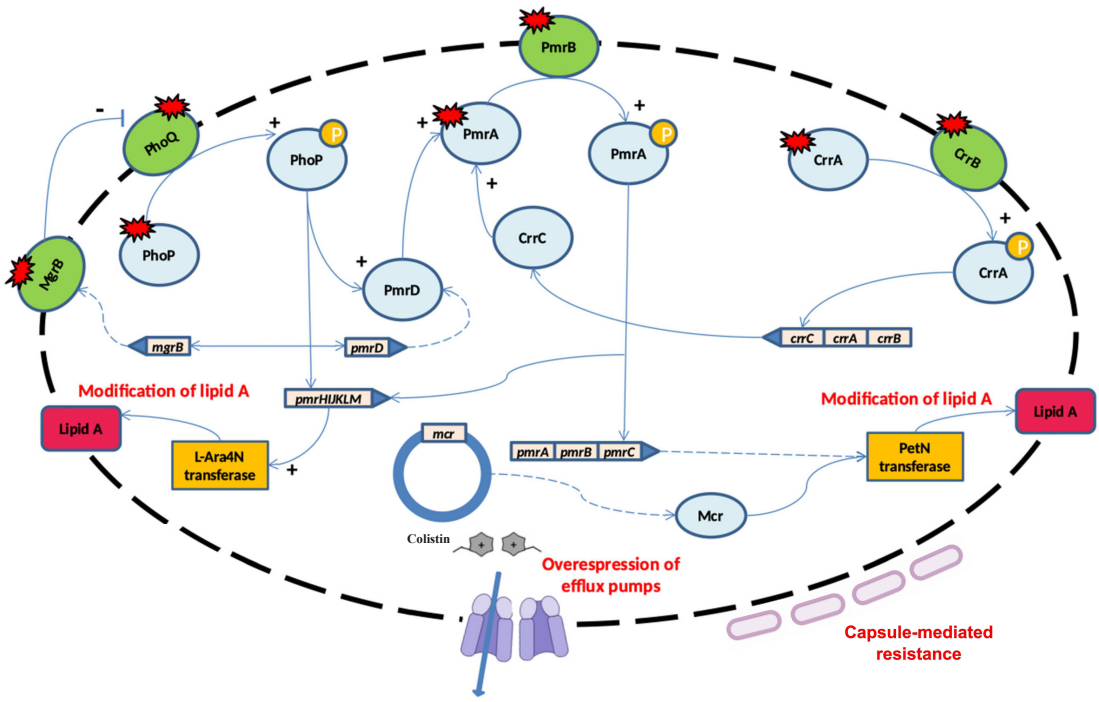

Figure 5. Mechanisms of colistin resistance among GNB (adapted from Diani *et al*., 2024).

For decades, these chromosomally mediated mechanisms were considered the predominant basis of resistance in colistin-non-susceptible isolates. The LPS modification by chromosomal mutations was the most well-characterized, prevalent and clinically significant mechanism of colistin resistance, involving genetic alterations that modify the lipid A component of LPS,

reducing its net negative charge and impairing colistin binding (Mondal *et al*., 2024; Gogry *et al*, 2021; Binsker *et al*., 2022, Diani *et al*., 2024). GNB developed resistance through mutations in key operons, the two-component systems (TCSs), particularly the PmrA-PmrB (PmrAB) and PhoP-PhoQ (PhoPQ) systems, as well as in regulatory genes, MgrB and CrrA-CrrB (CrrAB) (**Figure 5**). These systems regulate the addition of PEtN and L-Ara4N to lipid A. The two TCSs (PmrAB and PhoPQ) are responsible for the lipid A modification in response to colistin or low Mg^{2+} and Ca^{2+} levels, respectively, with the addition of PEtN (*pmrC*) and L-Ara4N (*pmrHFIJKLM*) to lipid A (Shahzad *et al*., 2023; Mondal *et al*., 2024; Elias *et al*., 2021). The MgrB exerts negative feedback over PhoPQ system, with its inactivation leading to TCSs PhoPQ overactivation and lipid A modification (Diani *et al*., 2024; Shahzad *et al*., 2023; Elias *et al*., 2021). CrrAB activates *pmrCAB* and *pmrHFIJKLM* in response to stimuli, causing LPS modifications similar to PmrAB (Elias *et al*., 2021). These modifications decrease the negative charge of the OM, reduce electrostatic interactions between colistin and LPS and ultimately diminish colistin susceptibility (Diani *et al*., 2024; Elias *et al*., 2021; Mondal *et al.,* 2024).

Two other chromosomal mediated resistance mechanisms act synergistically with LPS modification. The overexpression of efflux pumps reduces intracellular colistin accumulation, decreasing its antimicrobial efficacy, being their direct role still under debate (**Figure 5**) (Diani *et al*., 2024; Shahzad *et al*., 2023; Elias *et al*., 2021). The capsule-mediated resistance increases capsule production and shedding, forming a physical and electrostatic barrier that limits colistin access to the OM (Mondal *et al*., 2024; Diani *et al*., 2024; Elias *et al*., 2021; Shahzad *et al*., 2023).

2.4. Mobile colistin resistance – MCR

Chromosomal mutations were long considered the main mechanism of colistin resistance, until a significant shift occurred with the discovery of the first plasmid-mediated colistin resistance gene, designated as “mobile colistin resistance” (*mcr)* gene *mcr*-1 (Liu *et al*., 2016; Mondal *et al*., 2024; Diani *et al*., 2024). This mechanism is particularly concerning, as it relies on HGT via MGEs, enabling the rapid dissemination of *mcr* genes across bacterial populations (Luo *et al*, 2020; Binsker *et al*., 2022; Liu *et al*., 2024). The first *mcr* gene was identified in *E. coli* isolates from animals in 2015 (Liu *et al*., 2016). Since this discovery, multiple *mcr* genes (*mcr-1* to *mcr-10*) have been detected in diverse bacterial species, often carried on conjugative plasmids, facilitating widespread dissemination across different environments and hosts (Zhang *et al*., 2021; Hussein *et al*., 2021; Mmatli *et al*., 2022; El-Sayed Ahmed *et al*., 2020). MCR proteins are phosphoethanolamine transferases that modify lipid A, lowering colistin

affinity in a mechanism similar but independent of chromosomal mutations (Liu *et al*., 2016; Poirel *et al*., 2017).

2.4.1. Function and mechanism of action of MCR proteins

MCR proteins belong to the alkaline phosphatase superfamily and act as membrane-bound PEtN transferases (El-Sayed Ahmed *et al*., 2020; Liu *et al*., 2024). They mediate resistance to colistin by modifying lipid A through the addition of PEtN moiety to the bacterial OM, which reduces the net negative charge and consequently decreases colistin binding affinity (**Figure 5**). This modification follows a phosphoethanolamine transferase mechanism (Hussein *et al*., 2021; Zhang, 2024). First, the MCR enzyme binds phosphatidylethanolamine, the donor molecule of the phosphoethanolamine group, in the bacterial IM, in the presence of zinc (Hussein *et al*., 2021; Liu *et al*., 2024). Then, cleavage of the PEtN group occurs, followed by its transfer to the lipid A (**Figure 6**) (Mmatli *et al*., 2022). Finally, the modified lipid A, now bearing a PEtN group, is transported to the OM, where it interacts with adjacent lipid A molecules through hydrogen bonding and electrostatic repulsions, stabilizing the membrane. This structural change results in colistin resistance, as the increased cationic charge of lipid A reduces electrostatic interactions between colistin and its target site (Liu *et al*., 2024; Zhang, 2024).

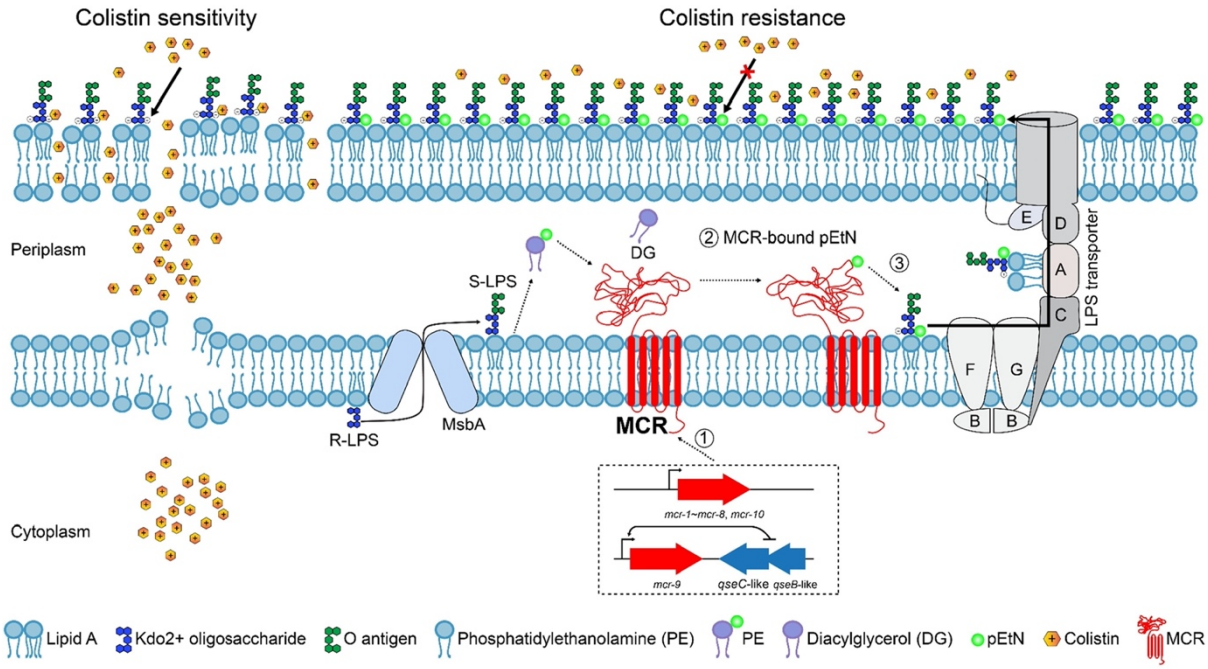

Figure 6. Action mode of MCR (Liu *et al*, 2024).

MCR-1, -2, and -4 confer high levels of colistin resistance, with 4- to 8-fold increases in minimum inhibitory concentrations (MIC). However, other variants, such as MCR-3 and MCR-5, have been associated with lower resistance levels, possibly due to differences in their structural configurations (Zhang *et al*., 2019). Additionally, *mcr-9* gene expression is inducible, meaning that external stimuli – such as subinhibitory concentrations of colistin – are required for its full activation (Liu *et al*., 2024).

2.4.2. *mcr* genes

The discovery of *mcr*-1 in 2015 in *E. coli* isolated from pigs in China was the first evidence of plasmid-mediated colistin resistance, shifting the paradigm of colistin resistance transmission (Liu *et al*., 2016). Subsequent research identified nine additional *mcr* genes (*mcr-2* to *mcr-10*) and their variants across different bacterial species, hosts, and geographical locations (**Table 1**). The potential origins of *mcr* genes have been linked to various bacterial genera that naturally harbor PEtN transferase enzymes, such as *Moraxella, Aeromonas, Shewanella, Kosakonia*, and *Buttiauxella* spp. (**Table 1**) (Liu *et al*, 2024; Hussein *et al*., 2021). These mainly environmental and commensal bacteria, inhabiting soil, freshwater, sediments, and the intestinal or respiratory tracts of animals, are intrinsically resistant to colistin, supporting the hypothesis that HGT events from environmental bacteria contributed to the emergence and dissemination of colistin resistance in pathogenic GNB (Liu *et al*, 2024; Zhang *et al*., 2021; Hussein *et al*., 2021).

Table 1. Characteristics of the first reported isolates of each *mcr* gene (*mcr-1* to *mcr-10*).

mcr gene	First Detection	Country	Bacterial Host	Host	Potential Origin	Reference
mcr-1	2015	China	*Escherichia coli*	Pig	*Moraxella* species	Liu *et al.,* 2016
mcr-2	2016	Belgium	*Escherichia coli*	Cattle, Pig	*Moraxella* species	Xavier *et al*., 2016
mcr-3	2017	China	*Escherichia coli*	Pig	*Aeromonas* species	Yin *et al*., 2017
mcr-4	2017	Italy	*Salmonella* Typhimurium	Pig	*Shewanella* species	Caratolli *et al*., 2017
mcr-5	2017	Germany	*Salmonella* Paratyphi *B*	Poultry	Unknown	Borowiak *et al*., 2017
mcr-6	2017	UK	*Moraxella* spp.	Pig	Unknown	AbuOun *et al*., 2017
mcr-7	2017	China	*Klebsiella pneumoniae*	Chicken	*Aeromonas* species	Yang *et al.,* 2018
mcr-8	2018	China	*Klebsiella pneumoniae*	Human, Pig	*Kosakonia* species	Wang *et al.,* 2019
mcr-9	2019	USA	*Salmonella* Typhimurium	Human	*Buttiauxella* species	Carroll *et al.,* 2019
mcr-10	2019	China	*Enterobacter roggenkampii*	Human	*Buttiauxella* species	Wang *et al.,* 2020

Most *mcr* gene families exhibit genetic diversity, with *mcr-1* and *mcr-3* showing the highest variability, comprising 37 (*mcr-1.1* to *mcr-1.37*) and 42 (*mcr-3.1* to *mcr-3.42*) allelic variants, respectively, deposited in the National Database of Antibiotic Resistant Organisms (NDARO) (**Figure 7**). Different allelic variants, differing by one or a few amino acids, have also been described for other *mcr* genes: *mcr-2* (8 variants), *mcr-4* (9 variants), *mcr-5* (5 variants), *mcr-8* (5 variants), *mcr*-9 (3 variants) and *mcr-10* (5 variants). In contrast, *mcr-6* and *mcr-7* have only a single known variant each (*mcr-6.1* and *mcr-7.1*, respectively) (El-Sayed Ahmed *et al*., 2020; Hussein *et al*., 2021; NDARO, 2021).

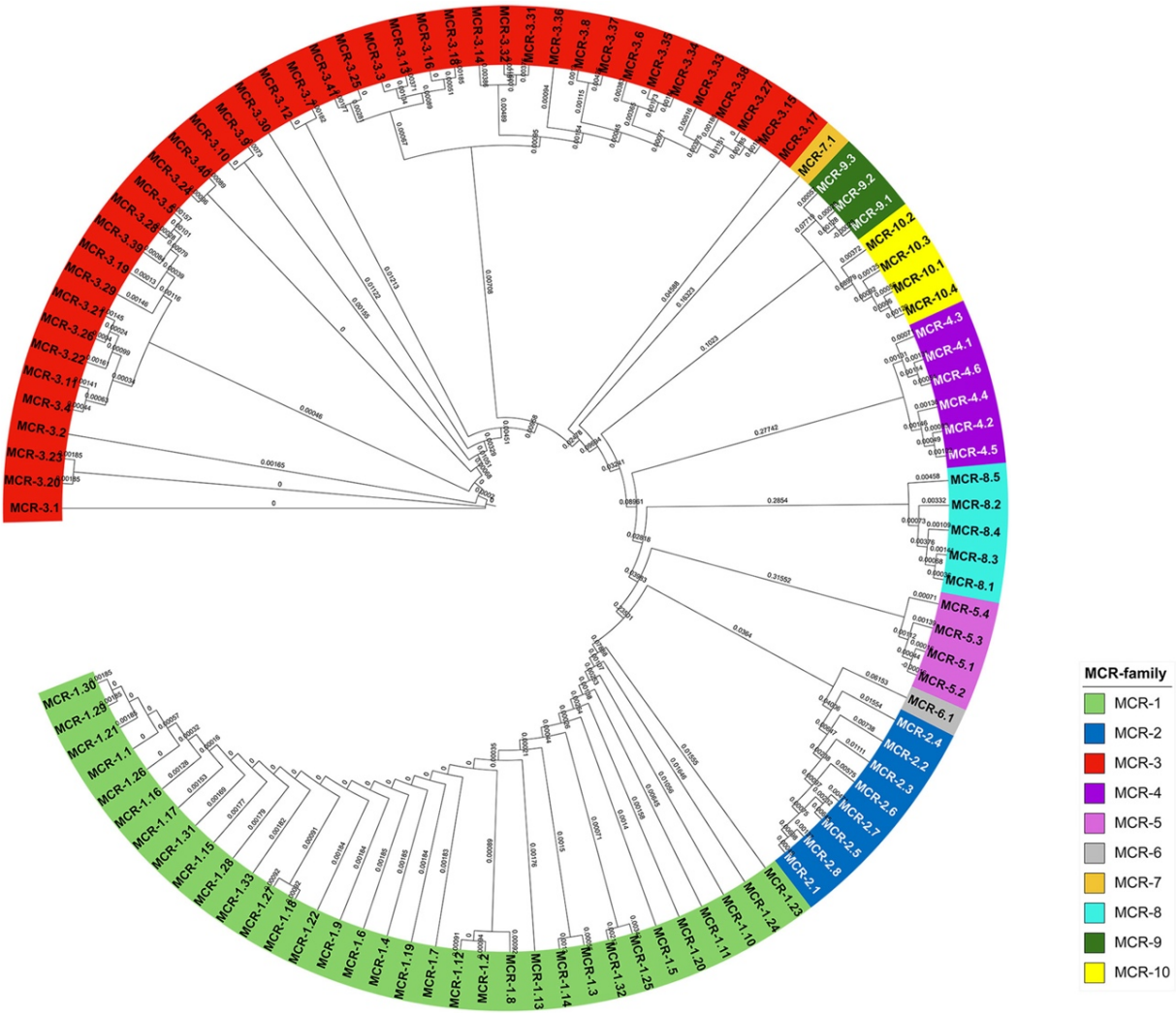

Figure 7. The phylogenetic tree of MCR-gene variants (Khuntayaporn *et al*., 2022). Only representative variants of MCR-1, -3, -4, -5, and -10 are shown; additional variants described in the text are not depicted.

2.4.3. Global spread of *mcr* genes

Although *mcr-1* was first identified in *E. coli* in 2015, retrospective studies have traced its presence back to the 1980s in *E. coli* isolates from poultry, linking its emergence to the introduction of colistin in food-animal production (Shen *et al*., 2016; Liu *et al., 2024;* Luo *et al.,* 2020). The earliest human-associated *mcr* gene was detected in *Shigella sonnei* from a clinical case in Vietnam in 2008 (Luo *et al*., 2020). To date, *mcr* genes have been identified in over 70 countries, with the highest prevalence in Asia, followed by Europe, highlighting the global spread of these resistant determinants (Liu *et al*., 2024*;* Mmatli *et al.,* 2022*).*

The widespread detection of *mcr* genes in diverse reservoirs - including food-producing animals, humans, wild animals, wastewater treatment plants, food products (meat and vegetables), and various environmental compartments, particularly aquatic ecosystems - underscores their extensive dissemination and the complex interspecies transmission dynamics involved (Bastidas-Caldes *et al*., 2022; Liu *et al*., 2024). Among these reservoirs, food-animal production exhibits the highest prevalence of *mcr* genes, supporting the hypothesis that their origin is closely linked to the selective pressure imposed by colistin use in livestock farming (Luo *et al*., 2020; Mmatli *et al*., 2022*).* Also, *mcr* genes are widely disseminated among different bacterial hosts, being predominantly found in *Enterobacteriaceae*, especially in *E. coli*, *K. pneumoniae,* and *Salmonella enterica,* in that order (Bastidas-Caldes *et al*., 2022; Mmatli *et al*., 2022*).*

Among them, *mcr*-1 is the most widely dispersed, having been detected across all five continents, with a notably higher prevalence in China (**Table 2**) (Mmatli *et al*., *2022;* Liu *et al*., 2024). As the first identified *mcr* gene, *mcr-1* has been the focus of more studies compared to other *mcr* genes, consequently, its high detection rate may be influenced by research bias (Mmatli *et al*., *2022*). The *mcr-2* gene has been primarily reported in Europe, North America and China, while *mcr-3* shows a strong presence in Asia, as well as in North and South America (**Figure 8**). The *mcr-4* and *mcr-5* genes are more frequently detected in Europe, often in *Salmonella enterica* and *E. coli* isolates, but have also been identified in North and South America, China, and Australia (**Figure 8**). In contrast, *mcr-6* gene has been rarely reported, mainly in *Moraxella* species. The *mcr-7* and *mcr-8* genes have been linked to *K. pneumoniae* infections, particularly in Asia (**Table 2**). Meanwhile, *mcr*-9 gene has been detected in *Enterobacter cloacae* complex and *S. enterica* across multiple continents, and *mcr*-10 has been identified in Europe, Asia, North and South America (**Figure 8**, **Table 2**) (Liu *et al*., 2024).

Despite the generally low prevalence of *mcr* genes in most countries, their global distribution is likely underestimated, as surveillance efforts have been concentrated in Asia - particularly

China - and in European countries. In contrast, LMICs, such as India and several African nations, have conducted limited research on *mcr* genes (Bastidas-Caldes *et al*., 2022; Liu *et al*., 2024*)*. Nonetheless, the detection of *mcr* genes in a wide variety of sample types, along with the broad dissemination of *mcr*-1, highlights the complex and rapid transmission dynamics of these genes (**Table 2**) (Liu *et al*., 2024; Bastidas-Caldes *et al*., 2022).

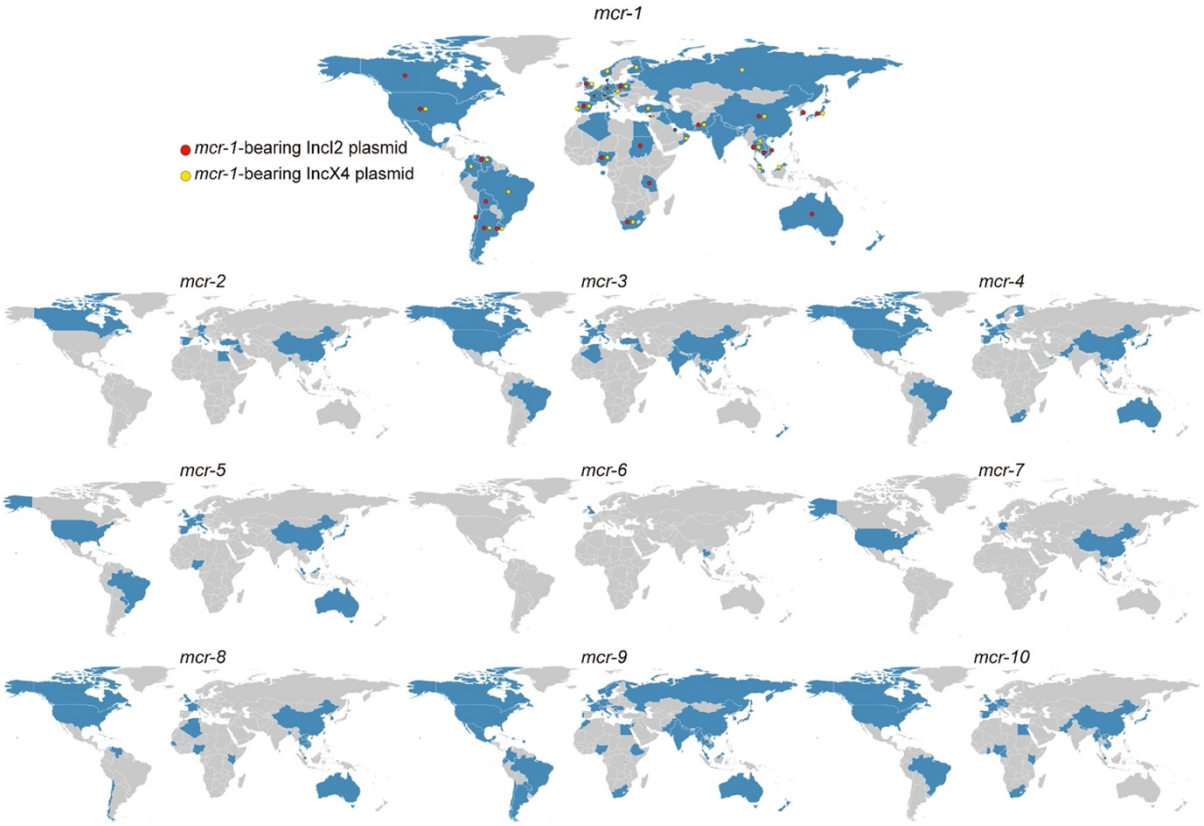

Figure 8. Geographical distribution of *mcr-1* to *mcr-10* (Liu *et al*., 2024).

Table 2. Characteristics of *mcr* genes (*mcr-1* to *mcr-10*), including associated plasmid types, host species, bacterial carriers, and countries where each *mcr* gene has been detected (Talat *et al*., 2023; Valiakos & Kapna, 2021; Mmatli *et al*., 2022; NDARO[a]; Liu *et al*., 2024)

***mcr* gene**	**Plasmids**	**Host**	**Bacteria**	**Countries**
mcr-1	IncA, IncB, IncC, IncD, IncE, IncF, IncFIA, IncFIB, IncFII, IncH, IncHI1, IncHI2, IncI2, IncK2, IncN, IncP, IncQ, IncR,IncX, IncX4, IncY, IncW	Human Livestock (swine, cattle, poultry, rabbit, sheep) Meat (pork, chicken, beef, shrimp, fish) Companion animals Environment Wild animals	*Acinetobacter baumannii,* *E. coli,* *K. pneumoniae,* *Salmonella spp.,* *Enterobacter spp.,* *Shigella spp.,* *Citrobacter spp.,* *Moraxella spp.,* *Providencia alcalifaciens,* *R. ornitholytica,* *C. sakazakii*	Algeria, Argentina, Australia, Bahrain, Bangladesh, Belgium, Bolivia, Brazil, Britain, Cambodia, Canada, Chile, China, Colombia, Czechia, Denmark, Ecuador, Egypt, Estonia, Finland, France, Germany, Greece, Hong , Kong, Hungary, India, Iraq, Iran, Italy, Japan, Laos, Lithuania, Lebanon, Malaysia, Mexico, Nepal, Netherlands, New Zeeland, Nigeria, Norway, Oman, Pakistan, Paraguay, Poland, Portugal, Qatar, Romania, Russia, Sao Tome and Principe, Singapore, South Africa, South Korea, Spain, Sudan, Switzerland, Taiwan, Tanzania, Thailand, Tunisia, Turkey, United Kingdom, United States of America, Uruguay, Venezuela, Vietnam
mcr-2	IncHI1B/IncFIB, IncX4	Human Livestock (swine, cattle, poultry)	*E. coli,* *K. pneumoniae,* *Salmonella spp.*	Bangladesh, Belgium, Canada, China, Egypt, Germany, Iraq, Italy, Japan, Spain, Switzerland, Turkey, United Kingdom
mcr-3	IncA/C, IncFI, IncFII, IncHI1, IncHI2, IncI-gamma/K1, IncN, IncP, IncQ1, IncR, IncX, IncY	Human Livestock (swine, cattle, poultry) Meat (pork, chicken) Companion animals Environment Wild animals	*Aeromonas* spp., *E. coli,* *K. pneumoniae,* *Salmonella spp.,* *Shigella spp.*	Algeria, Bangladesh, Brazil, Cambodia, Canada, China, Denmark, England, France, Germany, India, Iraq, Italy, Japan, Korea, New Zewland, South Korea, Spain, Thailand, Turkey, Vietnam, United Kingdom, United States of America
mcr-4	ColE, IncC, IncHI2, IncQ	Human Livestock (swine, cattle, poultry) Food	*Acinetobacter baumannii,* *Enterobacter cloacae,* *Enterobacter kobei,* *E. coli,* *Salmonella spp.,* *Shewanella* spp.	Australia, Belgium, Brazil, Canada, China, Czechia, Finland, France, Germany, Ireland, Italy, Japan, Netherlands, Norway, Pakistan, Singapore, South Africa, South Korea, Spain, Thailand, United Kingdom, United Arab Emirates, United States of America

mcr-5	IncFIA, IncFII, IncI-gamma/K1, IncHI1,IncI1, IncHI2, IncN, IncP, IncQ1, IncX1	Human Livestock (swine, cattle, poultry) Meat (pork, chicken)	*E. coli,* *Morganella morganii,* *Pseudomonas aeruginosa,* *Salmonella enterica,* *Aeromonas hydrophila,* *Cuproavidus gilardii*	Algeria, Belgium, Brazil, China, France, Germany, Hong Kong, Italy, Japan, Malaysia, Paraguay, Singapore, Spain, Taiwan, United Kingdom, United States of America, Uruguay
mcr-6	ColE, IncHI2	Swine	*E. coli,* *Moraxella spp.*	Thailand, United Kingdom
mcr-7	IncI2	Human Livestock (swine, poultry)	*E. coli,* *K. pneumoniae*	China, Germany, Thailand, United States of America
mcr-8	IncFIA/C, IncFIA, IncFIB, IncFII, IncQ, IncR	Human Environment Livestock (swine, poultry)	*Citrobacter freundii,* *Enterobacter cloacae,* *E. coli,* *Klebsiella* spp., *Raoutella* spp.	Algeria, Australia, Bangladesh, Cambodia, Canada, Chile, China, France, Hong Kong, Lebanon, Kenya, Laos, Netherlands, Nigeria, Senegal, Singapore, South Korea, Switzerland, Taiwan, Thailand, United Kingdom, Venezuela, Vietnam, United States of America
mcr-9	IncFI; IncFII, IncHI1, IncHI2, IncI1-I, IncI2, IncN; IncP	Human Environment Livestock (swine, poultry, cattle) Wild animals Meat (chicken, turkey, beef, pork) Vegetables Animal feed Companion animals	*Citrobacter freundii,* *Cronobacter spp.,* *Enterobacter spp.,* *E. coli,* *Klebsiella* spp., *Kluyvera intermedia,* *Morganella morganii,* *Providencia alcalifaciens,* *Raoutella* spp., *Salmonella* spp., *Serratia marcescens*	Argentina, Australia, Bangladesh, Belgium, Brazil, Cambodia, Canada, Chile, China, Czechia, Denmark, Dominican Republic, Ecuador, Egypt, Ethiopia, France, Germany, Greece, Guadeloupe, Hungary, India, Isreal, Italy, Japan, Jordan, Kuwait, Lebanon, Malaysia, Mexico, Myanmar, Nepal, Netherlands, New Zeland, Nigeria, Norway, Pakistan, Paraguay, Puerto Rico, Portugal, Qatar, Russia, Romania, Rwanda, Serbia, South Africa, South Korea, Sweden, Switzerland, Trinidad and Tobago, Thailand, United Kingdom, United States of America, Vietnam
mcr-10	IncFIA, IncFIB, IncFII	Human Environment Meat (chicken)	*Citrobacter freundii,* *C. sakazakii,* *Enterobacter roggenkampii,* *K. pneumoniae,* *Kluyvera ascorbata,* *Pluralibacter gergoviae,* *Raoultella* spp.	Australia, Brazil, Cambodia, Cameroon, Canada, China, Czechia, Egypt, France, Gambia, Germany, Ghana, Japan, Kenya, Laos, Myanmar, Nepal, Netherlands, Nigeria, Pakistan, Singapore, South Africa, Spain, Switzerland, United Kingdom, United States of America, Vietnam

a. NDARO db version 2025-07-16, accessed on 12 July 2025.

2.4.4. Role of mobile genetic elements in the dissemination of *mcr* genes

A critical factor enabling the widespread dissemination of *mcr* genes is their association with MGEs, particularly plasmids (Mmatli *et al*., 2022). Plasmids play a pivotal role in the HGT of *mcr* genes across bacterial species and ecological niches, facilitating their persistence and spread in diverse environments (Biswas, 2024; Zhang *et al*., 2021). More than 20 plasmid types carrying *mcr* genes have been reported worldwide (**Table 2**). Conjugative plasmids - especially those from incompatibility groups IncI2, IncX4, IncP, IncX, and IncFII - enhance the ability of *mcr* genes to circulate among bacterial populations in humans, animals, and environmental settings (Luo *et al*., 2020; Liu *et al*., 2024; Zhang *et al*., 2021).

The high transferability of these plasmids allows *mcr* genes to spread not only within bacterial species but also between species, having been detected in various pathogenic *Enterobacteriaceae*, including *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, and *Enterobacter cloacae*, thereby compounding the challenge of colistin resistance management (**Table 2**) (El-Sayed Ahmed *et al*., 2020; Liu *et al*., 2024). In addition to plasmids, insertion sequences, transposons and integrons also play key roles in the mobilization and stabilization of these resistance determinants (Luo *et al*., 2020). Among them, *mcr-1* is frequently associated with one or two copies of the insertion sequence *ISApl1* within IncI2, IncHI2, and IncX4 plasmids, enabling transposition. Over time, however, genetic rearrangements - including the loss of flanking *ISApl1* elements - have contributed to the stabilization of *mcr* genes in diverse plasmid backgrounds (El-Sayed Ahmed *et al*., 2020; Luo *et al*., 2020). Although *mcr* genes are primarily plasmid-borne, sporadic reports of their integration into the bacterial chromosomes suggest a potential for long-term persistence within bacterial populations (Luo *et al*., 2020; El-Sayed Ahmed *et al*., 2020).

The *mcr* genes, particularly when located on high-copy-number plasmids, impose a fitness cost on bacterial hosts. Overexpression of *mcr*-1 has been shown to impair bacterial viability by disrupting cell membrane integrity, altering key metabolic pathways, and imposing an energy burden on the host, resulting in cytotoxic effects and reduced bacterial growth rates (Liu *et al*., 2024; Mmattli *et al*., 2022). Studies have shown that maintaining *mcr-1*-carrying plasmids at low copy numbers and promoting efficient conjugative transfer help stabilize plasmid carriage over time. These adaptations enable *mcr-1*-carrying plasmids to persist and disseminate despite their initial detrimental effects on bacterial fitness (Mmattli *et al*., 2022; Liu *et al*., 2024). IncX4 and IncI2 plasmids are the main vectors of *mcr* genes, being their predominance linked to high conjugation efficiency, small plasmid size, and low fitness cost,

which favour stable maintenance in *E. coli* and *K. pneumoniae* (Sun *et al*., 2017; Matamoros *et al*., 2017). Consequently, IncX4 and IncI2 plasmids act as highly effective vehicles for the global spread of colistin resistance across human, animal, and environmental bacterial populations (Liu *et al.*, 2016; Sun *et al.*, 2017; Manageiro *et al*., 2020). In addition to facilitating *mcr* gene transfer, plasmids often co-harbour other antimicrobial resistance determinants, contributing to the emergence of MDR bacterial strains that further complicate treatment options (Luo *et al*., 2020; Zhang *et al*., 2021). Many *mcr*-carrying plasmids also carry resistance genes for β-lactams, carbapenems, quinolones, tetracyclines, and phenicols, thereby promoting resistance to multiple antibiotic classes (El-Sayed Ahmed *et al*., 2020; Luo *et al*., 2020). Also, metal and biocide resistance determinants, including those for zinc, copper, silver and organic acids, have been identified on *mcr*-carrying plasmids (Khine *et al*., 2023). This is particularly concerning, as the use of non-polymyxin antibiotics following colistin ban can exert selective pressure that maintains and disseminates *mcr* genes within bacterial populations (Luo *et al*, 2020; Khine *et al*., 2023). Given these co-selection dynamics, the persistence and spread of *mcr* genes may not depend solely on colistin use but could also be driven by resistance to other antimicrobial agents, such metals and disinfectants (Zhang *et al*., 2021, El-Sayed Ahmed *et al*., 2020). The emergence and subsequent global spread of *mcr* genes have raised significant concerns due to their contribution to colistin resistance, a last-resort antibiotic for treating MDR infections (Liu *et al*., 2024). The widespread detection of these genes across diverse geographic regions and sectors - including livestock, food products, clinical settings, and the environment - suggests that their transmission is driven by multiple factors. These include not only the mobility of *mcr*-carrying-plasmids but also the extensive use of colistin and other antimicrobial agents in food-animal production and the role of environmental reservoirs.

2.5. Food-animal production and its reliance on colistin

Food-animal production, particularly intensive farming, has historically relied on the use of antibiotics for therapeutic, prophylactic, and metaphylactic purposes, as well as for growth promotion in several countries, including EU member states until 2006 (EC, 2005; Jansen *et al*., 2022; Olaitan *et al*., 2021; Binsker *et al*., 2022). These practices in modern food-animal production have emerged in response to the growing challenges posed by population expansion, limited resources, and land constraints, leading to the intensification of food-production systems. Such systems are characterized by high-density housing, rapid turnover, selective breeding, high levels of biosecurity, preventive veterinary medical approaches, optimized nutritional density and digestive efficiency, and mechanized herd management, all achieved at lower cost and in a more sustainable manner (Cronin *et al*., 2014; Robertson,

2020). In this production systems, efficiency is maximized through climate-controlled environments, automated feed and water supply and accelerated growth cycles, with poultry and rabbit farming serving as prominent examples. In poultry systems, genetically selected broilers are reared over 35–45-day cycles in large barns and maintained at high stocking densities, despite the use of all-in/all-out strategies to limit disease spread (Mak *et al*., 2022). Rabbit production often operates under continuous systems, where breeding females remain housed for up to 1.5 years alongside their offspring, leading to prolonged contact in confined environments (van der Sluis *et al*., 2024).

This management approach, characterized by high-density and stress-prone conditions, combined with the gastrointestinal vulnerability of monogastric animals, increases pathogen exposure and promotes the emergence of bacterial infections, particularly from GNB such as *Salmonella enterica* and pathogenic *Escherichia coli* (Jansen *et al*., 2022; FAO, 2016; Caneschi *et al*., 2023). Gastrointestinal diseases remain a major cause of morbidity and mortality in swine, poultry, and rabbits, with *E. coli* being the most common etiological agent (Castro *et al*., 2022; dos Santos *et al*., 2024; Sargeant *et al*., 2019). Colibacillosis leads to diarrhoea, poor growth, and high mortality in piglets and neonatal rabbits, while in poultry it often causes systemic infections and increased deaths (Fairbrother & Nadeau, 2019; Nolan *et al*., 2013).

Despite improvements in biosecurity, vaccination, nutrition, and housing conditions, bacterial infections persist, making antimicrobial therapy a central component of disease management (Kasimanickam *et al*., 2021; FAO, 2016; EMA & EFSA, 2017; EC, 2005; Van Boeckel *et al*., 2015). Among the therapeutic options, colistin emerged as a key agent, widely used in swine, poultry, veal calves, and rabbits (Binsker *et al*., 2022; Jansen *et al*., 2022). Several factors contributed to this reliance on colistin: its potent bactericidal activity against a broad spectrum of GNB, poor systemic absorption (enabling localized intestinal action), the increasing resistance of pathogens to other antibiotic classes, reduced efficacy of alternative treatments, and the initial assumption that colistin resistance would be limited to chromosomal mutations (Binsker *et al*., 2022; EMA & EFSA, 2017; Jansen *et al*., 2022). As a result, colistin was extensively used in several countries for decades. However, the discovery of *mcr* genes in 2015 profoundly changed the scientific understanding and the regulatory approach to colistin use, as these genes were rapidly detected worldwide across multiple sectors and host species, including humans (Liu *et al*., 2016; Binsker *et al*., 2022; Olaitan *et al*., 2021).

2.6. Implemented measures in food-animal production to combat colistin resistance

The cross-sectoral impact of colistin-resistant bacteria - primarily *mcr*-carrying strains - originating from food-animal production, together with the global detection of *mcr* genes, has prompted widespread efforts to curb colistin use in livestock. These actions form part of broader AMR mitigation strategies aimed at preserving the efficacy of colistin as a last-resort antibiotic (Binsker *et al*., 2022; Caneschi *et al*., 2023). International organizations such as the WHO, the WOAH, and the Food and Agriculture Organization (FAO) have issued strong recommendations to phase out polymyxin as growth promoters and to restrict their use to targeted therapeutic applications under strict veterinary supervision (WHO, 2017; Binsker *et al*., 2022; Jansen *et al*., 2022; Rhouma *et al*., 2023).

Several HICs have responded proactively, especially the EU, which has implemented one of the most comprehensive regulatory frameworks for managing polymyxin use in veterinary medicine (Jansen *et al*., 2022). The first major step came in 2006 with Regulation (EC) No 1831/2003, banning the use of all antibiotics for growth promotion. Following the discovery of *mcr* genes, the EMA (European Medicines Agency) issued guidance in 2016 to reduce veterinary sales of polymyxin by 65%, setting a recommended threshold of no more than 5 mg per Population Correction Unit (PCU) (**Figure 9**) (EMA & EFSA, 2017). Importantly, these reductions were to be achieved without increasing the use of other critically important antimicrobials, such as fluoroquinolones or third- and fourth-generation cephalosporins (Jansen *et al*., 2022). In this way, EFSA (European Food Safety Authority) and EMA recommend a multifaceted approach to reduce colistin use in food animal production, highlighting biosecurity, vaccination, and optimized farm management to lower infection pressure (EMA & EFSA, 2017). Nutritional interventions - such as probiotics, prebiotics, organic acids, immunomodulators, and metals - are recommended to support gut health and promote growth, serving as potential alternatives to colistin (EMA & EFSA, 2017). Among these, the use of zinc and copper as antimicrobial substitutes has gained prominence due to their beneficial effects on intestinal function and productivity (Song *et al*., 2015; Gao *et al*., 2020). However, high-dose administration of these metals raises environmental concerns and may contributed to AMR through co-selection (EMA & EFSA, 2017). Notably, genes coding for decreased susceptibility to metals such as copper (*sil*±*pco*) have been identified on *mcr*-carrying plasmids, suggesting that metal exposure could facilitate the persistence and dissemination of colistin resistance within microbial communities (Khine *et al*., 2023). To mitigate environmental accumulation and co-selection risks, health agencies recommend the use of organic trace metals due to their higher bioavailability and lower required dosages (EMA

& EFSA, 2017). Further regulatory advances were established through Regulation (EU) 2019/6 and Regulation (EU) 2019/4, mandating veterinary prescription for all antibiotic use in animals and banning prophylactic group treatments (Jansen *et al*., 2022; Caneschi *et al*., 2023). These measures have required significant restructuring within the food-animal production sector, including the implementation of alternative strategies to reduce antimicrobial use while maintaining productivity, animal health and welfare (Binsker *et al*., 2022).

In 2020, colistin was formally classified by EMA as a Category B 'Restrict' antimicrobial - alongside fluoroquinolones and third- and fourth-generation cephalosporins - only permitted when no effective alternative exists, and only following susceptibility testing (**Figure 9**) (Jansen *et al*., 2022; Caneschi *et al*., 2023; Binsker *et al*., 2022). Complementary to these restrictions, the EU also implemented mandatory AMR surveillance programs to monitor trends in colistin resistance in food-producing animals and along the food chain (Jansen *et al*., 2022; Binsker *et al*., 2022). These coordinated actions contributed to a substantial decline in polymyxin sales in the EU - an 81% reduction from 11 mg/PCU to 2.1 mg/PCU between 2011 and 2022 (ECDC/EFSA/EMA, 2024). Nevertheless, variability persists between member states, with sales ranging from 0 mg/PCU to 10.2 mg/PCU. In Portugal, the reduction in polymyxin sales has mirrored the EU trend, with a 76.5% decline between 2011 and 2022, from 7.8 mg/PCU to 1.8 mg/PCU in food-producing animals (EMA, 2023). This was largely achieved through the voluntary adoption of antimicrobial reduction programs led by the animal production sector (Ribeiro *et al*., 2021).

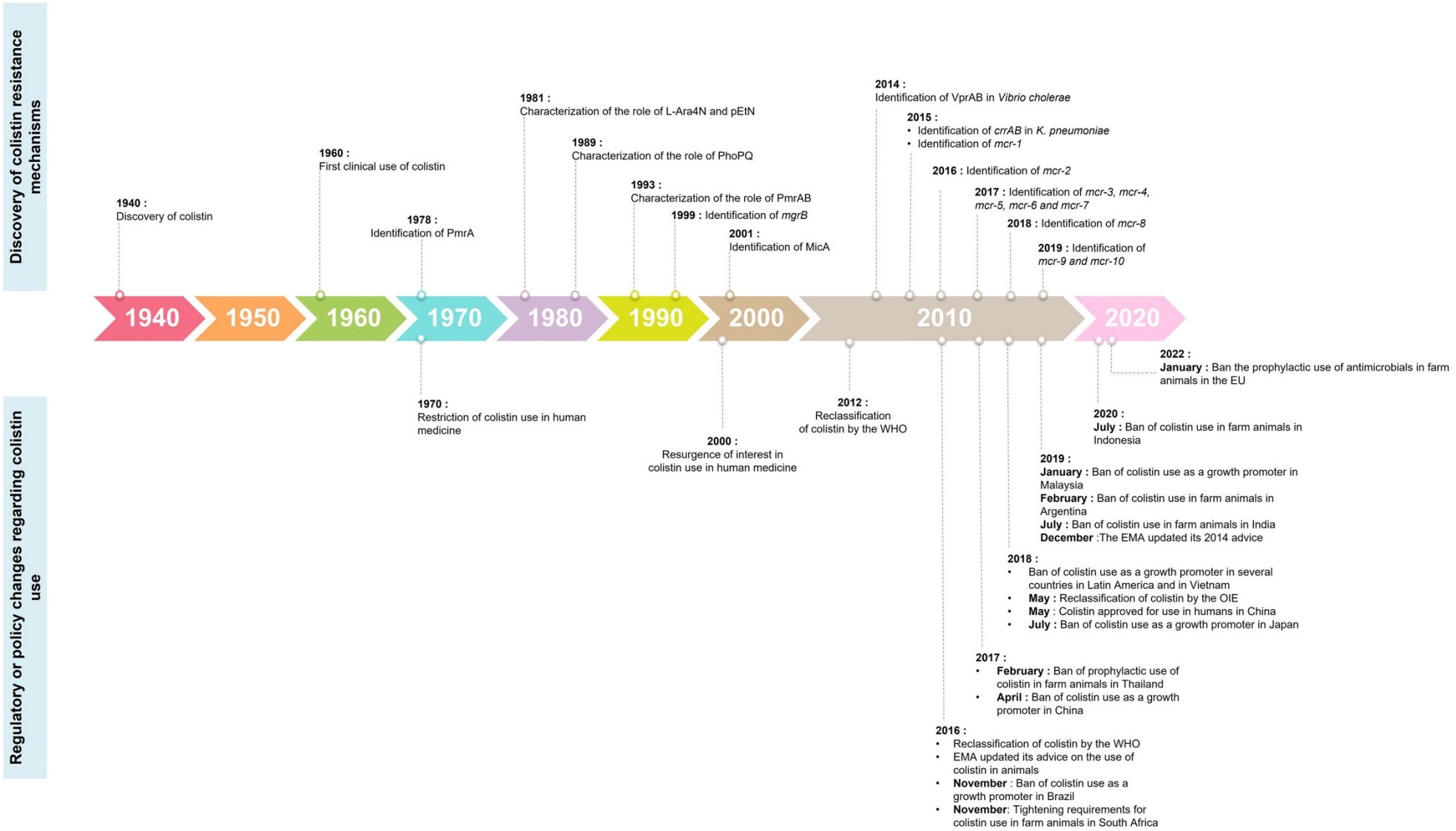

Figure 9. Colistin resistance and policy measures applied (Rhouma *et al.,* 2023).

In general, these measures have been associated with a significant decline in the prevalence of colistin-resistant *E. coli, particularly mcr-1*-positive isolates from both animal and human sources (Rhouma *et al*., 2023). However, their impact on environmental reservoirs remains largely unexplored. Few studies have investigated the persistence and evolution of colistin-resistant bacteria or *mcr* genes within farm environments following the implementation of these regulations. Moreover, the long-term effectiveness and sustainability of such policies require ongoing assessment (Rhouma *et al*., 2023; Binsker *et al*., 2022).

In many LMICs, however, the regulation and monitoring of polymyxins use remains inconsistent and often inadequate. Across Asia, Africa, and Latin America, colistin continues to be used without veterinary prescription, including for growth promotion, prophylaxis, and metaphylaxis (Binsker *et al*., 2022; WHO, 2017). While some countries - such as India and Vietnam - have begun to implement restrictions such as banning colistin as a growth promoter, enforcement is limited, and surveillance systems are still underdeveloped (Hennessey *et al*., 2025; Binsker *et al*., 2022).

This global disparity in antimicrobial stewardship poses a significant One Health challenge. Colistin resistance spreads across borders via food trade, movement of live animals, environmental dissemination, and human travel. As such, gaps in control in some countries undermine global progress against AMR (Binsker *et al*., 2022; Rhouma *et al*., 2023). Furthermore, several HICs continue to export colistin to LMICs, where its uncontrolled use contributes to ongoing selection and spread of resistance (Ardakani *et al*., 2023). Addressing this situation requires globally coordinated strategies, including sustained investment in surveillance, veterinary infrastructure, and regulatory capacity in LMICs (Binsker *et al*., 2022; Caneschi *et al*., 2023).

Although the use of some antimicrobials has been restricted exclusively to human medicine, colistin remains authorized in veterinary medicine, due to its role as a last-resort treatment for severe MDR infections in animals. Accordingly, colistin has not been included by EMA in the list of antimicrobials reserved exclusively for human use (EMA, 2019), underscoring the need for sustainable alternatives to reduce reliance, while ensuring animal health and food system resilience.

2.7. Implemented measures for colistin use in Human Medicine

Despite mitigation efforts in food-animal production, human exposure to colistin-resistant bacteria remains a significant public health concern, particularly due to the risk of severe systemic infections with limited therapeutic options (Barlaam *et al*., 2019). In this way, colistin presents a critical role against MDR GNB, been progressively recognized as a last-line treatment for life-threatening infections such as bloodstream infections caused by carbapenem-resistant Enterobacterales and other MDR pathogens. In response to these risks - alongside growing clinical reliance on colistin and the rising burden of AMR - several international health authorities and agencies have developed regulatory frameworks to reduce unnecessary polymyxin use, promote responsible prescribing, and strengthen AMR surveillance in the human health sector (Caneschi *et al*., 2023; Binsker *et al*., 2022). Among these initiatives, WHO launched in 2015, the Global Antimicrobial Resistance and Use Surveillance System (GLASS), playing a key role by collecting standardized global data on AMR and antibiotic consumption to inform evidence-based policy and clinical interventions (WHO, 2015b). In 2016, the WHO classified colistin as a 'Highest Priority Critically Important Antimicrobial' (HPCIA) for human medicine (WHO, 2024b; Rhouma *et al*., 2023). Following HPCIA designation, its use has been restricted to confirmed MDR-GNB infections with no available alternatives, while empirical or prophylactic use is strongly discouraged to preserve its efficacy (Andrade *et al*, 2020; WHO, 2024b). In alignment with international recommendations, antimicrobial stewardship programs have been implemented in many countries to restrict colistin use, typically to intensive care settings and severe infections such as bacteraemia, sepsis, and pneumonia, guided by susceptibility testing (WHO, 2015a; CDC, 2019; Giamarellou *et al*., 2023; Tamma *et al*., 2024). Nonetheless, colistin consumption in human healthcare has increased significantly in Europe between 2013 and 2022. Seven countries, including Portugal, reported significant rises. From 2020 to 2022, Portugal, Greece, and Spain consistently ranked among the top three EU countries in hospital polymyxin use - a trend that raised concerns about the potential for further AMR development, ultimately threatening the long-term viability of one of the last-resort antibiotics (ECDC, 2024; Andrade *et al*., 2020).

2.8. One Health dimensions of colistin resistance

Despite growing attention to *mcr* genes in clinical and food-animal production settings, the role of the environment and wildlife as reservoirs and vectors of colistin resistance remains critically understudied. Farm environments, through manure, wastewater, and contaminated bedding,

can serve as sources of environmental dissemination, while wildlife, including migratory species, may further contribute to the spread across ecosystems (UNEP, 2023; Bengtsson-Palme *et al*., 2018; Arnold *et al*., 2016). The food chain represents an important route of human exposure, with resistant bacteria entering through contaminated meat, crops, or water used in food production (Barlaam *et al*., 2019). The presence of *mcr* genes in soil and plants has been documented in Asia, South America, and Europe (Manageiro *et al*., 2020; Oliveira *et al*., 2019; Anyanwu *et al*., 2020). Surveillance and research in these non-clinical sectors remain limited, hindering our understanding of how colistin resistance emerges and circulates beyond farms (Bengtsson-Palme *et al*., 2018; UNEP, 2023; Rhouma *et al*., 2023).

The increasing reliance on colistin as a last-resort antibiotic in human medicine, combined with the circulation of *mcr* genes in food-producing animals and environmental reservoirs, represents a critical One Health challenge. This convergence elevates the risk of zoonotic and environmental transmission, threatening the long-term efficacy of this essential therapeutic option. Addressing this issue requires a comprehensive understanding of how agricultural practices, environmental reservoirs, and cross-resistance mechanisms collectively sustain *mcr* gene dissemination, in order to develop integrated strategies to safeguard colistin's clinical utility.

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CHAPTER 2 – Objectives and Outline of the Study

1. Statement of objectives

Supporting the hypothesis that *mcr* genes originated under the selective pressure imposed by colistin use in livestock farming, this study focuses on tracking colistin-resistant Enterobacterales across food-animal production systems, which represent a critical environment for the emergence and dissemination of colistin resistance. Intensive rearing practices in poultry and rabbit production further amplify selective pressures, fostering conditions that facilitate pathogen circulation and the spread of resistance due to high animal densities and the associated increased risk of infectious diseases, undermining the implementation of effective biosecurity measures. Although significant progress has been achieved in reducing colistin use through national and international policies and antimicrobial stewardship programs, **important challenges remain**. Of particular concern are the **long-term microbiological and ecological consequences of banning colistin in food-animal production**, and the **potential for other antimicrobials to sustain the dissemination of colistin-resistant bacteria, including *mcr*-carrying strains, via mechanisms of co-resistance and co-selection**. Moreover, **the environmental dimension of colistin resistance remains insufficiently explored**, with limited data on the persistence, spread, and evolution of *mcr* genes in soil, water, and wildlife. This constrains our understanding of their circulation across ecosystems. These knowledge gaps underscore the need for integrated, longitudinal research to unravel the interplay between antimicrobial practices and resistance dynamics in both agricultural and environmental contexts. As colistin resistance moves from farms to rivers, from food to hands, and from animals to humans, the case for a comprehensive **One Health framework** becomes increasingly compelling.

1.1. Study Hypothesis

The persistence of colistin-resistant bacteria, particularly those carrying *mcr* genes, in food-animal and related environmental systems is **hypothesized** to be sustained by co-selection pressures from other antimicrobials or metals, and by the contribution of multiple environmental reservoirs and transmission routes acting as interconnected drivers within a One Health framework.

1.2. General and specific objectives

The **general objective** of this thesis was to investigate the persistence and dissemination of colistin-resistant Enterobacterales, particularly *mcr*-carrying strains, in intensive poultry and rabbit production systems following the EU colistin ban. A One Health framework was adopted to identify and characterize multiple local and global drivers of colistin persistence and spread,

including co-selection pressures (antimicrobials and metals), environmental reservoirs, and transmission routes, that sustain their maintenance and spread, linking food-animal production, commercial pet food, and wildlife.

To achieve this goal, a One Health approach was applied, combining culturomics and genomics with sampling from food-animal production (n=180), commercial pet food (n=55), and wild gulls (n=14). Colistin-resistant *E. coli* and *K. pneumoniae* isolates (n=157) from these sources were characterized for genetic lineages, antimicrobial resistance determinants, mobile genetic elements (plasmids) and metal decreased susceptibility genes. Initial analyses in poultry and rabbit production provided insight into the persistence and dissemination of colistin resistance within food-animal systems following the colistin ban, while subsequent assessments of commercial pet food containing animal by-products and wild birds evaluated their potential role as external reservoirs and potential dissemination sources of colistin-resistant bacteria and *mcr* genes.

The **specific objectives** were as follows:

1 - To evaluate whether colistin withdrawal in poultry and rabbit production has led to the elimination of colistin-resistant Enterobacterales, including *mcr*-carrying strains, over time.

2 - To determine the occurrence, diversity and genomic characteristics of colistin-resistant *E. coli* and *K. pneumoniae* isolates recovered from poultry and rabbit production systems at multiple time points (short- and long-term) following colistin withdrawal.

3 - To identify and characterize potential drivers of colistin resistance persistence, including co-selection pressures (copper feed supplementation, use of other antimicrobials), environmental reservoirs (water, litter/nest material, feed), and farm management practices (vacancy periods, sanitation protocols). In addition, to determine their association with the maintenance and dissemination of antimicrobial resistant clones and plasmids within farms.

4 - To assess the presence, diversity and genetic context of colistin-resistant and *mcr*-carrying Enterobacterales in commercial pet food and in wild migratory birds, and to evaluate their potential role as reservoirs and vectors for the maintenance and dissemination of *mcr* genes.

5 - To integrate genomic, epidemiological and environmental data across food-animal production, commercial pet food, and wildlife, to elucidate cross-sector transmission pathways from local to broader environmental drivers, identify shared clones and plasmids between

different sectors, and determine the key drivers sustaining colistin resistance within a One Health framework.

2. Outline of the thesis

The research carried out to meet the specific goals of this thesis produced results organized in four scientific papers, all published in international peer-reviewed journals. Together, these studies provide a comprehensive view of colistin resistance dynamics across food production, the environment and public health, highlighting the interplay of local and global drivers within a One Health framework. The scientific data of this thesis were structured across the papers, according to the following rationale:

I. Influence of colistin withdrawal, farm management and co-selection drivers on the occurrence, diversity, and genomic features of colistin-resistant *E. coli* and *K. pneumoniae* across poultry and rabbit production systems

a. Mourão, J., **Ribeiro-Almeida, M.**, Novais, C., Magalhães, M., Rebelo, A., Ribeiro, S., Peixe, L., Novais, A., & Antunes, P. (2023). From farm to fork: Persistence of clinically relevant multidrug-resistant and copper-tolerant *Klebsiella pneumoniae* long after colistin withdrawal in poultry production. *Microbiology Spectrum*, *11*(4), e01386-23. doi: 10.1128/spectrum.01386-23

b. **Ribeiro-Almeida, M.**, Mourão, J., Rodrigues, I. C., de Carvalho, A. P., da Costa, P. M., Peixe, L., & Antunes, P. (2025). Persistence of *mcr-1*-carrying *E. coli* in rabbit meat production: Challenges beyond long-term colistin withdrawal. *International Journal of Food Microbiology*, 111248. doi: 10.1016/j.ijfoodmicro.2025.111248

II. Commercial raw-meat based pet food and wildlife as sources and reservoirs of colistin-resistant and *mcr*-carrying Enterobacterales

a. **Ribeiro-Almeida, M.**, Mourão, J., Magalhães, M., Freitas, A. R., Novais, C., Peixe, L., & Antunes, P. (2024). Raw meat-based diet for pets: a neglected source of human exposure to *Salmonella* and pathogenic *Escherichia coli* clones carrying *mcr*, Portugal, September 2019 to January 2020. *Eurosurveillance*, *29*(18), 2300561. doi: 10.2807/1560-7917.ES.2024.29.18.2300561

b. **Ribeiro-Almeida, M.**, Mourão, J., Novais, Â., Pereira, S., Freitas-Silva, J., Ribeiro, S., da Costa, P. M., Peixe, L., & Antunes, P. (2022). High diversity of pathogenic *Escherichia coli* clones carrying *mcr-1* among gulls underlines the need for strategies at the environment–livestock–human interface. *Environmental Microbiology*, *24*(10), 4702-4713. doi: 10.1111/1462-2920.16111

CHAPTER 3 – Results and Discussion

I. Influence of colistin withdrawal, farm management and co-selection drivers on the occurrence, diversity, and genomic features of colistin-resistant *E. coli* and *K. pneumoniae* across poultry and rabbit production systems

1. Mourão, J., **Ribeiro-Almeida, M.**, Novais, C., Magalhães, M., Rebelo, A., Ribeiro, S., Peixe, L., Novais, A., & Antunes, P. (2023). From farm to fork: Persistence of clinically relevant multidrug-resistant and copper-tolerant *Klebsiella pneumoniae* long after colistin withdrawal in poultry production. *Microbiology Spectrum*, *11*(4), e01386-23. doi: 10.1128/spectrum.01386-23

2. **Ribeiro-Almeida, M.**, Mourão, J., Rodrigues, I. C., de Carvalho, A. P., da Costa, P. M., Peixe, L., & Antunes, P. (2025). Persistence of *mcr-1*-carrying *E. coli* in rabbit meat production: Challenges beyond long-term colistin withdrawal. *International Journal of Food Microbiology*, 111248. doi: 10.1016/j.ijfoodmicro.2025.111248

1. From farm to fork: Persistence of clinically relevant multidrug-resistant and copper-tolerant *Klebsiella pneumoniae* long after colistin withdrawal in poultry production

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From Farm to Fork: Persistence of Clinically Relevant Multidrug-Resistant and Copper-Tolerant *Klebsiella pneumoniae* Long after Colistin Withdrawal in Poultry Production

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ABSTRACT Concerns about colistin-resistant bacteria in animal food-environmental-human ecosystems prompted the poultry sector to implement colistin restrictions and explore alternative trace metals/copper feed supplementation. The impact of these strategies on the selection and persistence of colistin-resistant *Klebsiella pneumoniae* in the whole poultry production chain needs clarification. We assessed colistin-resistant and copper-tolerant *K. pneumoniae* occurrence in chickens raised with inorganic and organic copper formulas from 1-day-old chicks to meat (7 farms from 2019 to 2020), after long-term colistin withdrawal (>2 years). Clonal diversity and *K. pneumoniae* adaptive features were characterized by cultural, molecular, and whole-genome-sequencing (WGS) approaches. Most chicken flocks (75%) carried *K. pneumoniae* at early and preslaughter stages, with a significant decrease ($P < 0.05$) in meat batches (17%) and sporadic water/feed contamination. High rates (>50%) of colistin-resistant/*mcr*-negative *K. pneumoniae* were observed among fecal samples, independently of feed. Most samples carried multidrug-resistant (90%) and copper-tolerant (81%; *silA* and *pcoD* positive and with a MIC_{CuSO4} of ≥16 mM) isolates. WGS revealed accumulation of colistin resistance-associated mutations and F type multireplicon plasmids carrying antibiotic resistance and metal/copper tolerance genes. The *K. pneumoniae* population was polyclonal, with various lineages dispersed throughout poultry production. ST15-KL19, ST15-KL146, and ST392-KL27 and IncF plasmids were similar to those from global human clinical isolates, suggesting chicken production as a reservoir/source of clinically relevant *K. pneumoniae* lineages and genes with potential risk to humans through food and/or environmental exposure. Despite the limited *mcr* spread due to the long-term colistin ban, this action was ineffective in controlling colistin-resistant/*mcr*-negative *K. pneumoniae*, regardless of feed. This study provides crucial insights into the persistence of clinically relevant *K. pneumoniae* in the poultry production chain and highlights the need for continued surveillance and proactive food safety actions within a One Health perspective.

IMPORTANCE The spread of bacteria resistant to last-resort antibiotics such as colistin throughout the food chain is a serious concern for public health. The poultry sector has responded by restricting colistin use and exploring alternative trace metals/copper feed supplements. However, it is unclear how and to which extent these changes impact the selection and persistence of clinically relevant *Klebsiella pneumoniae* throughout the poultry chain. We found a high occurrence of copper-tolerant and colistin-resistant/*mcr*-negative *K. pneumoniae* in chicken flocks, regardless of inorganic and organic copper formulas use

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and a long-term colistin ban. Despite the high *K. pneumoniae* isolate diversity, the occurrence of identical lineages and plasmids across samples and/or clinical isolates suggests poultry as a potential source of human *K. pneumoniae* exposure. This study highlights the need for continued surveillance and proactive farm-to-fork actions to mitigate the risks to public health, relevant for stakeholders involved in the food industry and policymakers tasked with regulating food safety.

KEYWORDS chicken meat, poultry sector, copper feed supplementation, antibiotic resistance, IncF plasmids, One Health, food safety

A major public health concern has been attributed to colistin-resistant bacteria, particularly those carrying mobilized colistin resistance (*mcr*) genes, due to implications for human, animal, and environmental health (1, 2). The wide use of colistin for decades in veterinary medicine imposed global restrictions on food animal production (3–5). Colistin in the European Union is categorized as "B—Restrict," which is reserved for clinical infections for which no alternatives are available, in compliance with 2022 regulations that ban routine antibiotic use in farming. Additionally, colistin restrictions align with the 2030 European goal of reducing farm animal antibiotic sales by 50%, with consequential effects on diverse environmental compartments (6–10). Diverse studies revealed a decreasing occurrence of *mcr*-carrying bacteria (mainly *Escherichia coli* and *Klebsiella pneumoniae*) in farms shortly after colistin restrictions (11, 12), whereas the persistence of colistin-resistant bacteria by mechanisms other than *mcr* is unknown. Furthermore, there are no studies evaluating the long-term impact of colistin restriction on the occurrence and diversity of colistin-resistant bacteria throughout food animal production environments and/or considering farm-level variation or food chain practices (4, 11). Studies on intensive poultry meat systems are crucial for informing strategies to reduce or replace antibiotics, given the European Union's prominent position as one of the top four global chicken meat producers and the increasing consumer demand for "greener" antibiotic-free food products (13–15).

Along the same line as colistin restriction is the development of nutritional alternatives to improve poultry health and growth, including the use of copper (Cu) in feeds (3, 15–17). Copper is a heavy metal widely used in poultry feed due to its requirement in many physiologic processes, immune-stimulating actions, and antimicrobial activity in the gut (15, 18, 19). In current commercial practice, inorganic trace mineral formulation feeds (ITMF) are supplemented with inorganic sources of Cu (e.g., Cu sulfate and Cu oxide) at high levels ($<$25 mg/kg; accordingly to EU Regulation 2018/1039), leading to potential environmental accumulation (20–22). Alternatively, organic trace mineral feed (OTMF) supplements (e.g., Cu chelated with amino acids, peptides, or proteins) have been associated with a higher Cu bioavailability in the digestive tract than that with ITMF and an animal performance improvement, with reduced fecal excretion and environmental release expected (19–23). However, the effect of different Cu-supplemented feeds on gastrointestinal microbiota composition is still unclear and needs to be further explored by experimental studies (18–20, 24). In addition, a link has been proposed between feed supplementation with Cu (and other metals) and the selection of antibiotic-resistant bacteria (24, 25). However, the impact of variable feed formulations (OTMF versus ITMF) on poultry gut bacteria, particularly in species often associated with colistin resistance, and throughout the whole food production chain has been poorly explored.

K. pneumoniae has been frequently associated with colistin resistance and *mcr* carriage in the clinical setting, but its occurrence in the animal food-environmental interface is underexplored (26). Besides, we have previously shown the colocalization of metal/Cu operons with the *mcr-1* gene in plasmids from *K. pneumoniae* isolated from chicken meat batches shortly after colistin withdrawal (12), suggesting a Cu coselection effect for the persistence of colistin resistance. In fact, *K. pneumoniae* is a versatile species with a natural ability to acquire antibiotic and metal resistance genes, enabling their survival and circulation among animal-environment-human ecosystems (27–29). However, it remains unclear how and to what extent restriction/replacement of colistin interventions in poultry production impacts the selection

and persistence of colistin-resistant *K. pneumoniae* (ColR-Kp) and, eventually, clones with a potential risk of foodborne and/or environmental transmission.

We used a One Health approach by spanning the poultry production chain (from 1-day-old chicks to meat) to better understand the impact of the long-term colistin ban on the occurrence of colistin-resistant and Cu-tolerant *K. pneumoniae* and its association with ITMF and OTMF feed formulations. We applied whole-genome sequencing (WGS) to characterize these isolates in terms of clonal diversity, antibiotic resistance, metal tolerance, and virulence determinants to understand the risks that exist in the poultry environment.

RESULTS

Occurrence of *K. pneumoniae* and colistin-resistant *K. pneumoniae* by samples. Our culture approach detected *K. pneumoniae* in 42% (n = 35/84) of the total tested samples within the poultry production chain. A high occurrence was found among fecal samples (82%; 78% [14/18 flocks] at the P1 stage [when chicks were 2 to 3 days old] and 88% [14/16 flocks] at the P2 stage [the day before slaughter]), corresponding in all but two of the flocks and all farms tested. However, a significant decrease in occurrence was detected between stage P2 and chicken meat batches (17% [3/18] at stage P3 [after slaughter], corresponding to flocks from 3 farms) ($P < 0.05$) (Fig. 1A). Only two flocks (2 farms) had *K. pneumoniae* isolates in all three stages (P1, P2, and P3), while the remaining ones were found in stage P1 and P2 (10 flocks) (75% [12/16 flocks] at P1 and P2 and/or P3) or only in stage P1 (1 flock), P2 (2 flocks), or P3 (1 flock). Similar occurrence rates were observed between OTMF and ITMF samples at each of the three sampling stages ($P > 0.05$) (Fig. 1A). Regarding poultry house environmental samples, three feed samples (2 farms) and one water sample (another farm) were contaminated with *K. pneumoniae*.

The ColR-Kp isolates were recovered from all farms and sample types, corresponding to 57% (20/35) of *K. pneumoniae*-positive samples. Most were fecal samples at stage P1 (50% [9/18 flocks]; 5 farms) and P2 (56% [9/16 flocks]; 6 farms), with no significant difference by feed type at the two stages ($P > 0.05$) (Fig. 1A). We noticed in the follow-up of flocks that ColR-Kp occurrence in chicken feces was maintained (n = 5 flocks) or increased (n = 4 flocks) from stage P1 to P2, with no significant difference in the ColR-Kp levels between the two stages ($P > 0.05$) (Fig. 1B). ColR-Kp was detected in only one of the three chicken meat batches contaminated with *K. pneumoniae* obtained from a farm which was also positive at stage P1 and P2. Regarding environmental samples, ColR-Kp was found in one water sample from one farm, also positive at stage P1 and P2. All isolates were negative for the screening of *mcr* genes but presented MICs for colistin ranging between 4 and ≥16 mg/L, which is compatible with the presence of other acquired resistance mechanisms (for results, see Whole-genome analysis of *K. pneumoniae* poultry-associated isolates).

Diversity of *K. pneumoniae* and colistin-resistant *K. pneumoniae* isolates. We recovered 100 *K. pneumoniae* isolates (50 from OTMF and 50 from ITMF flocks), corresponding to 35 positive samples. Of these isolates, 56 were colistin resistant, corresponding to 20 positive samples. Most isolates (90%) were recovered from P1 and P2 fecal samples (16 flocks, all farms) (Fig. 2). *K. pneumoniae* isolates were grouped into 26 capsular locus (KL) - types, with 12 dispersed in more than one farm (Fig. 2; see Table S1 in the supplemental material). Two KL type(s) represented 40% (n = 42) of isolates, independently of feed, and included colistin-resistant isolates KL109, which was widely dispersed (n = 24; 4 farms; P1, P2, and P3; farm B—flock 4 and flock 11 over 4 months), and, in contrast, KL106, which was present only in one farm (n = 18; water, P1 and P2) (Fig. 2). Although less frequent, other KL type(s) were shared by different farms or stages: KL12 (2 farms; P1), KL19 (2 farms; P1, P2, and P3), KL64 (4 farms; P1 and P2), KL111 (2 farms; feed, P1 and P3), KL27 (2 farms; feed and P1) and KL146 (2 farms; P2) (Fig. 2; Table S1). There were 22 different KL type(s) observed among the colistin-susceptible isolates (n = 44), while the colistin-resistant isolates (n = 56) exhibited 13 different KL type(s). Additionally, 9 KL type(s) were found to be present in both groups.

Antibiotic susceptibility of *K. pneumoniae* recovered from poultry production. Considering poultry and meat samples with *K. pneumoniae* (n = 31), antibiotic-resistant isolates were observed in all but one sample. Most of these (90%, n = 28/31) carried

A

Month/Year	Farm	Flock-Feed	P1-Three-day-old flock			P2-Pre-slaughter flock			P3-Poultry meat			Environmental samples (P1)		
			Kp	ColR-Kp	*silA* $^{+}$-Kp	Kp	ColR-Kp	*silA* $^{+}$-Kp	Kp	ColR-Kp	*silA* $^{+}$-Kp	Kp	ColR-Kp	*silA* $^{+}$-Kp
Oct/Nov 2019	A	2-ITM		1000			2100							
Oct/Nov 2019	A	1-OMT		<100			30000			<100				
Oct/Nov 2019	B	4-ITM		<100			18500							
Oct/Nov 2019	B	3-OTM		<100			<100							
Feb/Mar 2020	B	12-ITM		<100			<100							
Feb/Mar 2020	B	11-OTM		440			<100							
Nov/Dec 2019	C	5-ITM		<100			400							
Nov/Dec 2019	C	6-OTM		<100			100							
Nov/Dec 2019	D	8-ITM		12000			<100							
Nov/Dec 2019	D	7-OTM		15000			<100							
Feb/Mar 2020	E	10-ITM		<100		ND	ND	ND						
Feb/Mar 2020	E	9-OTM		<100		ND	ND	ND						
Sep/Oct 2020	E	18-ITM		<100			<100							
Sep/Oct 2020	E	17-OTM		<100			<100							
Jun/Jul 2020	G	14-ITM		<100			760							
Jun/Jul 2020	G	13-OTM		<100			<100							
Jul/Aug 2020	H	16-ITM		<100			10000							
Jul/Aug 2020	H	15-OTM		2460			7140							
Total nº flocks (%)			**14/18 (78%)**	**9/18 (50%)**	**13/18 (72%)**	**14/16 (88%)**	**9/16 (56%)**	**10/16 (63%)**	**3/18 (17%)***	**1/18 (6%)***	**2/18 (11%)**	**4/32 (13%)**	**1/32 (3%)**	**4/32 (13%)**
Total nº ITMF flocks (%)			7/9 (78%)	3/9 (33%)	6/9 (67%)	7/8 (88%)	5/8 (63%)	4/8 (50%)	1/9 (11%)*	0/9 (0%)*	1/9 (11%)	NA	NA	NA
Total nº OTMF flocks (%)			7/9 (78%)	6/9 (67%)	7/9 (78%)	7/8 (88%)	4/8 (50%)	6/8 (75%)	2/9 (22%)*	1/9 (11%)	1/9 (11%)	NA	NA	NA

B

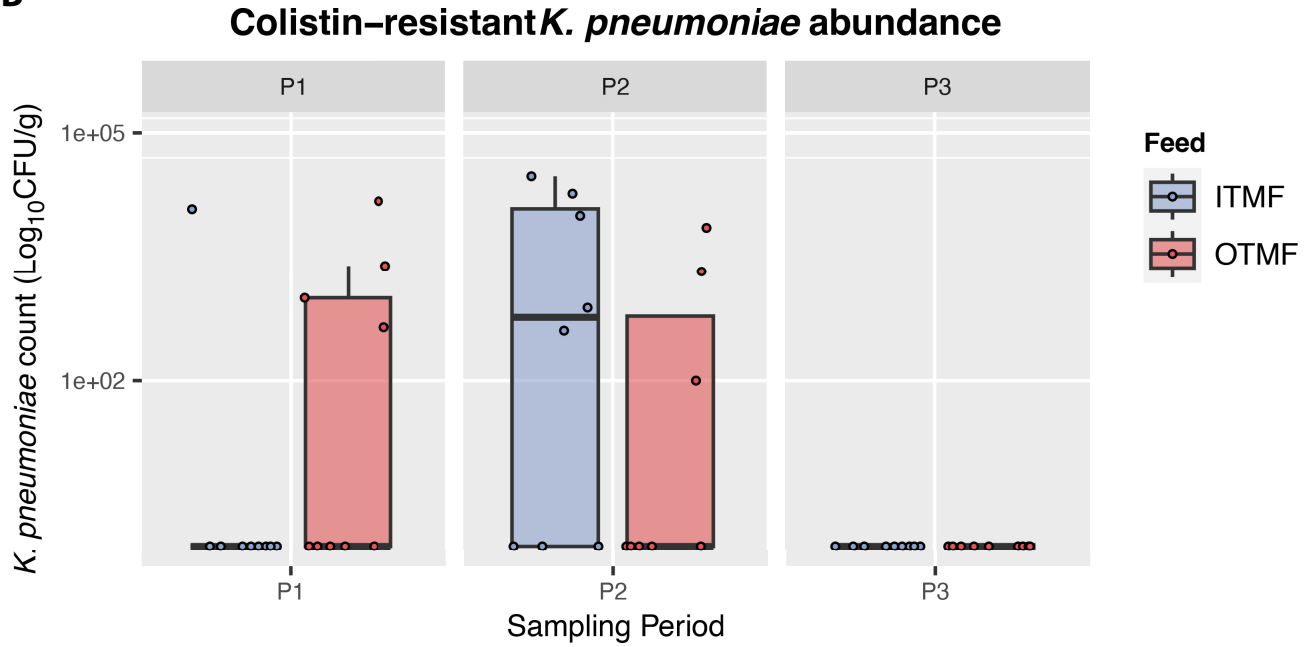

FIG 1 (A) Occurrence of *K. pneumoniae* (Kp-green), colistin-resistant *K. pneumoniae* (ColR-Kp-orange; the numbers represent an estimate [CFU/g] of ColR-Kp), and *silA*-carrying *K. pneumoniae* (silA^{+}-Kp - yellow) among flocks (feces, P1 and P2) and chicken meat (P3) by type of feed (ITMF or OTMF). Environmental samples 5, 7, and 8 were from feed, and sample 15 corresponds to water. *, $P < 0.05$ (Fisher's exact test), when P2 is compared with P3. ND, not determined; NA, not applicable. (B) Box plot of colistin-resistant *K. pneumoniae* counts among flocks (feces, P1 and P2) and chicken meat (P3) by type of feed (ITMF or OTMF). $P > 0.05$ was calculated using the Wilcoxon test for a comparison of P1 with P2 (both feeds). The median is represented by a black horizontal line, and blue (ITMF) or red (OTMF) data points represent the counts in each sample.

multidrug-resistant (MDR) isolates, corresponding to all farms and all but two flocks. Similar MDR and antibiotic resistance rates per sample were observed between both types of feed ($P > 0.05$) (Fig. 3). More than 50% of the samples presented at least one *K. pneumoniae* isolate with decreased susceptibility to ciprofloxacin (90%, $n = 28/31$) or resistance to tetracycline, sulfonamides, or trimethoprim (87%, $n = 27/31$ each), gentamicin (65%, $n = 20/31$), colistin (61%, $n = 19/31$), or chloramphenicol (55%, $n = 17/31$) (Fig. 3). Regarding the environmental samples with *K. pneumoniae* (feed [$n = 5$; 3 samples] and water [$n = 1$; 1 sample]) all carried MDR isolates. More than 15% of samples (including feces, chicken meat, and water) from diverse farms/flocks presented at least one isolate that was resistant to extended-spectrum cephalosporins.

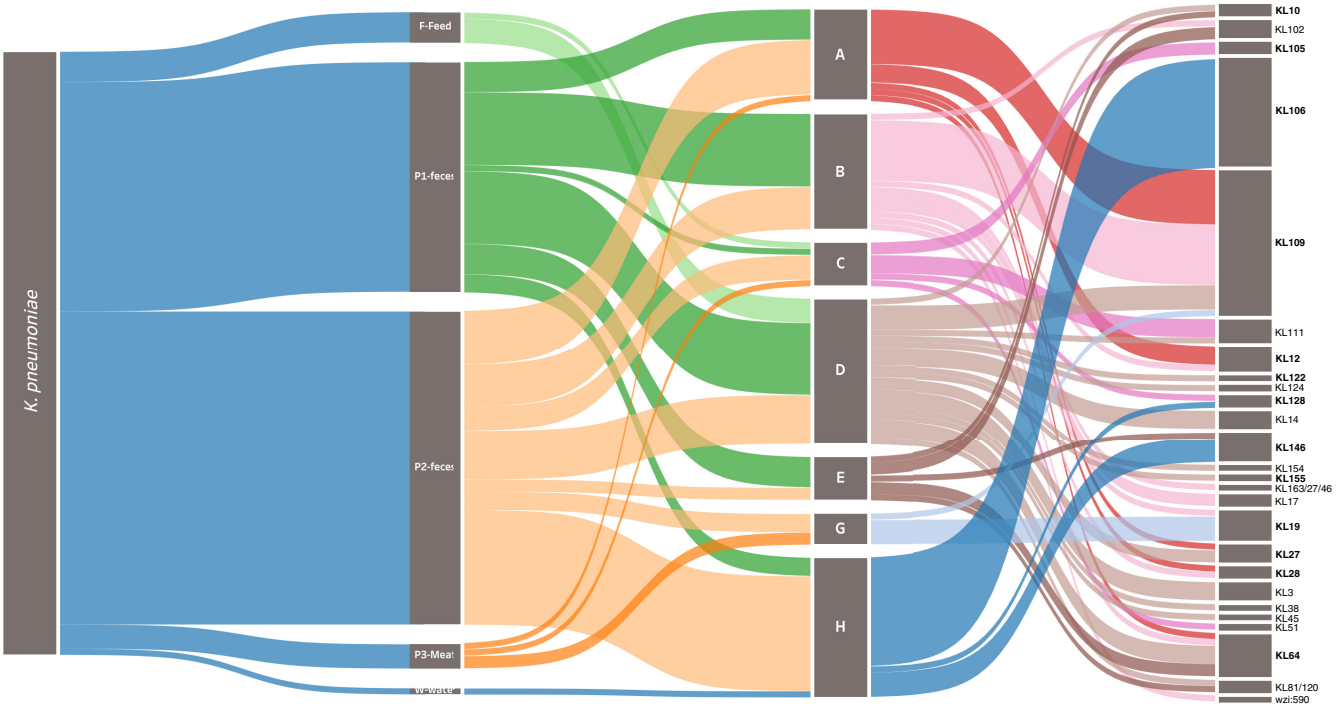

FIG 2 Sankey diagram representing, from left to right, the occurrence and diversity of *K. pneumoniae* isolates by sample, farm, and KL type(s). The width of each connection is proportional to the number of positive hits. The KL type(s) comprising colistin-resistant isolates are indicated in bold. The Sankey diagram was generated using Tableau Desktop 2021.4 (https://www.tableau.com/).

K. pneumoniae isolates resistant to colistin (n = 56/100) were mainly recovered in Simmons citrate agar with 1% inositol (SCAi) medium supplemented with this antibiotic, with or without the previous enrichment step (n = 24 and n = 30, respectively) (Table 1). High resistance rates to ciprofloxacin (86%), tetracycline (79%), sulfonamides (74%), and trimethoprim (75%) were observed as well as MDR (80%) independently of the selection strategy or colistin

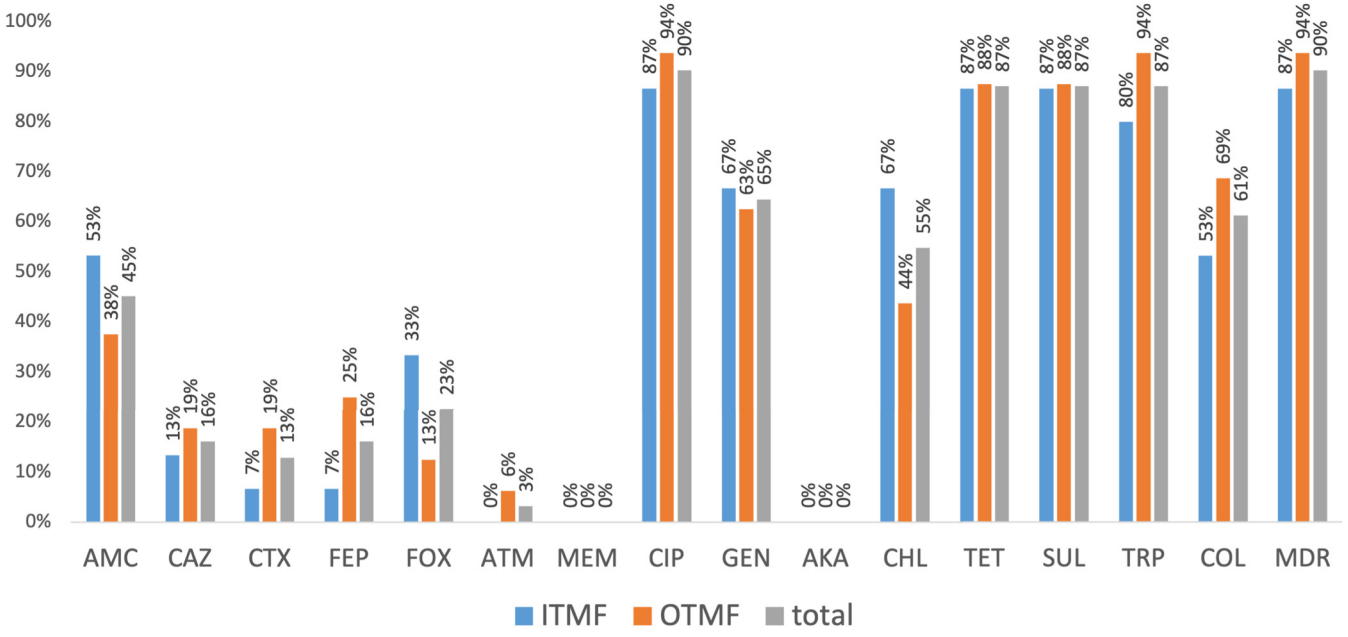

FIG 3 Occurrence of antibiotic-resistant *K. pneumoniae* isolates among positive poultry samples (P1, P2, and P3) by type of feed (ITMF - blue and OTMF - orange). $P > 0.05$ (Fisher's exact test). AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; ATM, aztreonam; MEM, meropenem; CIP, ciprofloxacin; GEN, gentamicin; AKA, amikacin; CHL, chloramphenicol; TET, tetracycline; SUL, sulfonamides; TRP, trimethoprim; COL, colistin; MDR, multidrug resistance.

TABLE 1 Antimicrobial susceptibility of *K. pneumoniae* isolates (n = 100), including colistin-resistant *K. pneumoniae* isolates (n = 56) recovered from poultry production samples

Isolate selection	*K. pneumoniae* (no. of isolates; no. of samples)	Antimicrobial resistance [no. of isolates (%)][a]																
		COL	AMC	CAZ	CTX	FEP	FOX	ATM	MEM	CIP	AKA	GEN	CHL	TET	SUL	TRP	MDR	*silA* and *pcoD* positive
SCAi without enrichment/direct	COL-R (1; 1)	1	1	1	0	0	1	0	0	1	0	1	1	0	1	1	1	2
	COL-S (6; 5)	0	5	0	0	0	1	0	0	6	0	2	4	6	6	6	6	5
	Total (7; 6)	1 (14)	6 (86)	1 (14)	0	0	2 (29)	0	0	7 (100)	0	3 (43)	5 (71)	6 (86)	7 (100)	7 (100)	7 (100)	7 (100)
SCAi with enrichment/BPW	COL-R (1; 1)	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
	COL-S (3; 3)	0	0	0	0	0	0	0	0	3	0	1	2	3	3	3	3	3
	Total (4; 4)	1 (25)	0	0	0	0	0	0	0	4 (100)	0	1 (25)	2 (50)	4 (100)	3 (75)	3 (75)	3 (75)	3 (75)
SCAi plus COL (3.5 μg/mL) without enrichment/direct	COL-R (30; 13)	30	13	9	0	2	13	0	0	28	0	15	14	23	22	21	24	16
	COL-S (21; 18)	0	3	0	1	1	2	0	0	16	0	5	8	14	11	12	15	17
	Total (51; 24)	30 (59)	16 (31)	9 (18)	1 (2)	3 (6)	15 (29)	0	0	44 (86)	0	20 (39)	22 (43)	37 (73)	33 (65)	33 (65)	39 (76)	33 (65)
SCAi plus COL (3.5 μg/mL) with enrichment/BPW	COL-R (24; 14)	24	10	7	2	3	6	0	0	20	0	16	10	21	21	21	21	13
	COL-S (14; 13)	0	3	2	2	1	1	1	0	11	0	4	3	11	10	11	10	11
	Total (38; 21)	24 (63)	13 (34)	9 (24)	4 (11)	4 (11)	7 (18)	1 (3)	0	31 (82)	0	20 (53)	13 (34)	32 (84)	31 (82)	32 (84)	31 (82)	24 (63)
All isolates	Total (100; 35)	56 (56)	35 (35)	19 (19)	5 (5)	7 (7)	24 (24)	1 (1)	0 (0)	86 (86)	0 (0)	44 (44)	42 (42)	79 (79)	74 (74)	75 (75)	80 (80)	67 (67)

[a]COL, colistin; COL-R, colistin-resistant *K. pneumoniae*; COL-S, colistin-susceptible *K. pneumoniae*; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; ATM, aztreonam; MEM, meropenem; CIP, ciprofloxacin; AKA, amikacin; GEN, gentamicin; CHL, chloramphenicol; TET, tetracycline; SUL, sulfonamides; TRP, trimethoprim; MDR, multidrug resistance; SCAi, Simmons citrate agar with 1% inositol; BPW, buffered peptone water.

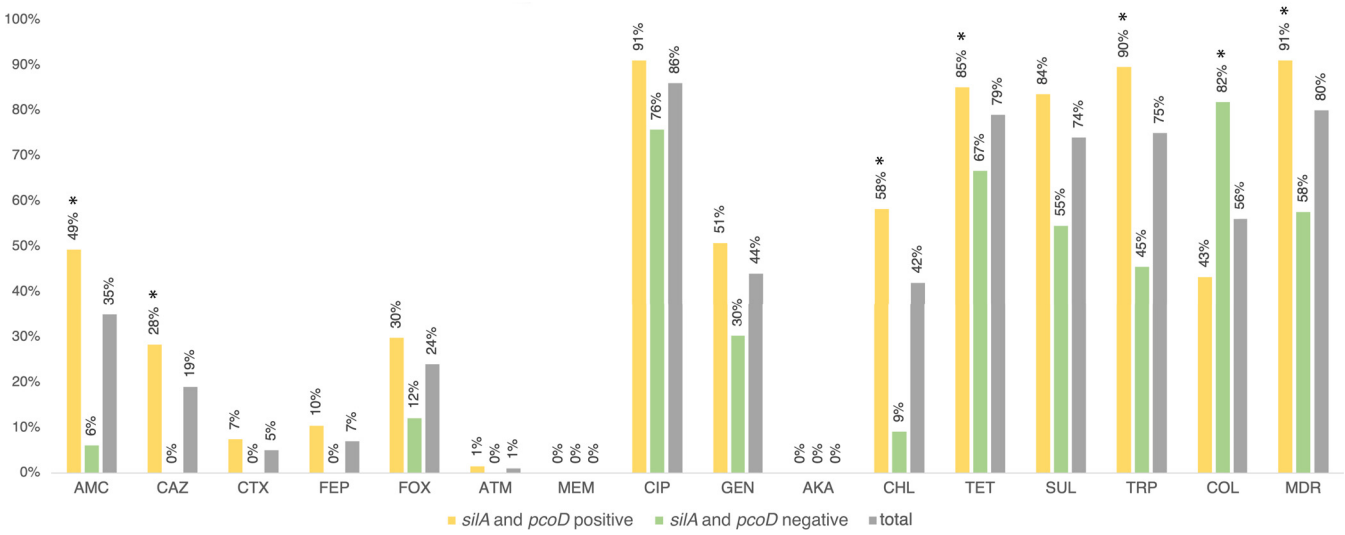

FIG 4 Percentage of antibiotic resistance detected among *silA* and *pcoD* positive - yellow (n = 67) and *silA* and *pcoD* negative - light green (n = 33) *K. pneumoniae* isolates. *, $P < 0.05$ (Fisher's exact test). AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; ATM, aztreonam; MEM, meropenem; CIP, ciprofloxacin; GEN, gentamicin; AKA, amikacin; CHL, chloramphenicol; TET, tetracycline; SUL, sulfonamides; TRP, trimethoprim; COL, colistin; MDR, multidrug resistance.

phenotype (Table 1). The MDR isolates belonged to different KL type(s) (n = 23). Among these isolates, the most frequent phenotypes were resistance to ciprofloxacin, tetracycline, sulfonamides, and trimethoprim (n = 62 isolates [62%]; 18 KL type(s)). Additionally, some of these isolates showed resistance to colistin (n = 33 isolates; 9 KL type (s)) and/or extended-spectrum cephalosporins (n = 26 isolates; 7 KL type(s)) (Table S1).

Copper tolerance of *K. pneumoniae* recovered from poultry production. The copper tolerance genes *silA* and *pcoD* were observed in isolates dispersed in all farms, most flocks (88%, n = 14/16), and poultry samples with *K. pneumoniae* (81%, n = 25/31) (Fig. 1A). Similar rates were observed between ITMF and OTMF samples among different sampling periods ($P > 0.05$). The *silA* and *pcoD* genes were detected in 67% (n = 67/100) of the *K. pneumoniae* isolates, regardless of their origin by feed source (70%, n = 35/50 ITMF versus 64%, n = 32/50 OTMF) or farm stage (n = 28 at P1 versus n = 30 at P2). The *silA* and *pcoD* positive isolates were also detected in chicken meat (n = 3; 2 samples at P3) and environmental samples (feed [n = 5; 3 samples] and water [n = 1; 1 sample]). The *silA* and *pcoD* positive isolates belonged to 25 of 26 KL type(s), compared to the *silA* and *pcoD* negative isolates, which belonged to only 9 KL type(s). Regarding antibiotic susceptibility, MDR was mostly found in *silA*- and *pcoD*-positive *K. pneumoniae* isolates (91%, n = 61/67 compared to 58%, n = 19/33 of isolates without *silA* and *pcoD* [$P < 0.05$]), with those also being more resistant to amoxicillin-clavulanic acid, ceftazidime (including extended-spectrum β-lactamase [ESBL] producers), chloramphenicol, tetracycline, and trimethoprim ($P < 0.05$) (Fig. 4). The correlation between colistin resistance and the absence of *silA* and *pcoD* can be explained by the predominance of the colistin-resistant KL109. More specifically, of the 33 isolates that lacked *silA* and *pcoD*, 23 of them corresponded to K type, which exhibited colistin resistance (Fig. 4; Table S1).

Copper phenotypic assays were performed in 85% of *K. pneumoniae* isolates carrying or not carrying copper tolerance genes representative of different farms, flocks, KL type(s), and antibiotic resistance profiles (Table 2). All *K. pneumoniae* isolates carrying the *silA* and *pcoD* genes (100%, n = 53/53) exhibited MICs to $CuSO_4$ of 16 to 32 mM (CuT phenotype, $MIC_{CuSO4} \geq 16$ mM). These data contrast with those of most *K. pneumoniae* isolates without acquired copper tolerance *silA* and *pcoD* genes (n = 30/32), showing MICs to $CuSO_4$ of 2 to 12 mM (Table 2). The MIC_{CuSO4} distributions were similar for isolates of the two feed sources (ITMF and OTMF).

Whole-genome analysis of *K. pneumoniae* poultry-associated isolates. The 20 sequenced *K. pneumoniae* isolates representing the most abundant KL type(s) were assigned

TABLE 2 $CuSO_4$ MICs in anaerobiosis for *K. pneumoniae* isolates ($n = 85$) by poultry feed type

Feed	*silA* and *pcoD* gene	Total no. of isolates[a]	No. of isolates with indicated MIC (mM)[b]											
			0.5	1	2	4	8	12	16	20	24	28	32	36
ITMF	+	29							7	7	12	3		
	−	14				1	4	8	1					
OTMF	+	24							4	6	12	1	1	
	−	18			1	5	1	10	1					
Total	+	53							11	13	24	4	1	
	−	32			1	6	5	18	2					

[a]The 85 isolates were selected to represent diverse farms, flocks, feeds, copper genotypes, and genomic backgrounds.

[b]*E. coli* ED8739 (plasmid pRJ1004 with *sil* and *pco* genes; MIC_{CuSO4}, 16 to 20 mM, anaerobiosis) and *Enterococcus faecium* BM4105RF (negative for all genes tested; MIC_{CuSO4}, 2 to 4 mM, anaerobiosis) were used as control strains in Cu assays (63). Corresponding $CuSO_4$ values in milligrams per liter for the millimolar values tested are as follows: 0.5 mM, 79.80 mg/L; 1 mM, 159.61 mg/L; 2 mM, 319.22 mg/L; 4 mM, 638.44 mg/L; 8 mM, 1,276.87 mg/L; 12 mM, 1,915.308 mg/L; 16 mM, 2,553.74 mg/L; 20 mM, 3,192.18 mg/L; 24 mM, 3,830.62 mg/L; 28 mM, 4,469.05 mg/L; 32 mM, 5,107.49 mg/L; and 36 mM, 5,745.92 mg/L.

to 12 sequence types (STs) (10 known and 2 new STs) and 14 lineages based on core genome and KL types, including globally dispersed ST11-KL105, ST15-KL19, ST147-KL64, and ST307-KL102. Based on the core genome multilocus sequence type (cgMLST) analysis and the proposed thresholds (<10 allele differences) (30–32), the *K. pneumoniae* isolates were clonally diverse, with 10 isolates distributed in five clusters (differing by 0 to 11 single nucleotide polymorphisms [SNPs]) (Fig. 5; Table S2). Those clusters included isolates from different sample types from the same farm, i.e., feces of poultry and derived meat ($n = 2$ ST6406/farm C and $n = 2$ ST6405/farm A), as well as water and poultry feces ($n = 2$ ST11/farm H). Also, we detected clones persisting over time in different farms ($n = 2$ ST15/farms E and H; $n = 2$ ST631/farms B and D). The phylogenetic relationship of our genomes with others from different sources, regions, and time frames available at Pathogenwatch was explored (Fig. 6), revealing fewer than 10 allele differences in all but four of the lineages (ST147, ST525, ST631, ST6405). Of note, isolates belonging to ST15-KL19, ST15-KL146, ST392-KL27, and ST1537-KL64 lineages revealed <21 SNPs with genomes from diverse origins (Fig. 6) (a threshold recently proposed for *K. pneumoniae* transmission in health care settings) (33). ST15 isolates were linked to genomes associated with human infections in the United Kingdom (ST15-KL19) and human infections and horses in Italy, France, and the United States (ST15-KL146). The ST392-KL27 isolate was linked to genomes from human infections and/or colonization in Spain, while ST1537-KL64 was detected in food products (chicken meat and salads) in France (Fig. 6).

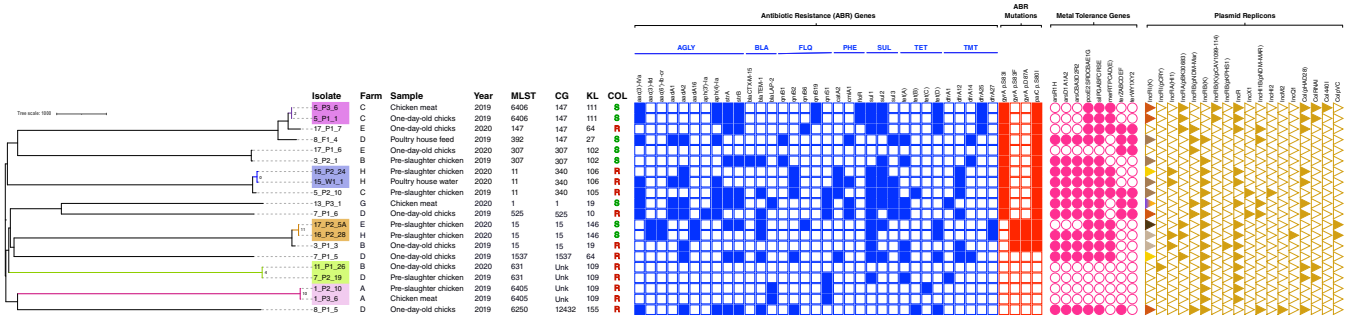

FIG 5 Neighbor-joining tree representing the phylogenetic relationships among the 20 *K. pneumoniae* genomes. The tree was constructed from the Pathogenwatch pairwise-distance matrix (i.e., based on SNPs called in 1,972 core genes). Scale bar units represent substitutions per variant site. The number of substitutions in our isolates compared to the 5 main clusters is represented in each branch. Associated metadata of all isolates were added using iTOL (https://itol.embl.de/). Each color-filled shape represents the presence of relevant antibiotic resistance, metal tolerance genes, and plasmid replicons associated with well-defined incompatibility groups. The different shades of colors represent the typing results for IncFII(K) plasmids (orange, $IncFII_{K2}$; brown, $IncFII_{K4}$; beige, $IncFII_{K5}$; dark brown, $IncFII_{K7}$; half violet/orange, $IncFII_{K8}$; yellow, $IncFII_{K21\text{-like}}$). Only known mutations conferring fluoroquinolone resistance are presented. *Klebsiella* intrinsic antibiotic resistance ($bla_{SHV\text{-}1}$, $bla_{SHV\text{-}11}$, $bla_{SHV\text{-}26}$, $bla_{SHV\text{-}28}$, *fosA*, *oxqAB*) and metal tolerance (*arsBCR*, *cusABFCRS*) genes are not represented. The isolate 17_P2_5A possesses all the genes of the *mer* operon except for *merE*, which is indicated by the use of curved brackets in merRTPCAD(E). AGLY, aminoglycosides; BLA, β-lactams; CG, clonal group; COL, colistin; FLQ, fluoroquinolones; KL, K locus; MLST, multilocus sequence typing; PHE, phenicols; SUL, sulfonamides; TET, tetracycline; TMT, trimethoprim; Unk, unknown.

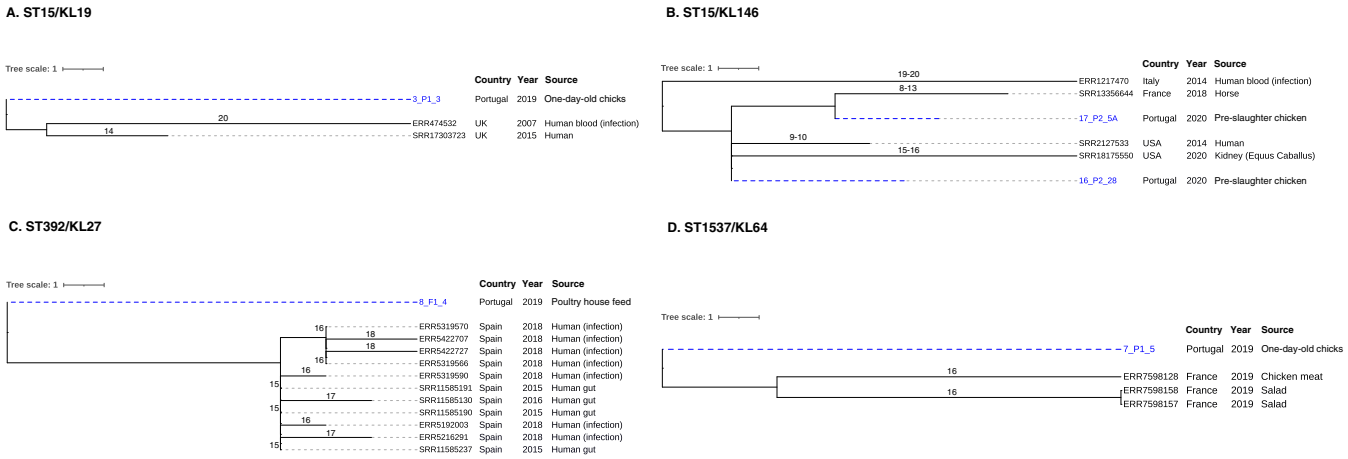

FIG 6 Neighbor-joining trees representing phylogenetic relationships among our *K. pneumoniae* genomes and those available in Pathogenwatch with fewer than 21 SNPs. (A) ST15/KL19; (B) ST15/KL146; (C) ST392/KL27; (D) ST1537/KL64. Genome selection was performed using cgMLST single linkage clustering to include the ones with fewer than 10 allele differences (threshold = 10). Then, these genomes were used to infer a neighbor-joining tree from the Pathogenwatch pairwise-distance matrix (i.e., based on SNPs called in 1,972 core genes). Scale bar units represent substitutions per variant site. The number of substitutions in our isolates and the ones available in Pathogenwatch are represented in each branch. All isolate-associated metadata (country, source of isolation, and collection date) were added using iTOL (https://itol.embl.de/).

WGS revealed a high load and diversity in antibiotic resistance and metal tolerance genes in comparison to virulence genes (Fig. 5; Table S3). All isolates carried the chromosomal *mrkABCDFHIJ* cluster encoding type 3 fimbriae, whereas only ST15 isolates carried the virulence accessory genes *kfuABC* (ferric uptake system) and the *kpiABCDEFG* genes (pilus system). Regarding the genomic analysis of colistin resistance, we detected 96 different chromosomal mutations (mostly missense; $n = 94/96$) in 72% of the genes ($n = 23/32$) encoding proteins previously implicated in colistin resistance compared with the reference strain *K. pneumoniae* MGH 78578 (described in detail in Fig. 7; see also Fig. S1). From these, 52 distinct mutations were present in all ColR-Kp isolates (9 lineages) across five operons/genes associated with colistin resistance mechanisms such as modifications of lipopolysaccharide (LPS)/lipid A (*arnABDFT*, *crrAC*, *phoQ*, *pmrACD*, *mgrB*), overexpression of efflux pumps (*acrB*), LPS loss (*lpxA*, *msbA*) or biosynthesis (*yciM*), and regulation (*rstB*) (Fig. 7). Diverse mutations implicated in colistin resistance were detected in seven of those genes (*acrB*, *arnB*, *arnD*, *arnT*, *mgrB*, *phoQ*, and *pmrC*), varying accordingly with the clone (Fig. 7). All but one of the isolates accumulated mutations in different gene clusters associated with colistin resistance (ranging from two to seven).

In addition to colistin resistance, we detected diverse acquired genes ($n = 36$) encoding resistance to seven different antibiotic classes, with 85% of genomes ($n = 16/20$) carrying genes conferring resistance to ≥4 classes but differing between lineages (0 to 6). The most frequent classes and genes conferring resistance to antibiotics were aminoglycosides (*strA/strB*), sulfonamides (*sul1/sul2*), tetracyclines [*tet*(A)/*tet*(D)], trimethoprim (*dfrA*), and phenicols (*catA* and *cmlA*) (Fig. 5; Table S3). Also, analysis of the genetic background of resistance to ciprofloxacin showed chromosomal mutations in the quinolone resistance determining region (QRDR) of topoisomerase genes *gyrA* (S83I, S87A, and S83F) and *parC* (S80I) (*gyrA* and *parC*, 70%, $n = 14/20$) and variable plasmid-mediated quinolone resistance (PMQR) genes *qnrB* ($n = 11$) and *qnrS* ($n = 6$) (Fig. 5). Extended-spectrum cephalosporin gene $bla_{\text{CTX-M-15}}$ was detected in only one ST307 isolate. Moreover, diverse acquired metal tolerance gene clusters encoding copper/silver (*pco/sil*), arsenic (*ars*), mercury (*mer*), and/or tellurite (*ter*) tolerance were detected in all but four of the genomes (Fig. 5). The *sil* and *pco* cluster (75%, $n = 15/20$) was frequently associated with operons *ars* (73%, $n = 11/15$), *mer* (67%, $n = 10/15$), and/or *ter* (40%, $n = 6/15$) (Fig. 5). Cooccurrence of antibiotic resistance genes and copper tolerance genes was detected in 75% ($n = 15/20$) of the genomes. Genomes carried an average of 5 plasmids (1 to 9 per genome) and a total of 18 different well-defined plasmid incompatibility groups (1 to 7 replicon types per isolate). The most common were IncFIB_K (80%; $n = 16/20$), followed by diverse IncFII_K (70%; $n = 14/20$) and IncR (70%; $n = 14/20$), most often in variable

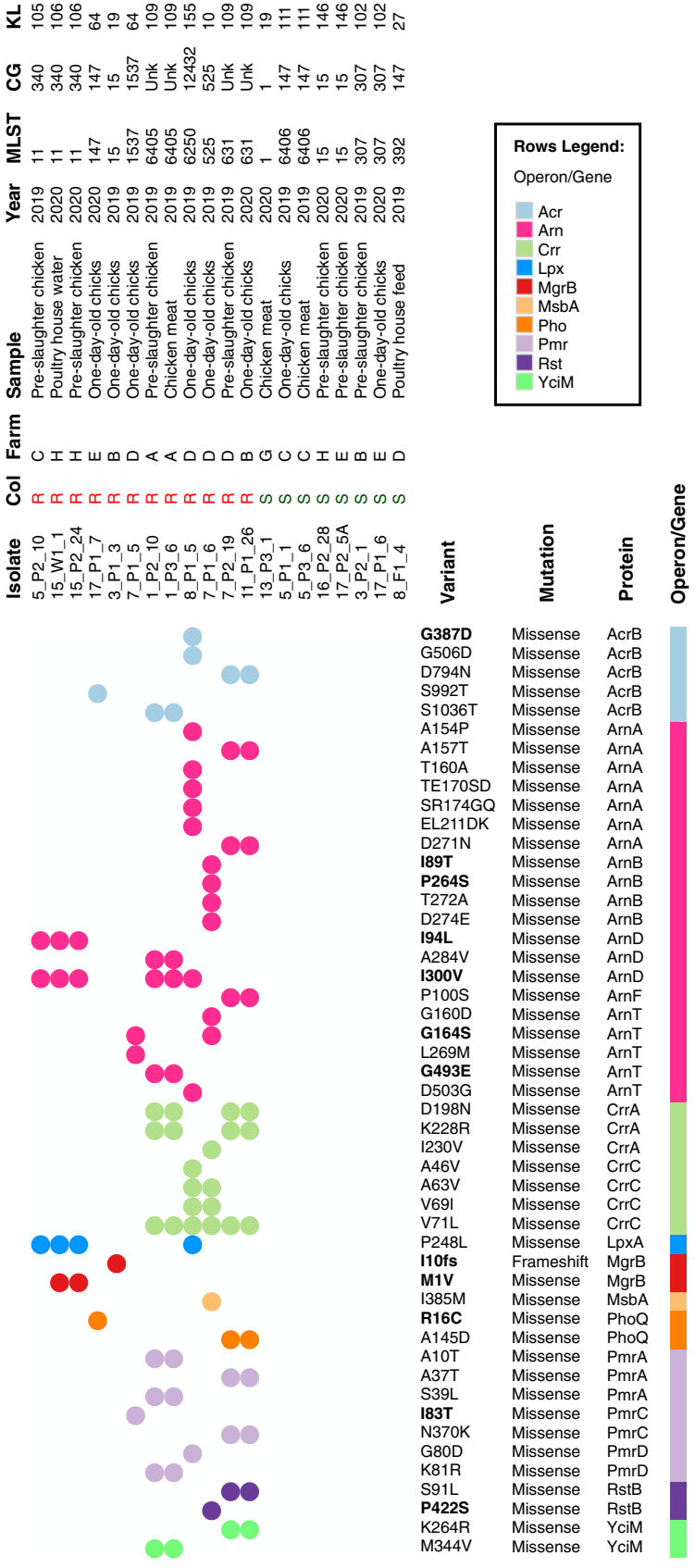

FIG 7 Heat map, generated using the Morpheus online web server (https://software.broadinstitute.org/morpheus/), representing the distribution of distinct mutations among the sequenced colistin-resistant

(Continued on next page)

combinations with FIA, other FIB types ($FIB_{pNDM-MAR}$, $FIB_{pCAV1099}$, FIB_{pKPHS1}), and/or HI1B (Fig. 5). Col plasmids were also frequent (65%; $n = 12/20$ genomes), while other classical incompatibility groups were rare (IncHI2, IncX, IncQ, and IncM). Copper tolerance genes *pco* and *sil* were located mainly in $IncFIB_K$ and $IncFII_K$ plasmids (73%; $n = 11/15$; ~80 to 270 kb) of different $IncFII_K$ groups by pMLST (2, 4, 5, 7, 8, and 21-like) along with variable antibiotic resistance genes [*aac(3)-IVa*, *aph(4)-Ia*, *aadA2*, *strA-strB*, *qnrB1*, *catA2*, *sul1*, *sul2*, *tetA*, *tetD*, *dfrA12*, *dfrA14*, bla_{TEM}, and $bla_{CTX-M-15}$] (Fig. 5). These plasmids carrying *pco* and *sil* were similar to others (MOB-recon; mash distance, 0.0012 to 0.0309) described in humans in multiple countries, occasionally carrying $bla_{CTX-M-15}$, bla_{DHA-1}, and/or *qnr* genes (Table S4).

DISCUSSION

This study first showed the absence of *mcr* but a high occurrence and diversity of colistin-resistant (*mcr*-negative) *K. pneumoniae* isolates after >2 years of colistin withdrawal in intensive chicken farms, independent of the type of Cu supplementation used in the feed formulation. Furthermore, we demonstrated the persistence of particular *K. pneumoniae* clones throughout the whole poultry production chain and suggested poultry as a potential foodborne/environmental source of *K. pneumoniae* lineages with clinical relevance.

The high rates of intensively raised flocks positive for *K. pneumoniae* in the early and preslaughter stages, together with the detection of *K. pneumoniae* with or without standard fecal indicators in feed or water samples (e.g., *Escherichia coli* and/or *Enterococcus* detected in 9 of 14 water samples from all farms) (data not shown), suggest diverse contamination events (e.g., hatchery farm, poultry house cleanliness and biosecurity level, water, feed, inanimate surfaces, and/or human handlers) occurring early and frequently along the production chain (34, 35). Environmental factors (e.g., type of litter, temperature, relative humidity, daily cycles, moment of sampling/age, season, and vacancy period) or the use of antimicrobial compounds (e.g., disinfectants, antibiotics, coccidiostats, or metals) are also known to have an impact on the composition of poultry gut microbiota (35–37). These factors could justify the differences found in fecal *K. pneumoniae* occurrence rates at the farm level in this study (82%, ranging from 25 to 100%) and compared to another recent study also using SCAi medium for bacterial recovery in chicken farms (26%) (38). However, despite the high rate of fecal samples carrying *K. pneumoniae*, resistant or not to colistin, our data revealed few positive chicken meat batches (17%), which is far below what was reported in studies from the United States (47%) and the European Union (60%) (39, 40). Besides, *Salmonella* was not detected in any sample (data not shown). These results demonstrate reduced meat cross-contamination and the effectiveness of sanitary measures during animal transport and at the slaughterhouse, thus reducing the consumer's risk of exposure (12, 37, 41).

Recent studies suggest that banning colistin in food animal production has had an encouraging outcome by limiting *mcr* spread (11, 12). Our results confirm this trend in poultry production by the absence of *mcr* in farms and meat isolates, although a high rate of samples carried ColR-Kp associated with a high diversity of chromosomal mutations, independently of the feed type used. This suggests the circulation of diverse colistin-resistant genotypes responding differently to the colistin ban. We detected mutations, alone or in

FIG 7 Legend (Continued)

Klebsiella pneumoniae genomes. Colored circles indicate the presence of a specific mutation, while each color represents the genes that are part of the same operon. Variants shown in bold were supported by the available literature as present in ColR-Kp and/or were predicted as deleterious by PROVEAN. *Klebsiella pneumoniae* isolates were grouped as colistin susceptible (S) when the MIC was ≤2 μg/mL or as colistin resistant (R) when the MIC was ≥2 μg/mL. When required, figures were minimally edited manually using Adobe Illustrator v25.3.1. Detailed resistance mechanisms: *acrB*, efflux pump; *arnABDFT*, lipid A modification with L-Ara4N addition; *crrA*, lipid A modification by upregulation of *pmrAB*/activation of the glycosyltransferase; *crrC*, connector protein; *lpxA*, inactivation of lipid A biosynthesis abolishing LPS synthesis; *mgrB*, inactivation of negative feedback regulator of the PhoP/PhoQ system; *msbA*, ABC transporter of lipid A; *phoQ* and *pmrA*, activation of LPS-modifying operation in the two-component systems; *pmrC*, lipid A modification with phosphoethanolamine; *pmrD*, connector protein; *rstB*, sensor protein; *yciM*, regulation of LPS biosynthesis. A, alanine; C, cysteine; D, aspartate; E, glutamate; G, glycine; I, isoleucine; K, lysine; L, leucine; M, methionine (start codon); N, asparagine; P, proline; R, arginine; S, serine; T, threonine; V, valine; Unk, unknown.

combination, in genes known to be involved in lipid A modifications and colistin resistance phenotypes (*arnABDFT*, *phoQ*, *pmrACD*, and *mgrB*), as described previously (42–50). However, a high variety of other nondescribed mutations were detected, supporting the urgent need for reliable genotypic-phenotypic correlations to explain colistin resistance mechanisms (51). Such a variety and frequency of chromosomal mutations in ColR-Kp isolates detected in different studies and environments (26, 43, 50, 51) suggest that they could play a role in adaptive features other than colistin resistance (e.g., *mgrB* inactivation in environmental *K. pneumoniae* survival and transmission or *phoPQ* or *pmrB* mutations in chlorhexidine tolerance) (52–54). Also, some of these mutations do not confer significant biological cost (55, 56), which may explain the occurrence of colistin-resistant *mcr*-negative *K. pneumoniae* isolates long after colistin withdrawal. Finally, other factors contributing to coselection (e.g., other antibiotics and growth promoters such as Cu) or maintenance (continuous contamination sources) of chromosomally mediated colistin-resistant strains in the livestock sector (26) could not be excluded.

In the context of antibiotic/colistin reduction and replacement, the use of diverse antimicrobial compounds may be expanded, making it difficult to understand the complexity of possible coselection events of the MDR strains. Some recent studies performed after colistin withdrawal revealed an increase in clinically relevant antibiotic resistance genes (e.g., $bla_{\text{CTX-M}}$ and bla_{NDM}) among food animal isolates (57, 58). In this study, these genes were not detected, but we showed high rates of resistance to multiple antibiotics in *K. pneumoniae* isolates from poultry, independently of the sample selection strategy (with or without colistin supplementation) or between sample groups (poultry life stages and feed type). Resistance rates were higher for antibiotics (e.g., ciprofloxacin and tetracycline) frequently used for therapeutics at poultry farms (12, 59), in contrast with other studies in poultry or other nonclinical environments (38, 60) where resistance rates were low. Such differences in the available studies could reflect local variation in the usage of several antibiotic classes, the diversity of routes for *K. pneumoniae* dissemination within and beyond the production environment, and the circulating clonal lineages (38, 60).

Other largely used feed ingredients with antimicrobial activity like metals/copper may also contribute to changes in poultry microbiota and/or even potentiate Cu-tolerant and MDR strain coselection (19, 20, 24), a factor unexplored for *K. pneumoniae*. Recent studies revealed correlations between metal environmental pollution (e.g., Cu at poultry farm samples) and an increase in antibiotic resistance genes (61, 62). We have also detected the plasmid-acquired copper tolerance *pco* plus *sil* cluster in isolates dispersed in all farms and in most of the flocks. Furthermore, we describe for the first time in *K. pneumoniae* a correlation between the presence of *pco* and *sil* genes and the Cu tolerance phenotype (MIC $\geq$ 16 mM in anaerobiosis). These data suggest the adaptability of this species to stressful/unfavorable farming environments, as previously detected for other zoonotic bacteria such as the emergent MDR clones of *Salmonella* (63). According to our data, the feeding regime (ITMF or OTMF) does not seem to contribute to the expansion of MDR or ColR-Kp. These data suggest that the similar whole-farm environment (e.g., litter quality, ventilation system, and temperature control) and management practices (e.g., sanitation and disinfection protocols, waste, and water control) overlap with the feeding regime. Thus, studies evaluating environmental factors (e.g., pH) (64) and subinhibitory concentrations of heavy metals to maintain antibiotic-resistant bacteria in poultry production and other related environments are urgent (61, 65, 66).

It is of note that metal tolerance operons (*pco* plus *sil*) were mainly located in multireplicon F type (FII_K plus FIB_K) plasmids carrying genes encoding resistance to several classes of antibiotics, including the critical extended-spectrum cephalosporins and/or fluoroquinolones, supporting the potential for diverse coselection events (67, 68). These mosaic F type plasmids are common in *K. pneumoniae* populations from different sources (68–73), suggesting a major role in both dissemination and persistence of antibiotic resistance and metal tolerance genes and *K. pneumoniae* adaptation to different niches. Besides, the similarity between plasmid backbones identified in poultry isolates and those described in *K. pneumoniae* collections from humans (see Tables S3 and S4 in the supplemental material) (72) suggests a common pool of shared plasmids between humans

and eventually different animal species (70), which deserves to be further explored with comprehensive comparative plasmidome analysis.

This is the first study tracking *K. pneumoniae* throughout the whole poultry production chain, enabling the identification of multiple transmission routes in flocks and the farm environment. The identification of closely related MDR *K. pneumoniae* isolates (e.g., ST11-KL106, ST15-KL146, ST631-KL109, ST6405-KL109, and ST6406/CG147-KL111) in multiple poultry stages and environments in the same or different farms suggests that food sector efforts should be made in improving the sanitary measures in the poultry houses, poultry workers, and the environment beyond (treatment of wastewaters and manure). The persistence of MDR *K. pneumoniae* clones in the poultry chain, which is according to previous studies conducted on farms (34, 35, 38) or at slaughterhouses (41), suggests the presence of adaptive environmental features other than antimicrobial resistance (e.g., biofilm formation). Most available studies assessing *K. pneumoniae* sources from a One Health perspective have included retail foods, livestock farms, or wastewater, while the poultry chain has been greatly understudied (27, 38–40, 59, 60, 74, 75). Despite the high diversity found, we highlight the commonality of KL types (KL64, KL102, KL105, and KL106) and lineages (ST15-KL19, ST15-KL146, and ST392-KL27) identified between poultry and human clinical isolates (this study) (28, 76). Although the immunogenicity of different KL types is not well understood, particular capsular types have been shown to influence the selection of certain *K. pneumoniae* lineages (77), which might provide an advantage regarding host immunity (78, 79). Finally, although human contamination cannot be excluded, our data suggest poultry as a reservoir and source of globally dispersed and human clinically relevant *K. pneumoniae* lineages with a potential risk for transmission to humans and other ecosystems (animals and the environment).

Conclusions. This study constitutes the first comprehensive analysis of *K. pneumoniae* from a poultry farm-to-fork perspective. Our data revealed, independently of the Cu feed type, a high rate of chicken fecal samples carrying a high diversity of *K. pneumoniae* isolates, including MDR, Cu-tolerant, and colistin-resistant/*mcr*-negative strains, long after colistin withdrawal. Furthermore, we propose that poultry may serve as a reservoir and source of human clinically relevant lineages and mosaic F type plasmids (with antibiotic resistance and metal tolerance genes), which could pose a potential risk of human exposure through the food chain (e.g., poultry carcasses and occupational exposure) and/or environmental release (e.g., farm or slaughterhouse wastewaters). Therefore, it is crucial to further explore the drivers contributing to the circulation and persistence of MDR *K. pneumoniae* in poultry production. This will help to identify novel mitigation strategies addressing producers' efforts, consumer concerns, and the EU farm-to-fork goals in the fight against antimicrobial resistance and toward improving food safety and environmental sustainability.

MATERIALS AND METHODS

Sampling strategy at the chicken farm and slaughterhouse processing plant. Our pilot study involved seven Portuguese intensive farming-based chicken farms with similar conventional indoor and floor-raised production systems, comprising all the key practices and requirements in compliance with EU legislation, as indicated by the operators (80). To ensure proper management, the farms were selected based on having grow-out poultry houses with similar conditions and individual feed silos, allowing for the division of each flock upon arrival (1-day-old chicks) into two groups, each receiving a different feed type (OTMF or ITMF). In all farms, colistin had been banned since January 2018, while copper was routinely used as an additive in poultry feed. The current inorganic formulation feed (ITMF) was supplemented with inorganic sources of Cu, and the organic formulation (OTMF) was supplemented with organic forms, both previously evidenced as capable of maintaining broiler performance under commercial conditions (23). Both mineral supplements were added to the same commercial starter and grower feeds adapted to different periods of the broiler's life, with copper concentrations decreasing ≈42% in OTMF versus ITMF feeds (both far below the maximum dose of 25 mg/kg of Cu, according to EU Regulation 2018/1039).

Eighteen chicken flocks (10,000 to 64,000 animals from each house; Ross 308 strain) fed with ITMF ($n = 9$) or OTMF ($n = 9$) were sampled at each of the seven farms between October 2019 and November 2020 (two farms were sampled twice in different seasons). Personal protective equipment such as gloves, boots, and coveralls were used. Pooled fresh chicken feces (≈50 g; total $n = 34$) were collected with sterile spatulas/shovels from the floor by walking in a zig-zag pattern in two separated poultry houses (ITMF or OTMF) in each farm. The collection period was at 2 to 3 days of life (P1 stage; $n = 18$ samples) and the day before slaughter when the chickens were 28 to 30 days old (P2 stage; $n = 16$ samples; 2 samples missing during 2020 COVID-19

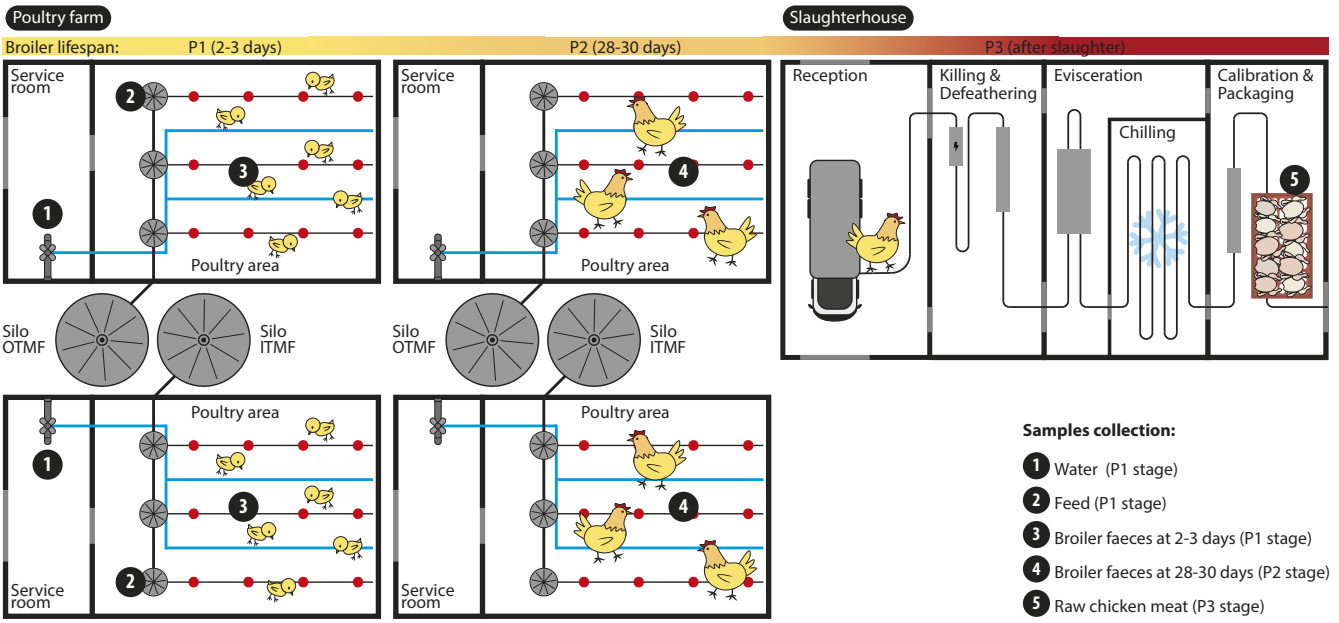

FIG 8 Sampling strategy at the chicken farm and slaughterhouse processing plant. Sample collection points are indicated by numbers.

lockdown). Environmental samples, including feed (≈50 g collected at the silo that supplied the feeding lines; n = 18) and water (≈1 L collected at the faucet that supplied the drinking water lines; n = 14), were also collected inside grow-out houses at each farm at the P1 stage (Fig. 8).

Raw chicken meat samples (n = 18 batches; recovered after slaughter and air chilling) of the same flocks were collected after slaughter (P3 stage) in the poultry production slaughterhouses, immediately before distribution for retail sale. Each meat sample included approximately 50 g of neck skin cut with a sterile scalpel from a pool of 10 carcasses from the same batch (each batch corresponded to one flock from the same farm slaughtered at the same time) (Fig. 8).

All previous solid and water samples were collected in sterile plastic bags or containers, transported at 4°C, and processed on the same day at the laboratory. Subsequent sample processing was performed by culture approaches, as described in the following sections.

Screening of *K. pneumoniae* and colistin-resistant *K. pneumoniae*. *K. pneumoniae* was selectively recovered, directly from the sample and after enrichment, in the Simmons citrate agar plates with 1% inositol (SCAi), a medium that selectively favors the growth of *Klebsiella* in potential *Escherichia coli*-rich samples (see Fig. S2 in the supplemental material). A common initial step consisted of weighing a portion of 25 g of tested solid samples (P1, P2, and P3 stages) or the 0.45-μm filter from filtration of 500 mL of water samples (P1 stage), suspended in 225 mL of buffered peptone water (BPW) supplemented with 3.5 mg/L of colistin. The direct culture method included spreading an aliquot of 100 μL of the BPW plus colistin after 1 h at room temperature (resuscitation step) on SCAi supplemented or not with colistin (3.5 mg/L). The enrichment approach involved the same procedure, but after a previous incubation of BPW plus colistin at 37°C for 16 to 18 h. All the SCAi plates were incubated at 37°C for 48 h. One to five colonies of each presumptive morphotype were selected for identification. Isolate identification was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (MALDI-TOF Vitek MS, bioMérieux, France) and by PCR for *K. pneumoniae* (81). In all the identified isolates, screening of colistin resistance genes (*mcr-1* to *mcr-5* and *mcr-6* to *mcr-9*) was assessed by two multiplex PCRs, as previously reported (82, 83). The MIC for colistin was determined by the reference broth microdilution (84). An estimation (in CFU per gram) of colistin-resistant *K. pneumoniae* in the poultry samples directly plated on SCAi plus colistin (see the procedure described above) was performed after counting typical *Klebsiella* colonies, species identification, and determination of colistin MIC.

Phenotypic and genotypic characterization of *K. pneumoniae*. Relatedness between isolates from different samples was inferred by Fourier transform infrared (FT-IR) spectroscopy with attenuated total reflectance (ATR) using a PerkinElmer Spectrum Two instrument. After growth under standardized culture conditions (37°C, 18 h), a colony was directly deposited on the ATR accessory of the FT-IR instrument and air dried. Spectra were acquired under standardized conditions (4,000 to 600 cm^{-1}, 4 cm^{-1} resolution, and 16 scan coadditions). The region corresponding to polysaccharides (1,200 to 900 cm^{-1}) was then compared between each other and with those from an in-house spectral database (allowing identification of 30 KL type(s) from well-characterized international *K. pneumoniae* clones), as previously described (76, 85). FT-IR-based assignments were confirmed using PCR of the *wzi* gene and further sequencing at Eurofins Genomics (https://www.eurofinsgenomics.eu/) to infer the KL type(s) at BIGSdb (https://bigsdb.pasteur.fr/klebsiella/) (86).

Antibiotic susceptibility profiles were determined by disc diffusion using 14 antibiotics (amoxicillin plus clavulanic acid, 30 μg; amikacin, 30 μg; aztreonam, 30 μg; cefepime, 30 μg; cefotaxime, 5 μg; cefoxitin, 30 μg; ceftazidime, 10 μg; chloramphenicol, 30 μg; ciprofloxacin, 5 μg; gentamicin, 10 μg; meropenem, 10 μg; sulfamethoxazole, 300 μg; tetracycline, 30 μg; trimethoprim, 5 μg). The interpretation was

performed using the guidelines of the European Committee of Antimicrobial Susceptibility Testing (EUCAST) (87) and, when this was not possible, by the Clinical and Laboratory Standards Institute (CLSI) guidelines (88). *Escherichia coli* ATCC 25922 was used as the control strain. MDR was considered when the isolates were resistant to three or more antibiotics of different families (in addition to ampicillin, to which all *K. pneumoniae* are intrinsically resistant).

All the isolates were screened for the *silA* and *pcoD* Cu tolerance genes by PCR, as previously reported (63). Copper susceptibility was studied in representative isolates (representing the diverse farms, flocks, feeds, and genomic backgrounds) by the agar dilution method (MIC_{CuSO4}, 0.5 to 36 mM; anaerobiosis), as previously published (63).

WGS and comparative analysis. Representative isolates (n = 20) of different farms, sources, time spans, and clones/KL type(s) were selected for WGS. The DNA was extracted with the Wizard genomic DNA purification kit (Promega Corporation, Madison, WI), with the final concentration measured with a Qubit 3.0 fluorometer (Invitrogen, Thermo Fisher Scientific, USA) and sequenced with an Illumina NovaSeq 6000 S4 PE150 XP system (Illumina, San Diego, CA) at Eurofins Genomics (https://eurofinsgenomics.eu/). Raw read quality control was assessed with default parameters using FastQC v0.11.9 (89) and MultiQC v1.12 (90). Good-quality raw reads were then *de novo* assembled using SPAdes v3.15.3 (91) with "–isolate" and "–cov-cutoff auto" flag options. We then used QUAST v5.0.2 (92) and CheckM v1.0.18 (93) within KBase (https://www.kbase.us/) to assess the quality and completeness of the assemblies.

The assemblies were annotated using the RAST server (94). Genome assemblies were then uploaded to Pathogenwatch v2.3.1 (https://pathogen.watch/) to determine species, capsular polysaccharide (K) and lipopolysaccharide O locus types and serotypes (29), MLSTs (95), and cgMLSTs. Pathogenwatch uses the calculated pairwise SNP distances between the genomes based on a concatenated alignment of 1,972 core genes to infer a neighbor-joining tree for phylogenetic analysis (96). Closely related genomes and the associated metadata (country, source of isolation, and collection date) were retrieved from all public genomes available at Pathogenwatch, after cgMLST single linkage clustering and selection of those with less than 10 allele differences (threshold = 10). Neighbor-joining trees were edited using iToL (97).

Snippy v3.2-dev (https://github.com/tseemann/snippy) was used to identify substitutions (SNPs) and insertions/deletions (indels) in genes (n = 32) putatively associated with colistin resistance (43, 51, 98), by aligning the raw read data from each isolate against the reference genome *Klebsiella pneumoniae* MGH 78578 (GenBank accession number CP000647). All gene mutations were further manually confirmed in the assembled genomes using the Geneious Prime 2023.0.1 software (http://www.geneious.com/). The deleterious effect of detected mutations on protein function was further assessed using the PROVEAN protein variation effect analyzer (99) or data from previous publications. We used the standard PROVEAN scores of less than or equal to −2.5 for a deleterious effect and greater than −2.5 for a neutral effect on protein function.

Kleborate v2.2.0 (100), integrated into Pathogenwatch, was used for the prediction of antibiotic resistance determinants (acquired and chromosomal mutations) and virulence traits (yersiniabactin YbST, colibactin CbST, aerobactin AbST, salmochelin SmST, and regulators of mucoid phenotype RmpA and RmpA2). ABRicate v1.0.1 (https://github.com/tseemann/abricate) with in-house databases was used to detect additional *K. pneumoniae* virulence genes from BIGSdb-Pasteur (https://bigsdb.pasteur.fr/klebsiella/), the chaperone-usher pilus system (*kpiABCDEFG*) (101), and relevant metal tolerance genes (*cusABFCRS*, *arsBCR*, *arsR1H-arsD1A1A2-arsCBA3D2R2*, *pcoGE1ABCDRSE2*, *silESRCFBAGP*, *merRTPCADE*, and *terZABCDEF-terWY1XY2* operons). AMRFinderPlus v3.10.30 (102) was used for the determination of antibiotic-resistant novel alleles.

In all 20 WGS-selected isolates, plasmid replicon typing was determined using PlasmidFinder (103, 104) and pMLST v2.0 (104) from the Centre for Genomic and Epidemiology (http://www.genomicepidemiology.org). $IncFII_K$ plasmids were further characterized according to reference 105 (https://pubmlst.org/organisms/plasmid-mlst). To confirm the location of metal tolerance genes and reconstruct putative plasmids based on draft assemblies, we used the MOB-recon tool v3.0.0 from the MOB-suite package (106, 107). The metal tolerance genes were considered part of a specific plasmid when identified by MOB-recon or when located on the same contig as the replicon/incompatibility determinant.

Statistical analysis. Differences in occurrence, antimicrobial resistance, copper tolerance, and bacterial count among *K. pneumoniae* isolates and flocks fed ITMF and OTMF as well as among P1, P2, and P3 stages were analyzed by Fisher's exact and Wilcoxon matched-pairs signed rank tests (α = 0.05) using Prism software, version 8.1.1 (GraphPad).

Data availability. The data for this study have been deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under BioProject accession number PRJEB60418.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, EPS file, 2.2 MB.

SUPPLEMENTAL FILE 2, EPS file, 1.9 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

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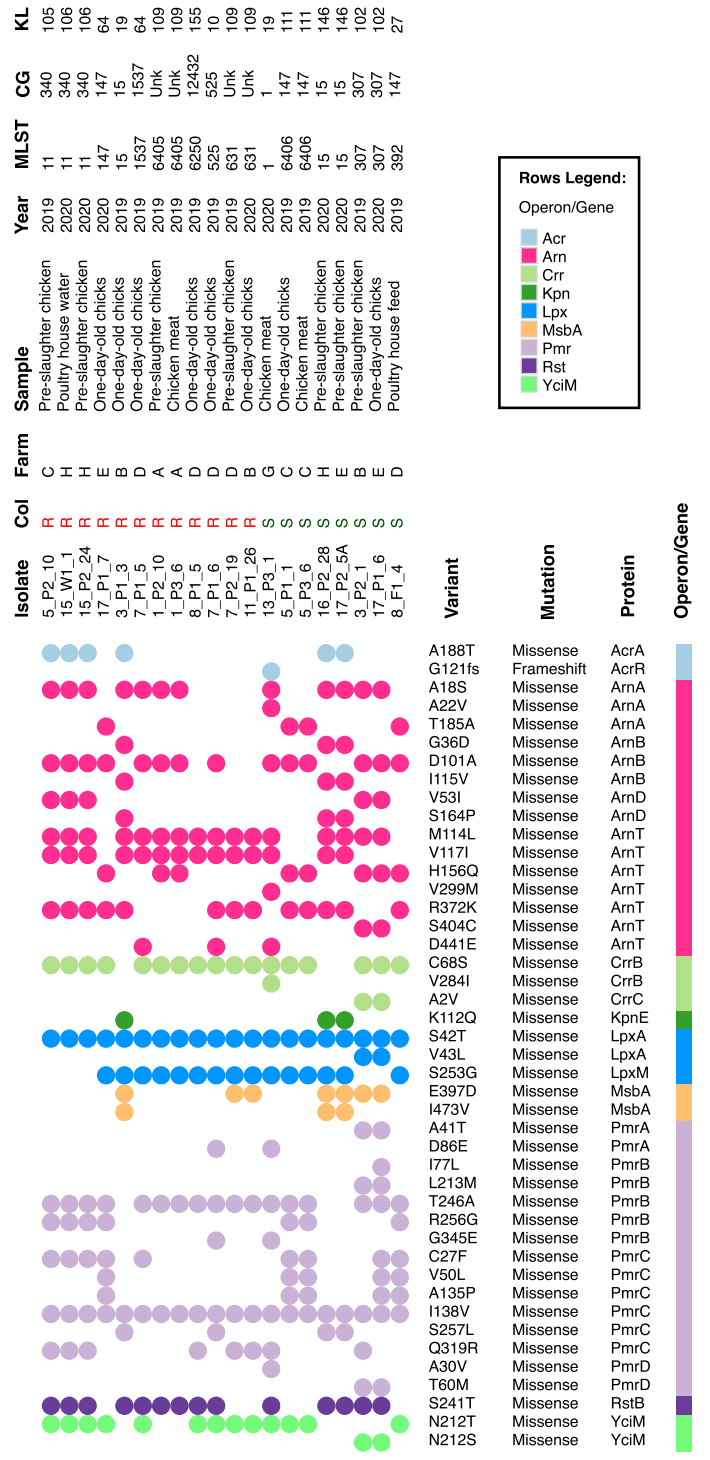

Fig. S1. Heatmap generated using Morpheus online web-server (https://software.broadinstitute.org/morpheus/) representing the distribution of colistin-associated mutations among the sequenced *Klebsiella pneumoniae* genomes. Coloured squares indicate the presence of a specific mutation, while each colour represents the genes that are part of the same operon. *Klebsiella pneumoniae* isolates were grouped into colistin-susceptible when MIC = <2 µg/mL or colistin-resistant when MIC = ≥2 µg/mL. When required, figures were minimally edited manually using Adobe Illustrator v25.3.1. Detailed resistance mechanisms: *acrA* – efflux pump; *acrR* – a transcriptional regulator of *acrAB* operon; *arnABDT* - lipid A modification with L-Ara4N addition; *crrB* – lipid A modification by upregulation of *pmrAB*/activation of the glycosyltransferase; *crrC* – connector protein; *kpnE* – efflux pump; *lpxA* – inactivation of lipid A biosynthesis abolishing LPS synthesis; *lpxM* – increased lipid A acylation; *msbA* - ABC transporter of lipid A; *pmrAB* - activation of LPS-modifying operation in the two-component systems; *pmrC* - lipid A modification with PEtn; *pmrD* – connector protein; *mgrB* - inactivation of negative feedback regulator of the PhoP/PhoQ system; *rstB* - sensor protein; *yciM* - regulation of LPS biosynthesis. Abbreviations: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine (start codon); N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Unk, unknown.

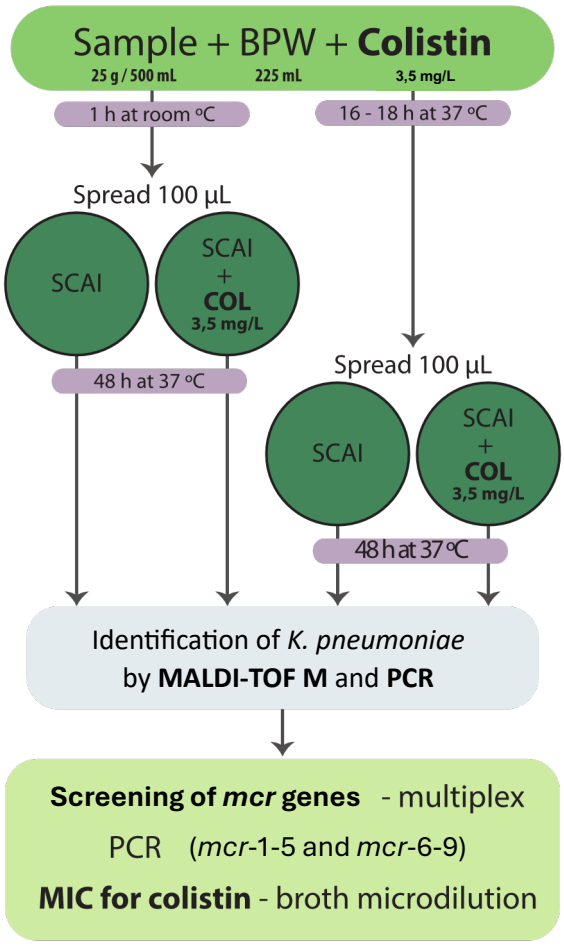

Fig. S2. Workflow for screening and counts of *K. pneumoniae* and colistin-resistant *K. pneumoniae*. BPW, buffered peptone water; COL, colistin.

Table S1. Characterization of the *Klebsiella pneumoniae* (n=100) recovered from poultry production over 2019-2020.

K/KL-type (no. isolates)	MLST-ST [a]	Farm (sample) [b]	*pcoD* and *silA* positive - no. isolates/total	Colistin MIC-mg/L (no. isolates)	Antibiotic resistance phenotype [c]
KL10 (n=2)	ST525 (n=1)	D (P1), E (P1)	2/2	>16 (n=1)	CHL, TET, SUL, TRP (AMC, CIP, GEN, COL)
				<2 (n=1)	
KL12 (n=4)	ND	A (P1), B (P1)	3/4	>16 (n=1)	(CIP, TRP, GEN, COL)
				<2 (n=3)	
KL14 (n=3)	ND	D (P2)	3/3	<2 (n=3)	AMC, CIP, TET, SUL, TRP (FOX, CHL)
KL17 (n=2)	ND	B (P1)	2/2	<2 (n=2)	AMC, TET
KL19 (n=5)	ST15 (n=1)	B (P1), G (P2, P3)	5/5	16 (n=1)	CIP, TET (AMC, GEN, CHL, SUL, TRP, COL)
	ST1 (n=1)			<2 (n=4)	
KL27 (n=3)	ST392 (n=1)	A (P1), D (F)	3/3	≥16 (n=1)	CIP, CHL, SUL, TRP (AMC, GEN, TET, COL)
				<2 (n=2)	
KL28 (n=2)	ND	A (P2), B (P1)	1/2	>16 (n=1)	(AMC, GEN, CHL, TET, SUL, TRP, COL)
				<2 (n=1)	
KL38 (n=1)	ND	D (P1)	1/1	<2 (n=1)	CIP, TET, SUL, TRP
KL45 (n=1)	ND	D (P2)	1/1	<2 (n=1)	CIP, GEN, TET, TRP
KL51 (n=1)	ND	C (P2)	1/1	<2 (n=1)	FOX, CIP, CHL, TET, TRP
KL64 (n=7)	ST147 (n=1)	A (P1), B (P2), D (P1, P2), E (P1)	6/7	4 – >16 (n=3)	CIP, TET, SUL, TRP (AMC, GEN, CHL, COL)
	ST1537 (n=1)			<2 (n=4)	
KL81 KL120 (n=2)	ND	D (P1), E (P2)	1/2	<2 (n=2)	CIP, TET, SUL, TRP (CHL)
KL102 (n=3)	ST307 (n=2)	B (P2), E (P1)	2/3	<2 (n=3)	CIP (FEP, CAZ, CTX, ATM, GEN, CHL, TET, SUL, TRP)
KL105 (n=2)	ST11 (n=1)	C (P2)	2/2	16 (n=1)	FOX, CIP, GEN, CHL, TET, SUL, TRP (COL)
				<2 (n=1)	
KL106 (n=18)	ST11 (n=2)	H (P1, P2, W)	15/18	≥16 (n=17)	FOX, CIP, CHL, SUL (AMC, FEP, CAZ, GEN, TET, TRP, COL)
				<2 (n=1)	
KL109 (n=24)	ST631 (n=2)	A (P2, P3), B (P1, P2), D (P1, P2), G (F	1/24	4 – >16 (n=24)	COL (FOX, CIP, GEN, CHL, TET, SUL, TRP)
	ST6405 (n=2)				
KL111 (n=4)	ST6406 (n=2)	C (P1, P3, F), D (P1)	4/4	<2 (n=4)	CIP, CHL, TET, SUL, TRP (AMC, GEN)
KL122 (n=1)	ND	D (P1)	1/1	>16 (n=1)	AMC, CIP, TET, SUL, TRP, COL
KL124 (n=1)	ND	D (F)	1/1	<2 (n=1)	CIP, TET, SUL, TRP
KL128 (n=2)	ND	C (P2), H (P2)	0/2	>16 (n=2)	CIP, SUL, TRP, COL (AMC, FOX, TET)
KL146 (n=5)	ST15 (n=2)	E (P2), H (P2)	5/5	4 – 16 (n=1)	CIP, GEN, TET, SUL, TRP (AMC, FEP, CAZ, CTX, COL)
				<2 (n=4)	
KL154 (n=1)	ND	D (P1)	1/1	<2 (n=1)	CIP, TET, SUL, TRP
KL155 (n=1)	ST6250 (n=1)	D (P1)	1/1	>16 (n=1)	AMC, GEN, TET, SUL, COL
KL16 KL27 KL46 (n=1	ND	B (P1)	1/1	<2 (n=1)	-
KL3 (n=3)	ND	D (P1, F)	3/3	<2 (n=3)	TET, TRP (AMC, CIP, SUL)
wzi590d (n=1)	ND	B (P1)	1/1	<2 (n=1)	-

[a] Multilocus Sequence Typing (MLST) obtained by Whole-Genome Sequencing (WGS), with sequence types indicated in bold. ND, not determined.

[b] Farms are designated by capital letters; Type of samples are designated by feces-P1, feces-P2, poultry meat-P3, feed-F and water-W.

[c] Abbreviations: AMC, amoxicillin+clavulanic acid; ATM, aztreonam; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; COL, colistin; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; GEN, gentamicin; SUL, sulphonamides; TET, tetracycline; TRP trimethoprim. A variable presence of antimicrobial resistance is presented between curved brackets - susceptible to all the tested antibiotics.

[d] *wzi* allele not associated with a known K/KL type.

Table S2. SNPs score matrix from the 1972 core-genome aligment of the sequenced *Klebsiella pneumoniae* (n=20) genomes analysed in this study.

Name	11_P1_26	13_P3_1	15_P2_24	15_W1_1	16_P2_28	17_P1_6	17_P1_7	17_P2_5A	1_P2_10	1_P3_6	3_P1_3	3_P2_1	5_P1_1	5_P2_10	5_P3_6	7_P1_5	7_P1_6	7_P2_19	8_F1_4	8_P1_5
11_P1_26	0	10318	10321	10321	11099	10126	11094	11096	11019	11022	10977	10133	10834	10285	10839	9889	9912	4	10951	10301
13_P3_1	10318	0	10195	10195	10832	10324	11082	10826	11046	11049	10714	10330	10871	10191	10872	9917	7929	10316	10985	10327
15_P2_24	10321	10195	0	0	11045	9953	10818	11042	11140	11143	11010	9961	10691	160	10693	9978	10054	10319	10856	9912
15_W1_1	10321	10195	0	0	11045	9953	10818	11042	11140	11143	11010	9961	10691	160	10693	9978	10054	10319	10856	9912
16_P2_28	11099	10832	11045	11045	0	10649	11822	11	11715	11718	206	10657	11659	11018	11663	10536	10694	11097	11744	10947
17_P1_6	10126	10324	9953	9953	10649	0	10413	10649	10863	10867	10550	45	10220	9962	10218	9942	10046	10124	10321	10276
17_P1_7	11094	11082	10818	10818	11822	10413	0	11831	12011	12014	11778	10422	320	10785	320	11048	10771	11092	725	11138
17_P2_5A	11096	10826	11042	11042	11	10649	11831	0	11695	11698	207	10657	11669	11016	11672	10540	10694	11094	11753	10951
1_P2_10	11019	11046	11140	11140	11715	10863	12011	11695	0	10	11602	10871	11813	11136	11822	10942	10969	11017	11849	10583
1_P3_6	11022	11049	11143	11143	11718	10867	12014	11698	10	0	11605	10874	11816	11140	11825	10945	10972	11020	11853	10586
3_P1_3	10977	10714	11010	11010	206	10550	11778	207	11602	11605	0	10558	11591	10993	11595	10480	10585	10975	11730	10888
3_P2_1	10133	10330	9961	9961	10657	45	10422	10657	10871	10874	10558	0	10229	9970	10227	9948	10054	10131	10329	10283
5_P1_1	10834	10871	10691	10691	11659	10220	320	11669	11813	11816	11591	10229	0	10657	2	10833	10500	10832	747	10966
5_P2_10	10285	10191	160	160	11018	9962	10785	11016	11136	11140	10993	9970	10657	0	10659	9963	10053	10283	10855	9897
5_P3_6	10839	10872	10693	10693	11663	10218	320	11672	11822	11825	11595	10227	2	10659	0	10832	10499	10837	745	10975
7_P1_5	9889	9917	9978	9978	10536	9942	11048	10540	10942	10945	10480	9948	10833	9963	10832	0	9945	9887	10924	10286
7_P1_6	9912	7929	10054	10054	10694	10046	10771	10694	10969	10972	10585	10054	10500	10053	10499	9945	0	9910	10675	10170
7_P2_19	4	10316	10319	10319	11097	10124	11092	11094	11017	11020	10975	10131	10832	10283	10837	9887	9910	0	10949	10299
8_F1_4	10951	10985	10856	10856	11744	10321	725	11753	11849	11853	11730	10329	747	10855	745	10924	10675	10949	0	11037
8_P1_5	10301	10327	9912	9912	10947	10276	11138	10951	10583	10586	10888	10283	10966	9897	10975	10286	10170	10299	11037	0

Given the dimension Table S3 and Table S4 are available at https://doi.org/10.1128/spectrum.01386-23

2. Persistence of *mcr-1*-carrying *E. coli* in rabbit meat production: Challenges beyond long-term colistin withdrawal.

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Persistence of *mcr-1*-carrying *E. coli* in rabbit meat production: Challenges beyond long-term colistin withdrawal

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ABSTRACT

Colistin, a last-resort antibiotic in human medicine, has been banned in European food animal production to mitigate antimicrobial resistance. This study investigates the long-term effects of the colistin ban on the occurrence and genomic features (WGS) of colistin-resistant, *mcr*-carrying *Escherichia coli* across intensive rabbit farms (8 farms, ~600 animals/farm, fecal and farm environmental samples) in the north and center of Portugal.

Colistin-resistant *E. coli* was detected in 25 % of groups from three farms in pre-slaughter fecal samples, with *mcr-1*-positive strains found throughout the lifecycle (does, offspring, and feed) in all fecal samples from one farm. A polyclonal multidrug-resistant (MDR) *E. coli* population carrying *mcr-1* persisted over three years, mostly in pre-slaughter rabbits but also in newly arrived younger does (GP). Comparative genomic analysis (cgMLST) revealed four clusters, with closely related strains between rabbit feces and feed (ST1196, ST40) and between feces and GP (ST1196), suggesting external reservoirs, biosecurity concerns, and cross-contamination. WGS also revealed high load and diversity in virulence (EPEC and ExPEC), antibiotic resistance and genes related to metal decreased susceptibility. All *mcr-1* genes were located on similar IncHI2 multireplicon plasmids, carrying *sil* + *pco* (copper) co-located with antibiotic resistance genes, and circulating in global sources. These results highlight that, despite colistin withdrawal, MDR *mcr*-carrying *E. coli* clones persist over three years in a single farm, underscoring complex co-selection pressure and biosecurity gaps. The findings underscore food safety risks via the food chain and environmental contamination. Enhanced biosecurity, feed monitoring, and One Health surveillance are essential to mitigate AMR dissemination and safeguard public health.

1. Introduction

The global health community faces an escalating crisis with the rise of antimicrobial resistance, particularly in Gram-negative bacteria (WHO, 2024a). This crisis has led to the re-emergence of colistin as a critically important antimicrobial that should be treated as a "last-resort" option, as indicated in the WHO's List of Medically Important Antimicrobials (WHO, 2024b). However, this reliance on colistin is under threat due to the widespread dissemination of mobile colistin resistance genes (*mcr*). Predominantly plasmid-borne in *Enterobacteriaceae*, these *mcr* genes have been identified across diverse ecosystems—from food-producing animals to human healthcare settings—raising significant concerns about their role in foodborne and environmental transmission (Liu et al., 2016; Sun et al., 2018; Zelendova et al., 2021).

In veterinary medicine, colistin has been extensively used over the past decades as a prophylactic and metaphylactic agent, particularly in rabbit farming, where the delicate balance of rabbit's intestinal microbiota is easily disrupted by stress and dietary challenges (Agnoletti et al., 2018; Freitas-Silva et al., 2018). It has been widely used to manage *Escherichia coli* infections in neonatal and post-weaning rabbits, which are highly susceptible to colibacillosis (Freitas-Silva et al., 2018; Silva

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et al., 2024). Rabbit farming in the European Union, the second-largest global producer of rabbit meat, is concentrated in the Iberian Peninsula, which serves as the primary contributor and a major consumer (Solans et al., 2019). These rabbit meat production systems typically operate under a continuous, closed production cycle, where all stages of rabbit development occur on the same farm, supported by a pelleted diet tailored to their nutritional needs (EFSA et al., 2020). However, concerns over colistin resistance have led to voluntary withdrawal of this antibiotic from the rabbit farming sector in some regions, including Portugal. Several studies have reported a decline in *mcr*-carrying bacteria shortly after colistin restrictions in various food-animal production settings, however there is limited European data on AMR in rabbit breeding (Rhouma et al., 2023; Ribeiro et al., 2021). Notably, no studies have evaluated the long-term impact of colistin restrictions on the occurrence and diversity of colistin-resistant bacteria throughout intensive rabbit meat productions (Massella et al., 2021).

This study aims to provide a comprehensive analysis of the occurrence, diversity and persistence of *mcr*-carrying *E. coli* across the entire intensive rabbit production chain to better understand the impact of long-term colistin ban. Whole-genome sequencing (WGS) was applied to characterize these isolates in terms of clonal diversity, genes associated with antibiotic resistance or metal decreased susceptibility, and virulence determinants. This approach was made to identify the drivers of resistance and environmental sources that could contribute to the spread of *mcr* genes within the rabbit farm environment.

2. Materials and methods

2.1. Study design

2.1.1. Farm selection and characterization

This study was conducted primarily between September 2020 and June 2021 (Period 1) on eight intensive rabbit farms located in the north and center of Portugal (arbitrarily designated as A to H) (Fig. 1). All selected rabbit farms had closed maternity and fattening lines, housing animals in conventional metal cages equipped with automatic feeders. Reproduction was carried out through artificial insemination. All farms implemented biosecurity measures, including wildlife control, footbaths, rabbits' vaccination, and disinfection practices. Five farms (A, B, D, E, F and H) had sanitary vacancy period of 6–7 days, while two farms (C and G) had of 2–3 days.

The rabbit farms, including A, B, E, F, and H (hosting 500–1000 reproductive females, known as does) and C, D, and G (hosting 1000–2000 does), were selected based on their geographical location and recent history of colistin use, with all farms having implemented a ban on colistin as both a prophylactic and therapeutic agent. Three of these farms (A, D, and E) had implemented a colistin ban within the two years before the study (2y_colban), while the remaining five (B, C, F, G, and H) had instituted the ban in the year immediately preceding the beginning of the study (1y_colban). Colistin had been the primary therapeutic agent against colibacillosis on all farms for several years, before being replaced by other antibiotics such as sulfonamides and tetracyclines in maternity, and valnemulin and tiamulin (pleuromutilins class), bacitracin and apramycin in fattening lines. Although farm selection was not randomized, a purposive sampling approach was applied to include farms with different durations since colistin withdrawal. This allowed the study to explore the potential persistence of *mcr*-positive bacteria under varied field conditions, while acknowledging that the absence of randomization may limit the generalizability of the findings.

All the feeds were supplemented with various metals, including copper sulfate and zinc oxide, to ensure that rabbits' physiological and nutritional requirements were met.

Farms that tested positive for *mcr*-carrying bacteria were subsequently resampled in the following years, specifically in 2022 (Period 2) and 2023 (Period 3).

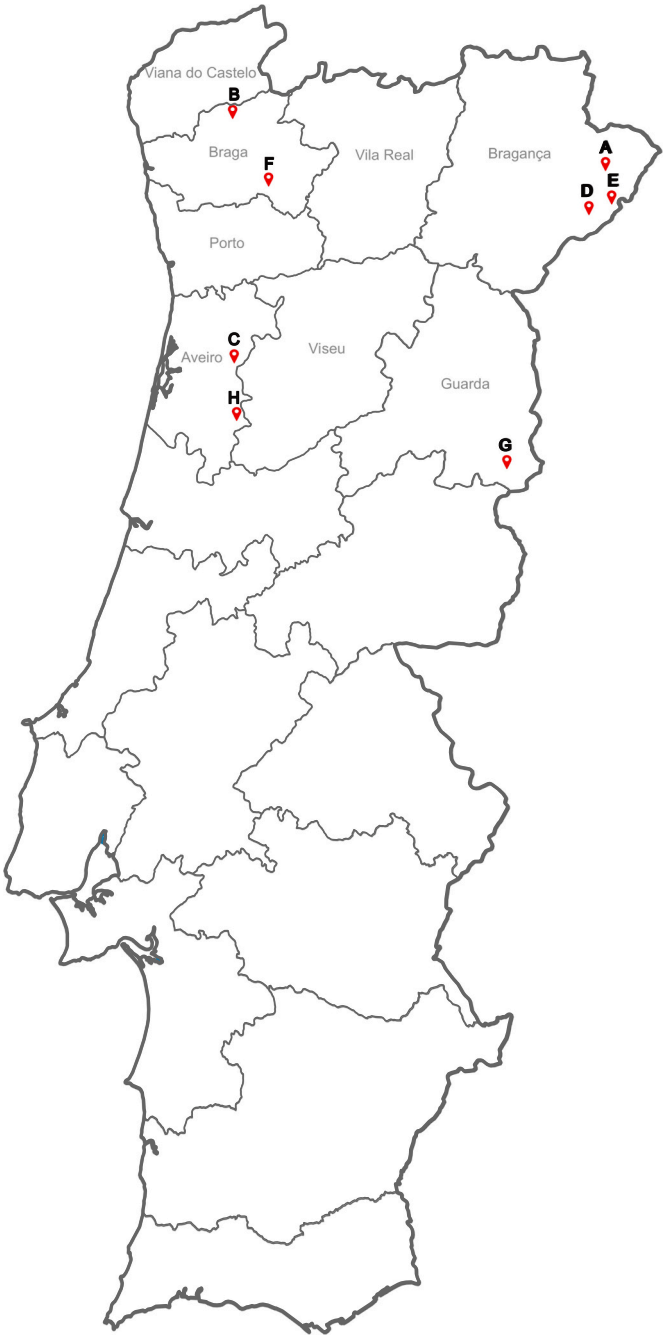

Fig. 1. Geographical localization of the sampled rabbit farms, farm A to H.

2.1.2. Animal group selection

At each farm, we selected two groups of healthy does from the maternity line, with each group consisting of six cages, and each cage housing one doe. Aseptic nets were placed under the rabbit cages to collect pooled fecal samples from these does. A total of 16 groups across the farms were categorized based on the reproductive history of the does: one group included six older does with a history of 12 to 14 births (referred to as "Old"), and the other group comprised six younger does with 2 to 4 births (referred to as "Young"). In the five 1y_colban farms, the older does had prior exposure to colistin, while the younger ones did not. Conversely, at the remaining three 2y_colban farms, neither the older nor the younger does have been exposed to colistin. At all farms, the offspring from each selected group (comprising six groups of 10 to 12 rabbits each, totaling 60 to 70 rabbits) were collectively transferred to six adjacent cages in the fattening line. This transfer facilitated the

collection of fecal sample pools.

2.2. Sample collection

During Period 1, two visits were made to each farm, where rabbit fecal pooled samples were collected using a non-invasive technique. Sterile spatulas were employed to collect freshly voided dropping feces (approximately 50 g each; total $n = 48$) into aseptic nets placed under the cages. Environmental samples were also collected in each farm, including stored feed for does, as well as for weaning and pre-slaughter rabbits (approximately 100 g; $n = 16$ samples), stored nest material (approximately 100 g; $n = 8$ samples), and water from the entrance of the farm (1 L; n = 8 samples), end of the maternity line (1 L; n = 8 samples), and end of the fattening line (1 L; n = 8 samples). The first visit (T1) took place the day after the weaning of the rabbits (aged 30–39 days). Pooled fecal samples were collected from the 16 groups of does (M; at the maternity line) and from their recently weaned offspring (R1; at the fattening line), along with environmental samples (Fig. 2; Table 1). The second visit (T2) took place before the slaughter of the rabbits (aged 60–80 days), and pooled fecal samples were collected from the 16 groups of pre-slaughter rabbits (R2; at the fattening line) (Fig. 2; Table 1).

Farms testing positive for *mcr*-carrying bacteria underwent re-sampling in subsequent years. In Period 2 (2022), the sampling strategy was consistent with the original approach, involving two subsequent visits with the same collection points and sample types. In Period 3 (2023), only a single visit was conducted, coinciding with the arrival date of new younger does (GP) (Table 1). All farms' received GP does from the same Portuguese supplier, except for farm C, which obtained does from a different national GP supplier. During this visit, pooled fecal samples were obtained from two groups of does (M, comprising both old and young) and their pre-slaughter-age offspring (R2), using previously described methods. Additionally, fecal samples were collected from individual GPs upon their arrival at the farm ($n = 10$ samples) (Table 1).

Personal protective equipment, including gloves, boots, and coveralls, was used. All samples were collected in sterile containers, transported at 4 °C, and processed on the same day at the laboratory. The subsequent cultural and molecular approaches are described in the following sections.

Table 1
Overview of samples collected in rabbit farms across the three sampling periods.

Period (farms)	Visit	Sample type	Sample source	Sample size
Period 1 2020/2021 (A–H)	T1	Fecal	Does[a] (M)	n = 16
			Post-weaning rabbits (R1)	*n* = 16
		Environmental	Feed	n = 16
			Nest Material	n = 8
			Water[b] (W)	*n* = 24
	T2	Fecal	Pre-slaughter rabbits (R2)	n = 16
Period 2 2022 (C)	T1	Fecal	Does[a] (M)	n = 2
			Post-weaning rabbits (R1)	*n* = 2
		Environmental	Feed (does)	n = 2
			Nest Material	n = 1
			Water[b]	*n* = 3
	T2	Fecal	Pre-slaughter rabbits (R2)	n = 2
Period 3 2023 (C)	T1	Fecal	Does[a] (M)	n = 2
			Pre-slaughter rabbits (R2)	n = 2
			New younger does (GP)	n = 10
		Environmental	Feed	n = 2
			Nest Material	n = 1
			Water[b]	n = 3

[a] In each farm, two groups of does were selected: older does ($n = 6$, with a history of 12 to 14 births) and their offspring, and younger does (n = 6, with a history of 2 to 4 births) and their offspring.
[b] In each farm, water samples were collected from three distinct areas: the entrance, maternity line, and fattening line.

2.3. Screening of mcr-carrying E. coli

The initial processing step involved weighing 25 g portion of each solid sample or collecting 0.45-μm filter from the filtration of 100 mL of water samples. These were then added to 225 mL of Buffered Peptone Water (BPW), with and without 3.5 mg/L of colistin. This was followed by a 1-h resuscitation step at room temperature. For the direct cultural method, 100 μL of BPW + sample and BPW + colistin+sample were each spread onto Tryptone Bile X-glucuronide agar plates (TBX) containing colistin (3.5 mg/L) and incubated at 37 °C for 24 h to detect *E. coli*.

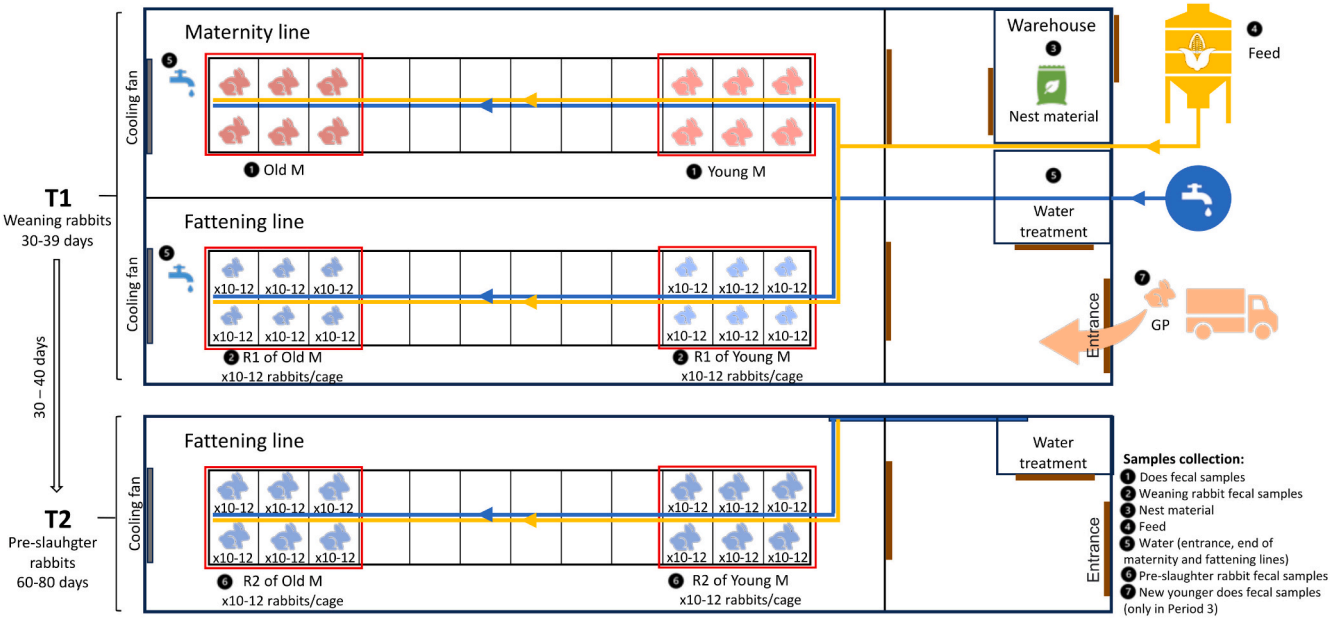

Fig. 2. Sampling strategy at rabbit farm plant. Sample collection points are indicated by numbers. M, does; R1, weaning rabbits; R2, pre-slaughter rabbits; GP, new younger does.

Concurrent with this, an estimation of *E. coli* (cfu/g) in each solid sample was conducted using the drop plate technique, which involved placing three 10 μL drops of a standard serial 10-fold dilution from BPW + sample onto TBX plates, followed by counting the typical *E. coli* colonies. Also, in each water sample, 100 mL was filtered using 0.45-μm filters and placed on TBX. For the enrichment cultural method, the BPW + colistin+sample was incubated at 37 °C for 16–18 h before spreading a 10 μL aliquot onto TBX plates with colistin (3.5 mg/L). Subsequently, one to five colonies of each presumptive morphotype on TBX + colistin plates were spread onto a CLED plate to isolate presumptive *E. coli* colonies.

Isolate identification was performed using Matrix-Assisted Laser Desorption-Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS VITEK MS, bioMérieux, France) and/or standard PCR (Wang et al., 1997). For all recovered isolates identified as *E. coli*, colistin resistance genes (*mcr-1* to *mcr-5* and *mcr-6* to *mcr-9*) were searched using two multiplex PCRs, respectively (Rebelo et al., 2018; Borowiak et al., 2020). The minimal inhibitory concentration (MIC) for colistin was determined in all *E. coli* isolates using the European Committee of Antimicrobial Susceptibility Testing (EUCAST) reference broth microdilution method (EUCAST, 2016).

2.4. Phenotypic and genotypic characterization of mcr-carrying E. coli

Antibiotic susceptibility profiles were determined by disk diffusion for the following antibiotics: amoxicillin-clavulanic acid (30 μg), ampicillin (10 μg), aztreonam (30 μg), cefepime (30 μg), cefoxitin (30 μg), ceftazidime (10 μg), cefotaxime (5 μg), imipenem (10 μg), ciprofloxacin (5 μg), gentamicin (10 μg), chloramphenicol (30 μg), tetracycline (30 μg), sulfonamides (300 μg), and trimethoprim (5 μg). Interpretation was conducted according to the guidelines provided by the EUCAST (EUCAST, 2022). When this was not possible, the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2022) were followed. Multidrug resistance (MDR) was considered when the isolates were resistant to three or more antibiotics from different families (Magiorakos et al., 2012). The relatedness of *mcr*-positive *E. coli* isolates from the same or different samples was investigated by determining their phylogenetic groups (PhG) using a standard multiplex PCR (Clermont et al., 2013) and *XbaI* pulsed-field gel electrophoresis (PFGE) following the protocol of the Centers for Disease Control and Prevention (CDC, 2017). Plasmids were classified and characterized using the PCR-based typing technique for the most common *mcr*-carrying plasmid replicons IncHI2, IncI2, and IncX4 (Carattoli et al., 2005; Johnson et al., 2012).

2.5. Whole-genome sequencing for characterization of mcr-carrying E. coli

Representative isolates of *mcr*-carrying *E. coli* ($n = 13$), selected from different samples, but prioritizing those from external-farm environment samples, PhG, PFGE profiles, and periods, were chosen for whole-genome sequencing (WGS) analysis. The DNA was extracted with the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI) and the final concentration was measured with Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA) and sequenced with Illumina NovaSeq 6000 S4 PE150 XP (Illumina, San Diego, CA) at Eurofins Genomics (https://eurofinsgenomics.eu/). The quality of the raw reads was assessed with FastQC v0.12.1 (Andrews, 2010) and MultiQC v1.14 (Ewels et al., 2016), using default parameters. High-quality raw reads were then de novo assembled using SPAdes v3.15.5 (Bankevich et al., 2012) and the assembly quality and completeness were assessed with QUAST v5.2.0 (Gurevich et al., 2013) and BUSCO v5.4.4 (Seppey et al., 2019), respectively. The assemblies were annotated using the RAST server (Aziz et al., 2008). An *in-house* database of proteins codified by acquired genes associated with metal decreased susceptibility – including ArsR1H-ArsD1A1A2-ArsCBA3D2R2, PcoGE1ABCDRSE2, SilESRCFBAGP, MerRTPCADE, and TerZABCDEF-TerWY1XY2Y3 – was used as a reference. The BLASTX 2.14.0+ (Camacho et al., 2009), with additional parameters "-evalue 0.001 -query_gencode 11", was employed to perform the sequence alignment of the bacterial genomes against this database. Tools from the Centre for Genomic and Epidemiology (http://www.genomicepidemiology.org) were used to evaluate *E. coli* antibiotic resistance genes (ResFinder v4.1) or known mutations (PointFinder v4.1) (Zankari et al., 2017; Bortolaia et al., 2020; Camacho et al., 2009), virulence genes (VirulenceFinder v2.0) (Joensen et al., 2015; Malberg Tetzschner et al., 2020; Camacho et al., 2009), plasmid replicons (PlasmidFinder v2.1) (Camacho et al., 2009; Carattoli et al., 2014) and plasmid typing (pMLST v2.0) (Carattoli et al., 2014). Tools from EnteroBase v5.1 were used to define Multilocus Sequence Typing (Zhou et al., 2020) and core genome Multilocus Sequence Typing (cgMLST) (Zhou et al., 2020). *E. coli* phylogenetic groups were confirmed using ClermontTyper (http://clermontyping.iame-research.center/).

To confirm the location of the *mcr-1* gene and generate hypothetical plasmid reconstructions from draft assemblies, we utilized the MOB-recon tool v3.1.0 from the MOB-suite package (Robertson et al., 2020; Robertson and Nash, 2018). In cases where the *mcr-1* gene was identified by MOB-recon in a plasmid or found on the same contig as the replicon/incompatibility determinant, they were regarded as part of a plasmid.

2.6. Comparative genomic analysis of mcr-1-carrying E. coli

A comparative genomic analysis was performed using core-genome MLST (cgMLST; *E. coli* scheme comprising 2513 loci) to compare our isolates among themselves and with genomes retrieved from Enterobase (https://enterobase.warwick.ac.uk/) (Achtman et al., 2022) as well as the Hierarchical Clustering (HierCC) of cgMLST (Zhou et al., 2020). These strains were used to develop a minimum spanning tree using the GrapeTree tool (Zhou et al., 2018) and the MSTreeV2 algorithm. Metadata of the included *E. coli* isolates were retrieved from Enterobase (isolate name, cgST, country, year, source). Additionally, a search of antibiotic resistance was conducted as described in the previous section.

3. Results

3.1. Detection of mcr-1 carrying E. coli by farm and samples

We detected *E. coli* in all farms and in all fecal samples collected at every stage of the rabbit production chain, with *E. coli* levels ranging from 1.0×10^5 to 1.5×10^9 cfu/g. In water samples, *E. coli* was detected in two farms (A and G), ranging from 3 to 100 cfu/mL. Colistin-resistant *E. coli* isolates were recovered from three (C, E, and F) out of the eight farms studied, with a common detection in pre-slaughter fecal rabbit samples (25 % – 4/16 groups; farm C – 2 groups, offspring of older and younger does; farm E – 1 group, offspring of younger does; and farm F – 1 group, offspring of older does). Notably, only farm C presented *mcr*-carrying *E. coli* isolates ($n = 28$), being those isolates detected in all does fecal samples (both age groups, older does: $n = 3$ and younger does: $n = 1$) and their offspring, at weaning (R1: $n = 13$, offspring of older and younger does) and pre-slaughter (R2: $n = 8$, offspring of older and younger does) stages (Table 2). Concerning environmental samples, only one feed sample from farm C was contaminated with colistin-resistant and *mcr*-carrying *E. coli* isolates (n = 3) (Table 2). No colistin-resistant *E. coli* or *mcr*-carrying *E. coli* were detected in water and nest material samples.

Our culture approach detected 32 colistin-resistant *E. coli* isolates (all with a $MIC_{COL} = 4$ mg/L), including 28 carrying the *mcr-1* gene. These 28 isolates were recovered using TBX + colistin plates, either following an enrichment step with BPW + colistin (n = 13 isolates from 5 samples), or after a 1-hour resuscitation step (BPW + sample: n = 8 isolates from 5 samples or BPW + colistin+sample: $n = 7$ isolates from 5 samples) (Supplementary Table S1).

Table 2
Detection and characterization of colistin-resistant and *mcr-1*-carrying *E. coli* (*n* = 70) from Farm C across the three sampling periods.

Period and year	Does group (age)[a]	Sample source[b] (no. of isolates)	PhG (no. of isolates)	PFGE profiles[c]	Antimicrobial phenotype other than colistin[d]
Period 1 2020/2021	G5 (Old)	Does (M) (n = 3)	B1 (n = 3)	G	AMP, CIP, GEN, TET, CHL, SUL, TRP
		Post-weaning rabbits (R1) (n = 7)	B1 (n = 6) E (n = 1)	A, B, D, E, F C	AMP, CIP, GEN, TET, CHL, SUL, TRP, (AMC)
		Post-weaning rabbits (R2) (n = 6)	B1 (n = 3) E (n = 3)	**H**, I, **J** K	AMP, CIP, GEN, TET, CHL, SUL
	G6 (Young)	Does (M) (n = 1)	B1 (n = 1)	**J**	AMP, CIP, GEN, TET, SUL, TRP
		Post-weaning rabbits (R1) (n = 6)	B1 (n = 5) E (n = 1)	**L**, M, N, O P	AMP, CIP, GEN, TET, SUL, TRP, (AMC, CHL)
		Post-weaning rabbits (R2) (n = 2)	B1 (n = 2)	**H**	AMP, GEN, TET, CHL, SUL
		Feed (n = 3)	B1 (n = 3)	**H**, **L**	AMP, CIP, GEN, TET, CHL, SUL, (TRP)
Period 2 2022	G17 (Old)	Does (M) (n = 7)	B1 (n = 5) E (n = 2)	T, U, X, NT V	AMP, CIP, GEN, TET, CHL, SUL, TRP
		Post-weaning rabbits (R1) (n = 6)	B1 (n = 4) E (n = 2)	**R**, S Q	AMP, CIP, GEN, TET, CHL, SUL, TRP
		Post-weaning rabbits (R2) (n = 7)	B1 (n = 7)	**R**, AC, AD, AE	AMP, CIP, GEN, TET, CHL, SUL, TRP, (AMC)
	G18 (Young)	Does (M) (n = 4)	B1 (n = 4)	AB, AG	AMP, CIP, GEN, TET, CHL, SUL, TRP
		Post-weaning rabbits (R1) (n = 3)	B1 (n = 1) E (n = 2)	W AH, **AI**	AMP, CIP, GEN, TET, CHL, SUL, TRP, (AMC)
		Post-weaning rabbits (R2) (n = 4)	B1 (n = 1) E (n = 3)	AF AA	AMP, CIP, GEN, TET, CHL, SUL, TRP, (AMC)
Period 3 2023	G19 (Old)	Post-weaning rabbits (R2) (n = 8)	B1 (n = 8)	**R**, AJ, AK, **AL**, AM, AN	AMP, CIP, GEN, TET, CHL, SUL, TRP
	G20 (Young)	Post-weaning rabbits (R2) (n = 2)	B1 (n = 2)	**AL**	AMP, CIP, GEN, TET, CHL, SUL, TRP
		New younger doe GP (n = 1)	B1 (n = 1)	**AI**	AMP, CIP, GEN, TET, SUL, TRP

[a] An older doe has a history of 12 to 14 births, while a younger doe has a history of 2 to 4 births.

[b] M: Doe's pooled fecal sample; R1: Post-weaning rabbit pooled fecal sample; R2: Pre-slaughter rabbit pooled fecal sample; GP: New younger doe's fecal sample.

[c] PFGE types were designated with letters from A to AN; NT represents non-typeable profiles. Identical PFGE patterns are highlighted in bold, and PFGE profiles sent for WGS analysis are underlined.

[d] AMC, Amoxicillin + Clavulanic acid; AMP, Ampicillin; CHL, Chloramphenicol; CIP, Ciprofloxacin; GEN, Gentamicin; SUL, Sulphonamides; TET, Tetracycline; TRP, Trimethoprim. The variable presence of antibiotic resistance is presented between curved brackets.

3.2. Follow-up study of the farm with mcr-1-carrying E. coli

In the subsequent year (Period 2), we found a similar occurrence of *mcr-1*-carrying *E. coli* on farm C, with all rabbit groups testing positive at all stages (does: *n* = 11 isolates; R1: *n* = 9; R2: n = 11) (Table 2). However, none of the environmental samples tested positive, contrary to what was found in Period 1 (one feed-positive sample). In the second follow-up (Period 3), only the two rabbit groups at the pre-slaughter (R2: *n* = 10 isolates) stage were positive for *mcr-1*-carrying *E. coli*. Interestingly, the *mcr*-1 gene was also present in *E. coli* recovered from newly arrived younger does (GP) at farm C for reproduction (Table 2).

A total of 70 *E. coli* carrying *mcr-1* were recovered from the 16 positive samples during the three periods at farm C. The three cultural tested approaches (Supplementary Table S1) were found to be complementary, with each showing a similar rate of detection of samples contaminated with *mcr-1* carrying *E. coli*.

3.3. Diversity and antimicrobial resistance of mcr-1-carrying E. coli

The 70 *E. coli* isolates harboring the *mcr-1* gene belonged to PhG B1 (*n* = 56) or E (*n* = 14). Despite originating from the same farm, they were grouped into 38 distinct PFGE profiles (B1, 30 profiles, and E, 8 profiles) (Table 2). Some isolates with identical PFGE profiles were identified across different samples (PFGE-L: post-weaning rabbit feces and feed; PFGE-J: pre-slaughter and does rabbit feces; PFGE-R: post-weaning and pre-slaughter rabbit feces) or groups (PFGE-H: pre-slaughter rabbit feces, G5, and G6; PFGE-AL: pre-slaughter rabbit feces, G19, and G20), within the same year. Furthermore, some PFGE-profiles, such as R and AI, were detected over time (PFGE-R - Period 2: G17- post-weaning rabbit feces, G17- pre-slaughter rabbit feces and Period 3: G19- pre-slaughter rabbit feces; PFGE-AI - Period 2: G18- post-weaning rabbit feces and Period 3: new younger doe's feces) (Table 2). Of note, the detection of this high diversity of PFGE profiles was only achievable using our cultural approach, with and without enrichment step (Supplementary Table S1).

All *mcr-1*-carrying *E. coli* obtained in this study presented a multidrug-resistant (MDR) profile, independently of the sample type, year, and PFGE profile (Table 2). All the isolates were resistant to ampicillin, gentamycin, tetracycline, and sulfonamides, with most of them additionally showing resistance to ciprofloxacin (97 %), chloramphenicol (96 %), and trimethoprim (89 %). However, no resistance to extended-spectrum cephalosporins or carbapenems was detected.

3.4. Whole-genome sequence analysis of mcr-1 carrying E. coli

The 13 sequenced isolates, representing both external and internal farm environments, were assigned to four sequence types (STs): B1-ST1196 (*n* = 7), B1-ST1589 (*n* = 3), B1-ST40 (*n* = 2), and E-ST1011 (n = 1). Comparative genomic analysis, based on the core genome multilocus sequence type (cgMLST) (Enterobase), revealed that the three STs from PhG B1 corresponded to three clusters, each comprising closely related strains (<20 allelic differences) detected in different samples and years (Fig. 3). Both clusters ST1196 and ST1589 included isolates from the 3 periods. Notably, in cluster ST1196, isolates from rabbit feces were genetically identical to the isolate found in feed (0

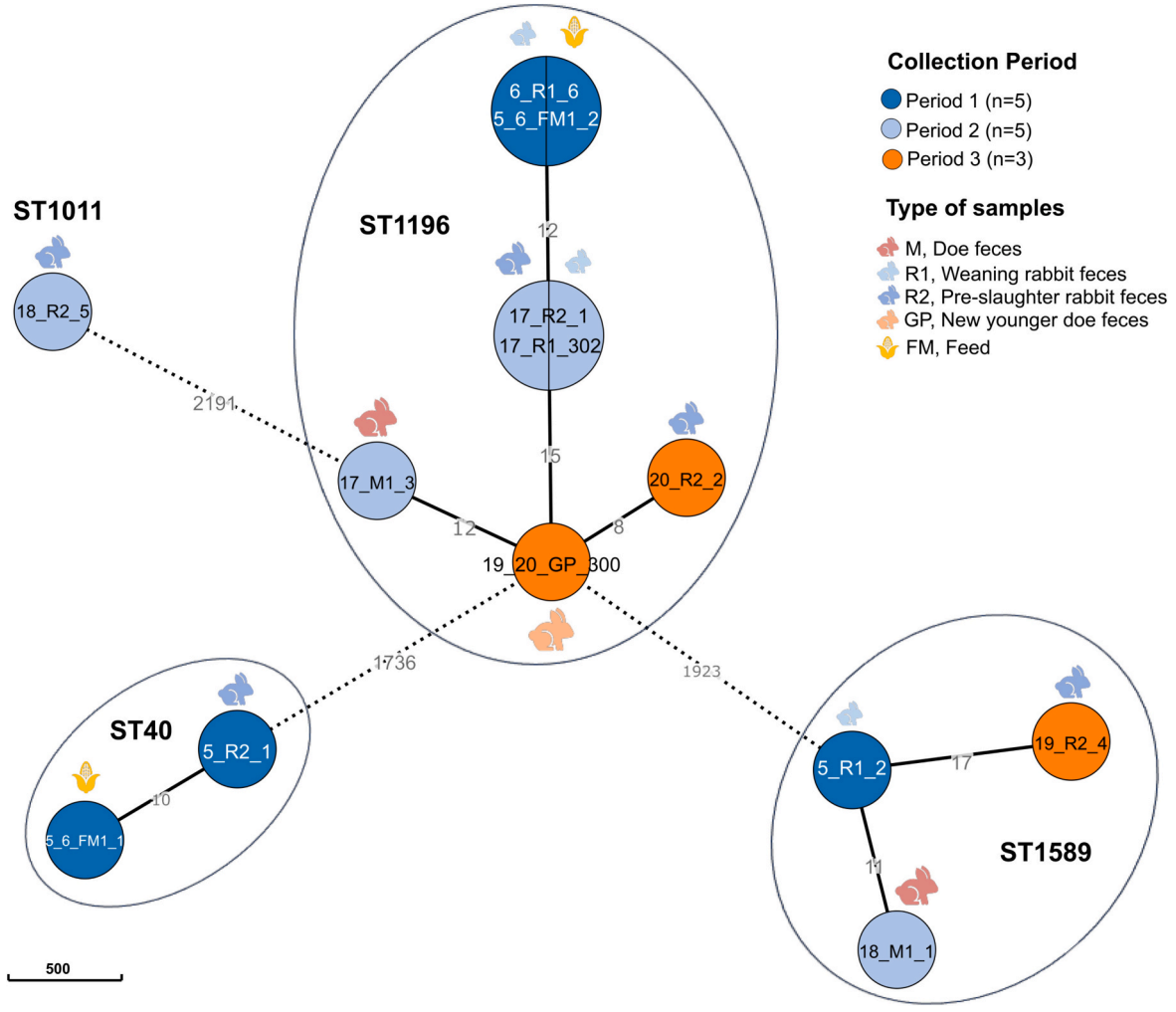

Fig. 3. Grape Tree of the 13 *mcr-1* carrying *E. coli* genomes obtained in farm C, over the three consecutive periods. The three clusters obtained were identified according to the ST (ST1196: HC20-cgST256181, ST40: HC20-cgST256413, ST1589: HC20-cgST256180). The core genome Minimum Spanning Tree (MST) was created within the Enterobase pipeline using the GrapeTree tool and the MSTreeV2 algorithm. The name of the isolates is indicated in each node. The scale bar corresponds to the number of cgMLST allelic differences.

allelic differences) and highly similar to newly arrived younger doe (GP) isolate (8–15 allelic differences). In the ST40 cluster, rabbit fecal isolates also showed high similarity to feed isolate (10 alleles differences) (Fig. 3). The phylogenetic analysis also revealed genetic relationships between our genomes and others from different sources, regions, and time frames, available in EnteroBase (until 6 October 2024). Based on the Hierarchical Clustering (HierCC), ST1196 genomes grouped within the HC50-3125 group, along with 179 globally dispersed isolates from diverse sources (humans, companion animals, livestock, environment), including a livestock isolate from Portugal (Supplementary Table S2). Also, ST40 genomes and ST1011 genomes obtained in this study, and present in distinct groups (ST40: HierCC HC50-136585 group, ST1011: HierCC HC50-6862 group), share HC50 with genomes from humans, environment, and livestock from Europe, including wild rabbits in Italy (ST40) and cat feces in Portugal (Lisbon) (ST1011) (Supplementary Tables S3, S4). In contrast, ST1589 genomes grouped within HC100, alongside human isolates from the Netherlands and China. Notably, three of these genomes carried both *mcr-1.1* and $bla_{CTX\text{-}M\text{-}65}$ genes (Supplementary Table S5).

WGS analysis revealed that all isolates were enriched in diverse virulence genes coding for adhesins, capsules, siderophores, toxins, protectins, bacteriocins, and various other putative virulence and/or colonization-associated markers. The ST1011 (*chuA*, *ompT*, *terC*, *traT*), ST1589 (*ompT*, *papC*, *terC*, *traT*) and ST40 (*cvaC*, *hlyF*, *iroN*, *ompT*, *sitA*, *terC*, *traT*) carried diverse extraintestinal pathogenic *E. coli* (ExPEC) virulence genes (Denamur et al., 2021; Malberg Tetzschner et al., 2020) (Fig. 4). The ST40 also carried enteropathogenic *E. coli* (EPEC) (*eae*, *espA*, *espB*, *espF*, *espJ*, *espY*, *tccP*, *tir*) virulence genes (Garmendia et al., 2005) (Fig. 4).

Concerning antibiotic resistance genes, in addition to *mcr-1* gene, diverse acquired genes ($n = 19$ encoding resistance to six classes were detected [aminoglycosides: *aac*(3)/*aadA*/ant(3″)/*aph*, beta-lactams: $bla_{TEM\text{-}1}$, phenicols: *catA*/*cmlA*, trimethoprim: *dfrA*, sulfonamides: *sul*1/*sul*2/*sul*3, and tetracyclines: *tet*(A)] (Table 2 and Fig. 4). Most of the genomes carried genes conferring resistance to the six detected antibiotic classes, except ST40 isolates which lack *dfrA* genes, and one ST1196 isolate without any phenicol resistance genes (Fig. 4). Resistance to ciprofloxacin, through chromosomal mutations in the quinolone resistance determining region (QRDR) of topoisomerase genes *gyrA* (S83L and D87N), *parC* (S80I), and *parE* (S458A), were identified in almost all isolates. The mutations of *gyrA* (D87N) and *parC* (S80I) genes were observed in all genomes except for ST40, which had another *gyr*A gene mutation (S83L). Also, all ST1589 genomes lack *parE* (S458A) gene mutation (Fig. 4). Diverse acquired metal gene clusters encoding decreased susceptibility to copper/silver (*pco/sil*), mercury (*mer*), and tellurite (*ter*) were detected in almost all the genomes, with ST1589 isolates lacking tellurite operons (Fig. 4).

The isolates, on average, presented 3 plasmids per genome (ranging

Fig. 4. Heatmap generated using the Morpheus online webserver (https://software.broadinstitute.org/morpheus/). It represents the distribution of virulence-associated (VirulenceFinder), genes coding for antibiotic resistance (ResFinder), and metal decreased susceptibility (*in-house* database), as well as plasmid replicons associated with well-defined incompatibility groups in 13 sequenced *Escherichia coli* genomes. Only known mutations conferring fluoroquinolone resistance are presented. Colored circles indicate the presence of genes/plasmid replicons sorted by category. Truncated genes are highlighted in light brown. The virulence-associated gene content associated with ExPEC is indicated in black bold and the virulence-associated gene content associated with EPEC is indicated in green bold. Plasmids carrying the *mcr-1.1*, which are also co-located with virulence and genes coding for antibiotic resistance and metal decreased susceptibility are indicated with bold outline circles.

from 2 to 6). These plasmids belonged to 9 distinct plasmid incompatibility groups, with 3 to 7 replicon or multireplicon types per genome (Fig. 4 and Supplementary Table S6). All the genomes carried the *mcr-1* in plasmids of the IncHI2 family, often in different combinations with FIA, FIB, or HI1B (Fig. 4). IncHI2/FIA was the most prevalent multireplicon associated with the *mcr-1* gene (46 %, $n = 6/13$), being dispersed across time (Periods 1 and 2) and clones (ST1011, ST1196, and ST1589), compared with IncHI2/FIB (13 %, $n = 2/13$) and IncHI2/HI1B (8 %, 1/13). Besides the *mcr-1* gene, these multireplicon plasmids co-carried other antibiotic resistance genes [e.g., *aac(3)*, *aadA1*, *aadA2*, *aph*, bla_{TEM-1}, *catA*, *cmlA*, *sul*, *tet(A)*] and gene clusters coding for metal decreased susceptibility (e.g., *pco*, *sil*, *ter*, *mer*) (Fig. 4). According to MOB-Recon, all *mcr-1*-carrying IncHI2 plasmids detected in this study shared the same primary (AA738), and with the exception of 18_R2_5 (AJ047), the same secondary (AJ049) MOB-cluster, demonstrating highly similar *mcr*-plasmid backbones (Supplementary Table S6). Additionally, these plasmids were also similar to others (mash distance = 0.006 to 0.020) described in animals and humans in different countries (CP019019, CP045449, MH522420, KR653209).

4. Discussion

This study provides a comprehensive investigation of *mcr*-carrying *E. coli* occurrence and diversity across intensive rabbit meat farming, encompassing a substantial number of animals (all pooled feces samples represent ~600 animals/farm) and several external sources (feed, water, nest material, and GP), following a long-term colistin withdraw. Over a three-year follow-up, we observed the persistence of MDR *mcr-1*-carrying-*E. coli* clones and plasmids with clinical relevance. The detection of genetically similar *E. coli* strains in rabbits' feces and external farm sources, such as feed and GP feces, alongside *mcr-1* genes on mobilizable multireplicon plasmids, suggests possible pathways for the introduction and spread of the *mcr-1* gene within farms, even without colistin use.

Intensive rabbit meat production was associated with increased antibiotic pressure, including the use of colistin, when compared to other livestock systems such as those involving ruminants (Silva et al., 2023; Massella et al., 2021). The delicate intestinal microbiota of rabbits, combined with the prevalence of gut health issues, the stress from intensive production and the challenges of adapting to high-density nutritional diets - particularly in the post-weaning phase - contributed to the use of antibiotics (Agnoletti et al., 2018; EFSA et al., 2020; EMA & ESVAC, 2022), such as pleuromutilins, bacitracin zinc, aminoglycosides, sulfonamides and tetracyclines on the studied farms. This, in turn, fosters the development of antibiotic-resistant bacteria, including *E. coli* resistant to colistin - a critical antibiotic used for decades for treating colibacillosis in rabbits (Freitas-Silva et al., 2018; Agnoletti et al., 2018; Cunha et al., 2017).

Portugal, a significant producer and consumer of rabbit meat, experienced concerns since these animals have the highest consumption of antimicrobial agents (Silva et al., 2024), and a study conducted by Freitas-Silva et al. (2018), before the colistin ban, detected *mcr-1* genes in necropsied rabbits from Portuguese intensive farms. These findings, combined with the increased scrutiny on antibiotic use in food-producing animals in the last years, prompted the veterinary community, in alignment with European legislation, to implement a voluntary withdrawal of colistin use by Portuguese rabbit producers in 2019 (DGAV, 2022). In our study, conducted more than two years after the voluntary colistin withdrawal, colistin-resistant *E. coli* was detected in three out of the eight farms tested. Importantly, the detection of these colistin-resistant strains was independent of both the duration of colistin withdrawal (whether the farms had undergone withdrawal for 1 or 2 years) and the categorization of does into older or younger groups. In contrast, *mcr-1*-carrying *E. coli* was only found in one of the three farms with colistin-resistant strains, and was detected in both older does, which had been previously exposed to colistin, and younger does, which had never been exposed to the antibiotic. This represents a lower occurrence compared to findings from a study in Italy conducted in 2014–2015, when colistin was still in use and 47 % of the intensive rabbit production farms ($n = 15/32$) were positive for *mcr*-carrying *E. coli* (Agnoletti et al., 2018). These differences may suggest an impact of the colistin withdrawal on reducing *mcr*-mediated resistance, however additional factors could also play a role. Despite limited comprehensive baseline studies on rabbit farms before the colistin ban, our findings align with recent studies suggesting that banning colistin in diverse food-animal productions has been beneficial in limiting the spread of *mcr* genes (Rhouma et al., 2023; Ribeiro et al., 2021).

While several studies have demonstrated the presence of *mcr-1*-carrying *E. coli* in food-producing animals (Freitas-Silva et al., 2018; Ribeiro et al., 2021; Agnoletti et al., 2018), investigations into the role of feed and other production/environment factors remain limited, despite their potential impact in food-animal intestinal microbiota (Davies and Wales, 2019). In this study, the detection of closely related *mcr-1*-carrying *E. coli* clones over a three-year period in one farm in rabbit feces, spanning early and pre-slaughter stages, as well as in feed and new arriving GP (external environment), suggests that external sources may continuously reintroduce those strains, indicating multiple sources of contamination/dissemination. These events appear linked to farm management practices, including cleaning protocols, water quality, feed handling, and the vacancy period. Once introduced, these strains likely contribute to cross-contamination within the farm, facilitated by workers, equipment and surfaces. The closed intensive rabbit production system, where full all-in/all-out practices are often unfeasible, combined with hygiene failures, the long lifespan of does (1½ years, 12–14 births) and extended contact with offspring (30–35 days), as observed in our study, further amplify the risk of bacterial transmission, including antimicrobial resistant strains (van der Sluis et al., 2024; EFSA et al., 2020; EFSA et al., 2021). Farm C exhibited specific biosecurity challenges, notably the absent of a quarantine room, which increases the risk of introducing and spreading new bacterial strains from incoming animals (Davies and Wales, 2019; van der Sluis et al., 2024). Additionally, the short sanitary vacancy period (2 to 3 days) limited the opportunity for proper disinfection of cages and hindered effective movement controls for does, allowing antimicrobial-resistant strains to persist within the farm environment.

The persistence of some *E. coli* clones throughout the rabbits' lifecycle and across years, also indicates their resilience and ability to adapt to the farm environment, including antimicrobial usage practices. The ban of colistin in certain farms has, in fact, led to the use of other antimicrobials such as tetracyclines, sulfonamides, pleuromutilins (valnemulin and tiamulin), aminoglycosides, bacitracin and zinc to maintain animal health and growth, as reported by other authors (Mourão et al., 2024; Silva et al., 2023). The use of these antimicrobials imposes a selective pressure that may promote the persistence of resistance genes, driving diverse co-selection events (Wales and Davies, 2015; James et al., 2023). This dynamic could still explain the occurrence of MDR *E. coli* carrying *mcr-1*-plasmids even with colistin ban, in this and other studies (Khine et al., 2023; Cao et al., 2020).

The detection of *mcr-1* genes predominantly located in multireplicon IncHI2 plasmids, which also carried genes coding for decreased susceptibility to copper, (*sil* + *pco*) and genes conferring resistance to multiple antibiotic classes (aminoglycosides, beta-lactams, phenicols, sulfonamides and tetracyclines), supports the occurrence of co-selection events. Mosaic IncHI2 plasmids, like those observed in this study, are frequently found in *mcr*-positive Enterobacterales circulating in various sources in Portugal and globally (Freitas-Silva et al., 2018; Ribeiro et al., 2021; Ribeiro-Almeida et al., 2022; Lima et al., 2022; Zelendova et al., 2021). The high similarity of IncHI2 plasmid backbones from isolates/clones in our rabbit farm to those described in other settings - such as humans in China and United States of America (Lu et al., 2019; Lindsey et al., 2015) and poultry in Switzerland (Zurfluh et al., 2014) - highlights their role in AMR spread and bacterial adaptation to diverse niches.

These plasmids offer survival advantages, persisting even without colistin pressure (Lima et al., 2022). Our findings challenge the idea that the colistin ban in Europe shifted the location of *mcr-1* genes from MDR plasmids like IncHI2 to specialized ones like IncX4 (Garcias et al., 2024), highlighting IncHI2's continued role in resistance dissemination.

In our comparative genomic analysis using WGS, we identified MDR *mcr-1*-carrying *E. coli* STs shared between rabbit and human clinical isolates. Additionally, certain rabbit farm strains exhibited a significant overlap of accessory genes, including fluoroquinolone resistance mutations and/or virulence genes (e.g., ExPEC, EPEC), with clinically relevant human clones reported in previous studies (Massella et al., 2021; Majewski et al., 2021; Maluta et al., 2014; Liang et al., 2021). These findings provide evidence of the potential transmission of such strains from food-animals to humans (Silva et al., 2023; Massella et al., 2021), emphasizing rabbit meat farming as an underexplored reservoir and source of clinically relevant strains.

5. Conclusions

This study demonstrates that intensive rabbit farms can be a source of clinically relevant *E. coli* carrying resistance genes to last-resource antibiotic colistin, even after long withdrawal. The persistence of the same *mcr*-carrying *E. coli* clones and plasmids over three years in a single farm underscores the complex environmental transmission routes of MDR bacteria (e.g., feed, GP at arrival, and cross-contamination), as illustrated in Fig. 5, and the likelihood of co-selection events driven by other antimicrobial compounds, such as copper in feed formulations and non-colistin antibiotics. These findings have significant implications for public health and food safety, emphasizing farm environment as contamination sources.

Raising awareness of the diverse internal and external farm drivers of MDR strains (e.g., feed, water, nesting material, new animals, workers) and implementing robust biosecurity practices, such quarantine for GP at arrival, vaccination programs, vacancy periods, higher microbiological control of feed and safe farm environments, are essential. These measures, combined with stringent antibiotic use control, are critical to effectively fight colistin resistance under a One Health strategy.

CRediT authorship contribution statement

Marisa Ribeiro-Almeida: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Joana Mourão:** Writing – review & editing, Visualization, Software, Methodology, Investigation, Formal analysis. **Inês C. Rodrigues:** Writing – review & editing, Investigation. **André Pinto de Carvalho:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Paulo Martins da Costa:** Writing – review & editing, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Luísa Peixe:** Writing – review & editing, Supervision, Funding acquisition. **Patrícia Antunes:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Ethics statement

Ethical review and approval were not required because the samples were taken from farm environmental points (e.g., nets beneath rabbit cages) conducted by the local veterinarian, under the auspices of the rabbit farm producers.

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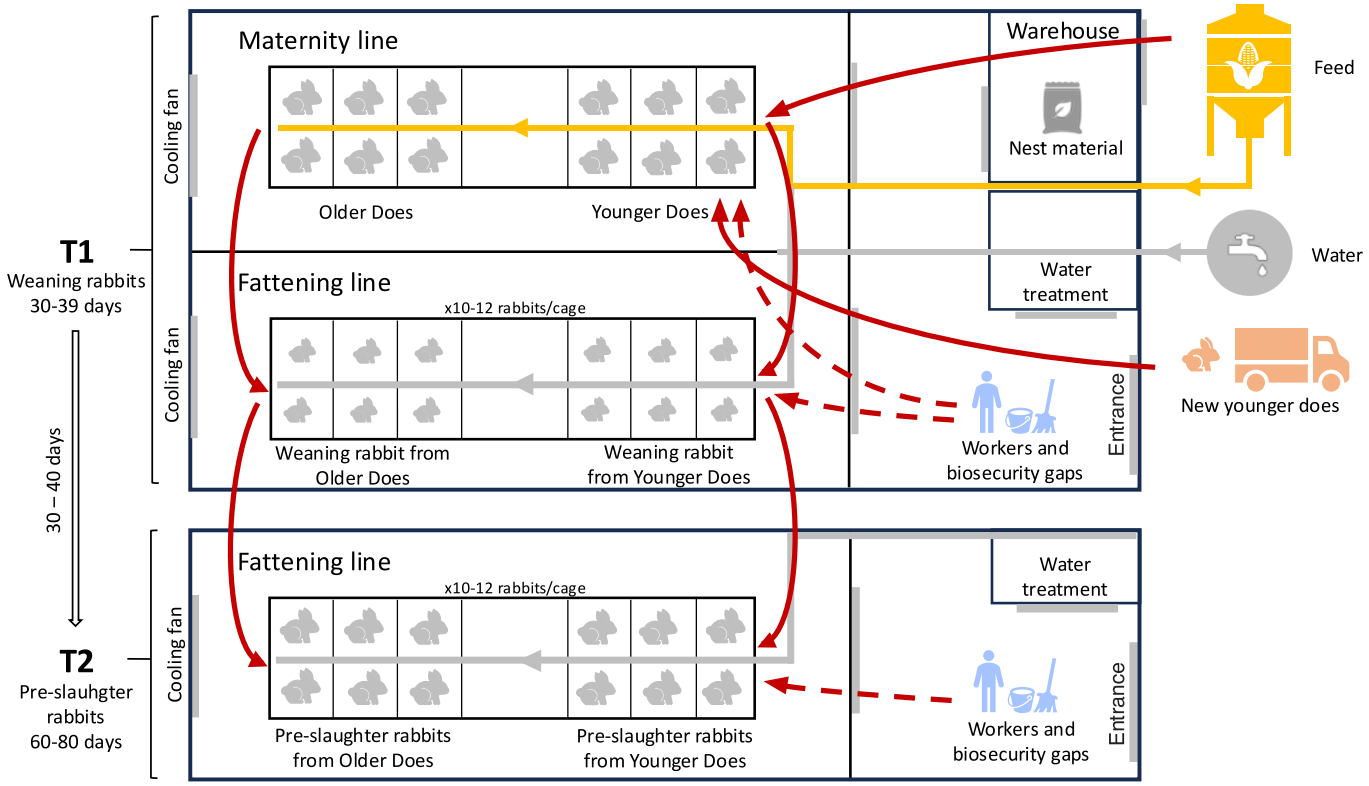

Fig. 5. Potential transmission pathways of *mcr*-positive *E. coli* in rabbit farms across spatial and temporal dimensions. Solid lines represent the transmission routes identified in this study, while dashed lines represent extrapolated transmission pathways.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2025.111248.

Data availability

The raw reads of thirteen *E. coli* (5_R1_2 - Uberstrain number (Un) ESC_GB8560AA, 5_6_FM1_1 – Un ESC_GB8577AA, 5_6_FM1_2 – Un ESC_GB8578AA, 5_R2_1 – Un ESC_GB8579AA, 6_R1_6 – Un ESC_GB8576AA, 17_M1_3 – Un ESC_HB9024AA, 17_R1_302 – Un ESC_HB9198AA, 17_R2_1 – Un ESC_HB9199AA, 18_M1_1 – Un ESC_HB7262AA, 18_R2_5 – Un ESC_HB9200AA, 19_20_GP_300 – Un ESC_GB8552AA, 19_R2_4 – Un ESC_GB8551AA and 20_R2_2 – Un ESC_HB9201AA) were deposited at the Enterobase (https://enterobase.warwick.ac.uk/).

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Table S1. Detection of *mcr*-1 carrying *Escherichia coli* (n=70) from farm C across the 3 periods according to the cultural method

Period and Year	Doe group (age) [a]	Direct [b] sample type (no. of isolates; PFGE profile) [c]		Post-enrichment [b] sample type (no. of isolates; PFGE profile) [c]
		Direct BPW [d]	**Direct BPW+COL [d]**	**BPW+COL [d]**
Period 1 2020/2021	G5 (Old)	M (n=1; **G**) R1 (n=2; E, F)	M (n=1; **G**) R1 (n=2; A) R2 (n=1; H)	M (n=1; **G**) R1 (n=3; B, C, D) R2 (n=5; I, **J**, K)
	G6 (Young)	M (n=1; **J**) R1 (n=2; O) R2 (n=2; **H**)	R1 (n=2; P, **L**)	R1 (n=2; M, N)
	G5+G6_Feed	ND	FM (n=1; **H**)	FM (n=2; **L**)
Period 2 2022	G17 (Old)	M (n=3; V, X) R1 (n=3; Q, **R**) R2 (n=1; AC)	M (n=3; T, U) R2 (n=1; **R**)	M (n=1; NT), R1 (n=3; S), R2 (n=5; **R**, AE)
	G18 (Young)	R1 (n=2; **W**, AH)	R1 (n=1; **W**)	M (n=4; AB, AG) R2 (n=4, AA, AF)
Period 3 2023	G19 (Old)	R2 (n=4; **AI**, AM, AN)	R2 (n=3; **R**, AJ, AK)	R2 (n=1; **AL**)
	G20 (Young)	ND	R2 (n=2; **AL**)	ND
	G19+G20_GP	GP (n=1; **AI**)	ND	ND
Positive samples (no. isolates)	16 (70)	11 (22)	10 (17)	11 (31)

[a] An older doe has a history of 12 to 14 births, while a younger doe has a history of 2 to 4 births. GP: New younger doe's fecal sample.

[b] Isolates obtained from TBX+colistin (3.5 mg/L) plates.

[c] PFGE types were designated with letters from A to AN; NT represents non-typeable profiles. Identical PFGE patterns are highlighted in bold.

[d] M: Doe's pooled fecal sample; R1: Post-weaning rabbit pooled fecal sample; R2: Pre-slaughter rabbit pooled fecal sample; FM: Doe's feed.

Given the dimension Table S2, S3, S4, S5 and S6 are available at https://doi.org/10.1016/j.ijfoodmicro.2025.111248

II. Commercial raw meat-based pet food and wildlife as sources and reservoirs of colistin-resistant and *mcr*-carrying Enterobacterales

1. **Ribeiro-Almeida, M.**, Mourão, J., Magalhães, M., Freitas, A. R., Novais, C., Peixe, L., & Antunes, P. (2024). Raw meat-based diet for pets: a neglected source of human exposure to *Salmonella* and pathogenic *Escherichia coli* clones carrying *mcr*, Portugal, September 2019 to January 2020. *Eurosurveillance*, *29*(18), 2300561. doi: 10.2807/1560-7917.ES.2024.29.18.2300561

2. **Ribeiro-Almeida, M.**, Mourão, J., Novais, Â., Pereira, S., Freitas-Silva, J., Ribeiro, S., da Costa, P. M., Peixe, L., & Antunes, P. (2022). High diversity of pathogenic *Escherichia coli* clones carrying *mcr-1* among gulls underlines the need for strategies at the environment–livestock–human interface. *Environmental Microbiology*, *24*(10), 4702-4713. doi: 10.1111/1462-2920.16111

1. Raw meat-based diet for pets: a neglected source of human exposure to *Salmonella* and pathogenic *Escherichia coli* clones carrying *mcr*, Portugal, September 2019 to January 2020.

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Research

Raw meat-based diet for pets: a neglected source of human exposure to *Salmonella* and pathogenic *Escherichia coli* clones carrying *mcr*, Portugal, September 2019 to January 2020

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Background: The pet industry is expanding worldwide, particularly raw meat-based diets (RMBDs). There are concerns regarding the safety of RMBDs, especially their potential to spread clinically relevant antibiotic-resistant bacteria or zoonotic pathogens. **Aim:** We aimed to investigate whether dog food, including RMBD, commercially available in Portugal can be a source of *Salmonella* and/or other *Enterobacteriaceae* strains resistant to last-line antibiotics such as colistin. **Methods:** Fifty-five samples from 25 brands (21 international ones) of various dog food types from 12 suppliers were screened by standard cultural methods between September 2019 and January 2020. Isolates were characterised by phenotypic and genotypic methods, including whole genome sequencing and comparative genomics. **Results:** Only RMBD batches were contaminated, with 10 of 14 containing polyclonal multidrug-resistant (MDR) *Escherichia coli* and one MDR *Salmonella*. One turkey-based sample contained MDR *Salmonella* serotype 1,4,[5],12:i:- ST34/cgST142761 with similarity to human clinical isolates occurring worldwide. This *Salmonella* exhibited typical antibiotic resistance (bla_{TEM}+*strA-strB*+*sul2*+*tet(B)*) and metal tolerance profiles (*pco*+*sil*+*ars*) associated with the European epidemic clone. Two samples (turkey/veal) carried globally dispersed MDR *E. coli* (ST3997-complexST10/cgST95899 and ST297/cgST138377) with colistin resistance (minimum inhibitory concentration: 4 mg/L) and *mcr-1* gene on IncX4 plasmids, which were identical to other IncX4 circulating worldwide. **Conclusion:** Some RMBDs from European brands available in Portugal can be a vehicle for clinically relevant MDR *Salmonella* and pathogenic *E. coli* clones carrying genes encoding resistance to the last-line antibiotic colistin. Proactive actions within the One Health context, spanning regulatory, pet-food industry and consumer levels, are needed to mitigate these public health risks.

Introduction

The pet industry has evolved in recent decades due to increasing pet populations, stronger human–pet bonds and demand for high-quality pet food products [1,2]. Processed pet food manufactured with various processing methods (e.g. grinding, cooking, extrusion and dehydration) has traditionally been considered microbiological safe and nutritionally suitable for feeding pets [1,3]. However, since some pet owners consider unprocessed food healthier, raw meat-based diets (RMBDs) for dogs have gained popularity [1,2,4]. The RMBDs are mainly composed of uncooked or minimally processed meat, bones and organs, with freezing as the primary treatment, and are considered to be more natural than conventional processed pet food [1,5]. Nevertheless, the scientific evidence supporting RMBD benefits is scarce, and many veterinary professional organisations (e.g. the World Small Animal Veterinary Association) and international public health agencies (e.g. the United States (US) Centers for Disease Control and Prevention (CDC)) view them as potential health hazards for both animals and humans [1,5]; awareness of this issue appears less evident in Europe [6]. The

KEY PUBLIC HEALTH MESSAGE

What did you want to address in this study and why?

Raw meat-based diets (RMBDs) are increasingly popular among pet owners. However, the potential role of RMBDs has been neglected as a new source of bacteria resistant to last-resort antibiotics which could affect people co-living with pets. We wanted to analyse if dog food from brands sold in the European Union represents a possible source of *Salmonella* or other bacteria resistant to the important antibiotic colistin.

What have we learned from this study?

Conventionally processed pet food is a safer option than RMBDs. This is because RMBDs of European brands can carry multidrug-resistant bacteria, including globally disseminated pathogenic *Salmonella* and *E. coli* harbouring genes encoding resistance to colistin, an antibiotic critically important for human medicine. These hazards are frequent in food-animal production and are causing infections in humans worldwide.

What are the implications of your findings for public health?

The detection in RMBDs of a predominant pandemic *Salmonella* clone and pathogenic *E. coli* carrying mobile colistin resistance genes may pose a potential risk of human exposure. This can occur through handling of pet food and/or environmental release by pets. These findings indicate a need for proactive actions involving the pet industry, food safety agencies, and pet owners to mitigate risks for public health.

safety concerns associated with RMBDs are related to the potential contamination of raw ingredients with zoonotic pathogenic bacteria and parasites [1,3,4]. Such contamination could lead to the spread of these pathogens to both pets and humans cohabitating with pets, through direct contact with the pet or its feed, or indirectly through contact with contaminated household surfaces or hands during feed preparation.

In the European Union (EU), legal requirements for the use of animal by-products and derived products not intended for human consumption are established, including those to produce processed or raw pet food, helping to ensure microbiological safety [7]. Nevertheless, since 2020, there have been more than 20 notifications or recalls of pet food and RMBD in the EU due to the detection of zoonotic pathogens, particularly *Salmonella* and pathogenic *Escherichia coli* [8], and also cases of human infections with *Salmonella* and Shiga toxin-producing *E. coli* (STEC) linked to exposure to RMBDs [9-11]. Several studies have also established a correlation between the microbiota of pets and their owners, including the presence of antibiotic-resistant strains, with pet food as a potential source [12,13]. However, certain antibiotic-resistant bacteria and genes of public health concern, such as the *mcr* gene conferring resistance to the last-line antibiotic colistin, have not been extensively studied in pet food and RMBDs [1,14-16]. Consequently, these antibiotic-resistant strains and genes have not been recognised as notable food safety issues in the context of the pet food industry [6]. To address this knowledge gap, we aimed to investigate the occurrence of and further characterise *Salmonella* and other *Enterobacteriaceae* resistant to critical antibiotics, such as colistin, in dog food, including RMBDs, that is available in stores in Portugal to investigate if they represent a possible source of these hazards to public health.

Methods

Sampling strategy

We visited physical locations such as major supermarkets and pet stores in the Porto metropolitan area and conducted an online search to gather information on the primary canine food types and brands commercially accessible in Portugal. Over 5 months (September 2019 to January 2020), 55 dog food samples (22 wet, 14 raw-frozen, eight dry, seven treats and four semi-moist), corresponding to 50 different dog food items (four food types were acquired 2–3 times) and 25 brands commercialised in Portugal, were collected from 12 retail stores (eight supermarkets, three specialised stores and one veterinary clinic) in the Porto region; further details about the samples are appended in Supplementary Table S1. Most samples were obtained from brands marketed globally, including in the EU (21/25). The 14 raw-frozen dog food samples were a combination of fruits, vegetables and different types of meat (muscle/viscera). They were categorised into two groups based on the main meat type: poultry (n = 8; chicken, turkey, duck, goose) and ruminant (n = 6; veal, steer, deer) samples. The RMBDs were from the two international brands available in Portuguese stores (brand A types produced in the EU and brand B in the United Kingdom (UK)). The samples were processed according to their type, as previously described [17]. For the pre-enrichment step at 37 °C for 16–18 h, 25 g of each sample were homogenised (2 min in a Stomacher blender) with 1:10 buffered peptone water (BPW).

Detection of non-typhoidal *Salmonella*

Salmonella detection was performed using the International Standard Organisation [18] method for foodstuffs. Briefly, after the pre-enrichment, 0.1 mL and 1 mL of the BPW were transferred to Rappaport–Vassiliadis medium with Soya (RVS) and Muller–Kauffmann tetrathionate-novobiocin (MKTTn) broths, respectively, for selective enrichment (RVS at 41.5 °C for 24 h and MKTTn at 34–38 °C for 24 h). These broths were then streak-plated on xylose lysine deoxycholate agar and CHROMagar *Salmonella* Plus. Presumptive *Salmonella* colonies recovered from both selective agar plates (up to five colonies per plate) were confirmed by biochemical tests (e.g. API-20 E, bioMérieux, Marcy l'Etoile, France), by agglutination with *Salmonella* O poly antisera and serogroup-specific antisera (Becton Dickinson, New Jersey, US) and by PCR for *invA* gene detection and *Salmonella* serotypes of particular concern in the EU (Enteritidis, Typhimurium and 1,4,[5],12:i:-) [19].

Screening of *mcr*-carrying *Enterobacteriaceae*

After BPW enrichment, 100 µL and 10 µL were spread on Tryptone Bile X-glucuronide agar plates (TBX) and Simmons citrate agar + inositol (SCAi) with and without colistin (3.5 mg/L) and incubated (TBX at 37 °C for 24 h; SCAi at 37 °C for 48 h) for *E. coli* and *Klebsiella* spp. detection, respectively. From each plate, between one and five colonies of each morphotype were spread on a CLED medium for further identification by matrix-assisted laser desorption-ionisation-time of flight mass spectrometry (MALDI-TOF VITEK MS, bioMérieux) and standard PCRs for *E. coli* and *K. pneumoniae* [20]. Colistin resistance genes (*mcr-1* to *mcr-5* and *mcr-6* to *mcr-9*) were identified in *E. coli*, *K. pneumoniae* and *S. enterica* isolates using a multiplex PCR published previously [21]. Amplified simplex PCR products were purified using the NZYGelpure kit (NZYTech, Lisbon, Portugal) and sequenced at Eurofins Genomics (Konstanz, Germany).

Phenotypic and genotypic characterisation of *Enterobacteriacea*

We used disk diffusion to test susceptibility to the following antibiotics: amoxicillin 10 µg, amoxicillin/clavulanic acid 30 µg, cefepime 30 µg, cefoxitin 30 µg, ceftazidime 30 µg, cefotaxime 30 µg, meropenem 10 µg, ciprofloxacin 5 µg, pefloxacin 5 µg (only for *Salmonella*) nalidixic acid 30 µg, gentamicin 10 µg, streptomycin 10 µg, kanamycin 30 µg, tobramycin 10 µg, chloramphenicol 30 µg, tetracycline 30 µg, sulfonamides 300 µg, trimethoprim 5 µg and fosfomycin 200 µg (only for *E. coli*). Colistin minimum inhibitory concentration (MIC) was determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference cation-adjusted Mueller–Hinton broth microdilution method [22]. *Escherichia coli* ATCC 25922 served as the control. Interpretation followed the EUCAST guidelines [23], and for nalidixic acid and tetracycline, we used the Clinical and Laboratory Standards Institute clinical breakpoints [24]. Multidrug resistance (MDR) was defined as resistance to antibiotics from three or more different families. Phylogenetic groups (PhG) of *E. coli* were determined using a standard multiplex PCR [20]. In addition, Shiga toxin-producing *E. coli* (STEC) was assessed by PCR for *stx1* and *stx2* virulence genes [25].

Whole genome sequencing for characterisation of *Salmonella* and *mcr-1*-carrying *Escherichia coli*

We selected one isolate per sample of *Salmonella* and *mcr-1*-positive *Enterobacteriaceae* for WGS. We extracted DNA using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, US) and measured its concentration with a Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Massachusetts, US). The HiSeq (2 × 151 bp) Illumina platform (Illumina, San Diego, US) was used for sequencing at Eurofins Genomics. Raw reads quality was assessed with FastQC v0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) using default parameters. High-quality reads were then de novo assembled using SPAdes v3.15.5 (https://github.com/ablab/spades) within Unicycler v0.5.0 (https://github.com/rrwick/Unicycler). Assembly quality and completeness were assessed with QUAST v5.0.2 (https://quast.sourceforge.net) and BUSCO v5.0.0 (https://github.com/WenchaoLin/BUSCO-Mod), respectively. Draft genomes were annotated on the RAST server (https://rast.nmpdr.org). For metal tolerance genes search (*arsRD2A2BCA1D1-arsR1HD1A1A2CBA3D2R2*, *pcoGE1ABCDRSE2*, *silESRCF-BAGP*, *terFEDCBAZ-terY3Y2XY1W* and *merEDACPTR*), we used ABRicate v1.0.1 (https://github.com/tseemann/abricate) with an in-house database. We used tools from the Centre for Genomic and Epidemiology (http://www.genomicepidemiology.org) to evaluate *E. coli* and *Salmonella* antibiotic resistance genes (ResFinder v4.1, https://cge.food.dtu.dk/services/ResFinder) or known mutations (PointFinder v4.1, https://bitbucket.org/genomicepidemiology/pointfinder_db.git), virulence genes (only for *E. coli*, VirulenceFinder v2.0, https://cge.food.dtu.dk/services/VirulenceFinder), plasmid replicons (PlasmidFinder v2.1, https://cge.food.dtu.dk/services/PlasmidFinder), plasmid typing (pMLST v2.0, https://cge.food.dtu.dk/services/pMLST) and Multilocus Sequence Typing (MLST v2.0, https://cge.food.dtu.dk/services/MLST). *Salmonella* serotypes were confirmed with the *Salmonella* In Silico Typing Resource (SISTR) (https://github.com/phac-nml/sistr_cmd) and *E. coli* PhGs were validated using ClermontTyper (http://clermontyping.iame-research.center).

For confirmation of *mcr-1* gene location and hypothetical plasmid reconstructions, we used the MOB-recon tool v3.1.0 from the MOB-suite package (https://github.com/phac-nml/mob-suite). If the *mcr*-1 gene was identified in a plasmid by MOB-recon or on the same contig as the replicon, it was considered plasmid-associated. The PLSDB-plasmid database (https://ccb-microbe.

Table 1

Distribution of *Salmonella enterica*, *Escherichia coli* and *Klebsiella pneumoniae* among samples of commercially available raw-frozen dog food, Portugal, September 2019–January 2020 (n = 14)

Food type	Sample number	Brand[a]	Main ingredients[b] (% of the most prevalent ingredients)	Collection date	Bacteria detected		
					S. enterica	*E. coli (mcr-1)*	*K. pneumoniae*
Ruminant-based	6	A	**Veal** (59%); salmon (20%); vegetables; salmon oil	Sept 2019	–	+	–
	46			Nov 2019	–	+	–
	53			Jan 2020	–	+	–
	18	A	**Steer** (79%); vegetables; salmon oil	Oct 2019	–	+	+
	45			Nov 2019	–	+	–
	26	B	**Deer** (80%); vegetables; fruit	Nov 2019	–	+ *(mcr-1)*	–
Poultry-based	15	A	**Chicken** (60%); lamb (19%); vegetables	Oct 2019	–	+	–
	16	A	**Chicken** (60%); veal (19%); vegetables	Oct 2019	–	+	+
	55			Jan 2020	–	+	–
	17	A	**Turkey** (60%); lamb (20%); vegetables	Oct 2019	–	+	–
	54			Jan 2020	+	+	–
	25	B	**Duck** (80%); vegetables; fruit	Nov 2019	–	+	+
	51	B	**Turkey** (50%); goose (30%); vegetables; fruit	Jan 2020	–	+	+
	52	B	**Turkey** (40%); salmon (20%); white fish (20%); vegetables	Jan 2020	–	+ *(mcr-1)*	–

–: not detected; +: detected.

[a] Raw food samples were from either of two international brands distributed worldwide, here randomly designated as A and B.

[b] The most prevalent ingredient in each food type is shown in bold.

cs.uni-saarland.de/plsdb) was used for comparative genomic analysis. Alignment of *mcr-1*-carrying plasmids with closely related IncX4 ones was conducted using the BRIG tool (v0.95) (https://github.com/happykhan/BRIG).

Comparative genomic analysis of *Salmonella* and *mcr-1*-carrying *Escherichia coli*

We conducted a comparative genomic analysis using core-genome MLST (cgMLST) between our isolates and genomes queried from Enterobase as well as the hierarchical clustering of cgMLST (HierCC) (https://enterobase.warwick.ac.uk). These strains were used to develop a minimum-spanning tree using GrapeTree (https://achtman-lab.github.io/GrapeTree/MSTree_holder.html) and MSTreeV2. Metadata of the included *Salmonella* and *E. coli* isolates were retrieved from Enterobase (isolate name, cgST, country, year, source). In addition, we conducted a search of antibiotic resistance, metal tolerance and virulence genes as described in the previous section.

Statistical analysis

Occurrence rates and antibiotic-resistant *E. coli* variations across food types were assessed using Fisher's exact test ($\alpha = 0.05$). The 95% confidence intervals (CI) for proportions were calculated using Wilson CI. Both analyses were computed using Prism v 9.1.1 (GraphPad, Boston, Massachusetts, US).

Results

Detection and characterisation of *Salmonella*

In our study of 55 pet food samples (41 processed and 14 raw), only raw samples tested positive for Gram-negative bacteria, including the zoonotic pathogen *Salmonella*, along with bioindicators *E. coli* and *Klebsiella pneumoniae* (Table 1). We detected *Salmonella* in one of the raw samples (7%; 95% CI: 1.3–31.5; n = 1/14), a raw-frozen batch (EU, brand A), predominantly containing turkey (Table 1).

Six *Salmonella* isolates, all identified as *Salmonella enterica* serotype 1,4,[5],12:i:- (Typhimurium monophasic variant) were successfully isolated from the same sample. They exhibited the MDR ASSuT profile conferring resistance to amoxicillin (A, encoded by the bla_{TEM} gene), streptomycin (S, *strA-strB*), sulphonamides (Su, *sul2*), and tetracycline (T, *tet(B)*). In addition, they had an integrative and conjugative element (ICE) with copper/silver (*pco*, *sil*) and arsenic (*ars*) tolerance clusters/operons (Table 2), typical of the widespread clinically relevant 'European clone' [26].

The *S.* 1,4,[5],12:i:- strain belonged to sequence type ST34 (Table 2), which is commonly observed in Europe, as evidenced by the data available on EnteroBase (https://enterobase.warwick.ac.uk/species/index/senterica). Using the EnteroBase cgMLST scheme, the pet food isolate was classified as cgST142761, which grouped into a distinct cluster (Hierarchical Clustering-HierCC HC5–142761 group) among the globally dispersed ST34 clone (Figure 1A). This isolate exhibited

Table 2

Genomic characterisation of *Salmonella enterica*, and the *mcr-1*-carrying *Escherichia coli* isolates recovered from samples of commercially available raw-frozen dog food, Portugal, September 2019–January 2020 (n = 3)

Isolate name	Species	Brand (main ingredient)	Serotype	PhG MLST cgMLST[a]	Antimicrobial resistance genes; chromosomal mutations	*mcr-1* location PL-Inc (kb)	Virulence genes[b]	Acquired metal tolerance genes
R54_S100	*Salmonella enterica*	A (turkey)	1,4,[5],12:i:-	NA ST34 cgST142761	bla_{TEM-1B}, *aac(6')-Iaa*, *str*A, *str*B, *sul2*, *tet*(B)	NA	NS	*sil*ERSCFBAGP, *pco*GE1ABCDRSE2, *ars*RD2A2BCA1D1, *mer*EDACPTR
R26_1_100	*Escherichia coli*	B (deer)	O153:H37	B1 ST297 cgST193137	bla_{TEM-1B}, bla_{CARB-2}, *aad*A1, *aad*A2, *cml*A1, *mcr-1.1*, *sul3*, *dfr*A16; *gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I	PL-X4 (33)	*gad*, *lpfA*,***ompT***, *ter*	*ars*R1BCR2
R52_8	*Escherichia coli*	B (turkey)	O101:H37	A ST3997 cgST193139	bla_{TEM-1B},*mcr-1.1*, *str*A, *str*B, *tet*(B), *mdf*(A); *gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I	PL-X4 (33)	*cma*, ***cvaC***, ***etsC***, *gad*,***hlyF***, *hra*, ***iroN***, ***iss***, ***ompT***, *terC*, ***traT***	*ars*R1BC

APEC: avian pathogenic *Escherichia coli*; cgMLST: core genome multilocus sequence typing; ExPEC: extraintestinal pathogenic *Escherichia coli*; MLST: multilocus sequence typing; NA: not applicable; NS: not searched; PhG: *E. coli* phylogenetic group; PL: plasmid.

[a] cgMLST obtained from Enterobase (https://enterobase.warwick.ac.uk) for both species.

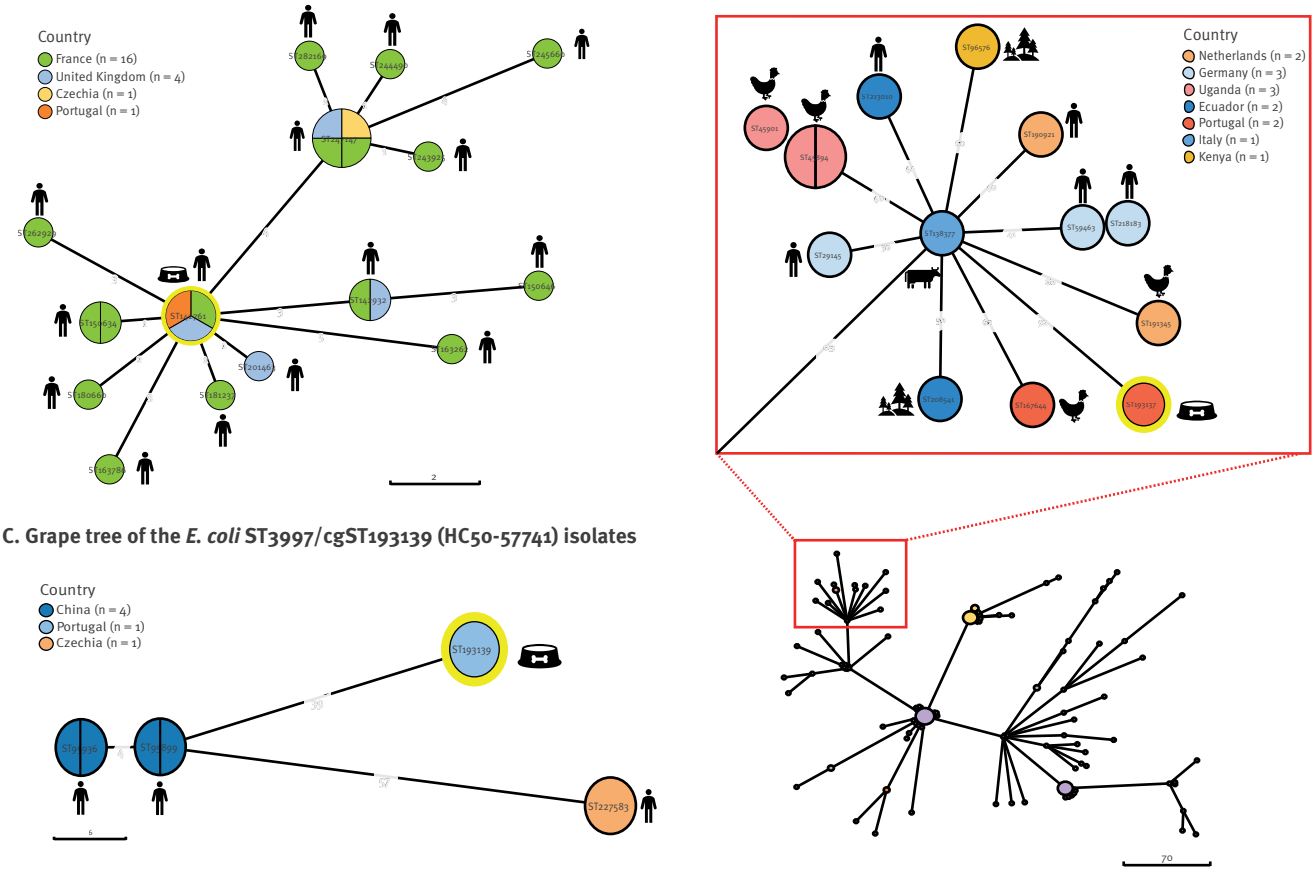
[b] The virulence genes associated with ExPEC are highlighted in bold, while those associated with APEC are underlined; genes that belong to both are indicated in bold and underline.

Figure 1

Phylogenetic trees of *Salmonella* Typhimurium and *Escherichia coli* isolates from raw pet food samples, Portugal, 2020 (n = 3) and related available genomes on EnteroBase up to 8 June 2023 (n = 22 *Salmonella* Typhimurium, n = 20 *E. coli*)

A. Grape tree of *Salmonella* Typhimurium isolates and the monophasic variant *S.* 1,4[5],12:i:- ST34/cgST142761 (HC5-142761)

B. Grape tree of *E. coli* ST297/cgST193137 (HC100-3512) isolates; inset: pet food isolates and related genomes



C. Grape tree of the *E. coli* ST3997/cgST193139 (HC50-57741) isolates

The core genome minimum spanning tree was created within the EnteroBase pipeline using the MSTreeV2 algorithm and GrapeTree tool. The cgST is indicated in each node. The yellow circle highlights the pet food isolate of each Grape tree. For the geographical analysis, the cgST was annotated using the country data (the number of genomes by country is indicated within parentheses). The scale bar corresponds to the number of cgMLST allelic differences.

a close genetic relationship (HC5) with 21 isolates of human origin (Czechia, France and UK; 2018–2022), with no apparent clustering based on European geographical location (Figure 1A); Supplementary Table S2 contains further strain details.

Detection and characterisation of colistin-resistant Enterobacteriaceae

We detected *E. coli* (n = 59 isolates; none STEC) in all raw-frozen food samples (100%; 95% CI: 79–100; n = 14/14). Four of them were also contaminated with *K. pneumoniae* (n = 5 isolates) (Table 1). A considerable percentage (71%; 95% CI: 45–88; n = 10/14) of the samples carried MDR *E. coli* isolates, independently of the main ingredients and PhGs (Table 3). Antibiotic resistance rates were similar between samples with poultry or ruminant-based ingredients (p > 0.05). More than half of the samples contained at least one *E. coli* with resistance to amoxicillin (79%; 95% CI: 52–92; n = 11/14 samples), ciprofloxacin (50%; 95% CI: 27–73; n = 7/14), nalidixic acid (57%; 95% CI: 33–79; n = 8/14), streptomycin (71%; 95% CI: 45–88; n = 10/14), tetracycline, sulphonamides or trimethoprim (64%; 95% CI: 39–84; n = 9/14 each); For further antibiotic resistance details, we refer to Supplementary Figure S1.

Colistin-resistant *E. coli* isolates (n = 4) were present in two batches (14%; 95% CI: 4–40; n = 2/14) from the same pet food brand (UK, brand B), one with deer and the other with turkey as the main ingredient (Table 3). All isolates carried the *mcr-1* gene with MIC = 4 mg/L and were recovered in TBX medium supplemented with colistin. They were co-resistant to amoxicillin, streptomycin, ciprofloxacin, nalidixic acid, streptomycin and sulphonamides. These *mcr-1*-carrying *E. coli* isolates belonged to B1-ST297 and A-ST3997-ST10 complex-Cplx (Table 2). The ST297 (cgST193137), classified as an extraintestinal pathogenic *E. coli* (ExPEC), shared a common HC100 cluster (Hierarchical Clustering-HierCC HC100–3512 group) with 162 genomes and the lowest number of allelic differences with 13 genomes from globally dispersed sources and countries (Germany,

Table 3

Characteristics of *Escherichia coli* recovered from samples of commercially available raw-frozen dog food, Portugal, September 2019–January 2020 (n = 59 isolates)

Food type	Sample ID (brand)	Main ingredients[a] (% of the most prevalent ingredients)	PhG (number of isolates)	Antibiotic resistance profile[b]	*mcr-1* gene detection	MDR (sample ID)[c]
Ruminant-based	6, 46, 53 (A)	**Veal** (59%); salmon (20%); vegetables; salmon oil	A (2)	AML (STR, SUL, TMP)	–	+ (53)
			B1 (8)	–	–	–
			B2 (2)	–	–	–
			D (1)	–	–	–
	18, 45 (A)	**Steer** (79%); vegetables; salmon oil	A (2)	(AML, STR, CHL, TET, SUL, TMP)	–	+ (45)
			B1 (4)	(AML, STR, NAL, TET)	–	+ (18)
	26 (B)	**Deer** (80%); vegetables; fruit	A (2)	TET	–	–
			B1 (4)	(AML, AMC, CIP, NAL, STR, CHL, COL, TET, SUL, TRP)	+	+
Poultry-based	15 (A)	**Chicken** (60%); lamb (19%); vegetables	B1 (4)	AML, TET, SUL, TMP (AMC, CHL, CIP, NAL, STR, KAN)	–	+
	16, 55 (A)	**Chicken** (60%); veal (19%); vegetables	A (10)	(AML, CIP, NAL, STR, TET, SUL, TMP)	–	+
			C (2)	AML, CIP, NAL, STR, TET, SUL, TMP (FOX)	–	+ (55)
	17, 54 (A)	**Turkey** (60%); lamb (20%); vegetables	B1 (2)	–	–	–
			D (2)	STR, TET, SUL, TMP (AML, CIP, NAL)	–	+ (17)
	25 (B)	**Duck** (80%); vegetables; fruit	A (1)	–	–	–
			B1 (1)	–	–	–
	51 (B)	**Turkey** (50%); goose (30%); vegetables; fruit	A (1)	NAL, TET	–	–
			B1 (7)	CIP, NAL, TET (AML, GEN, STR, KAN, TOB, CHL, SUL, TRP)	–	+
	52 (B)	**Turkey** (40%); salmon (20%); white fish (20%); vegetables	A (3)	AML, AMC, STR, TET (CIP, NAL, KAN, GEN, COL, SUL, TRP) AML, AMC, CIP, NAL, GEN, STR, TET, SUL, TRP	+	+
			B1 (1)		–	+

–: not detected; +: detected; AMC: amoxicillin + clavulanic acid; AML: amoxicillin; CHL: chloramphenicol; CIP: ciprofloxacin; COL: colistin; FOX: cefoxitin; GEN: gentamicin; ID: identification number; KAN: kanamycin; MDR: multidrug resistance; NAL: nalidixic Acid; PhG: *E. coli* Phylogenetic Group; STR: streptomycin; SUL: sulphonamides; TET: tetracycline; TOB: tobramycin; TRP: trimethoprim.

[a] The most prevalent ingredient in each food type is represented in bold.

[b] Variable presence of antibiotic resistance is presented in brackets. (–) indicates isolates susceptible to all tested antibiotics.

[c] The sample ID number is indicated only when more than one food sample type was analysed.

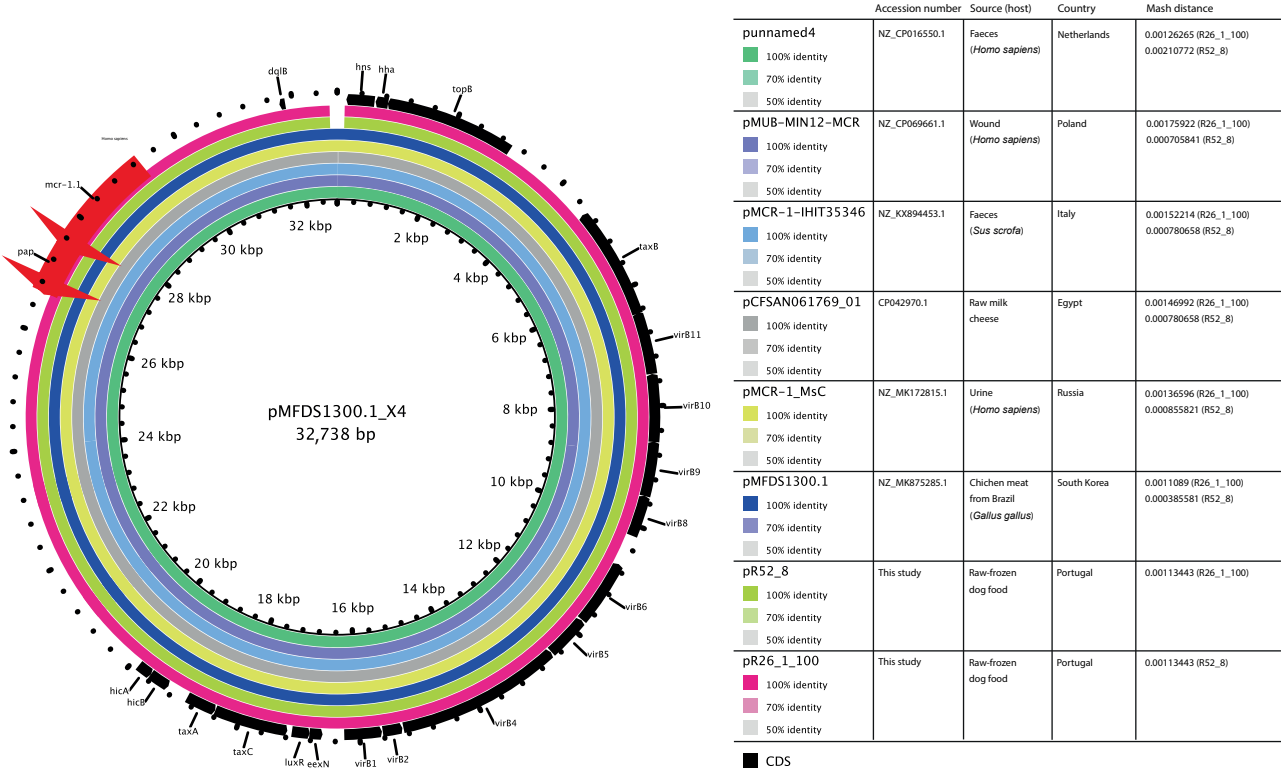
Italy, the Netherlands, Ecuador, Kenya and Uganda; 2014–2021), including from a Portuguese poultry farm (Figure 1B); Supplementary Table S3 provides further strain details. The ST3997-ST10 Cplx (cgST193139) presented virulence genes associated with avian pathogenic *E. coli* (APEC) and shared a single cluster at the HC50 level with five isolates from humans in Europe (Czechia, 2020) and Asia (China, 2017) (Figure 1-C); for further strain details we refer to Supplementary Table S4. Whole genome sequencing revealed that in both *E. coli* strains from the same pet food brand, the *mcr-1.1* gene was located on similar IncX4 plasmids (99.89% identity). These plasmids shared a common genetic environment near the *mcr*-1 cassette, contained the *pap2* gene (membrane-associated lipid phosphatase) and lacked the ISApl1 element [16]. Comparative genomics revealed that these IncX4 plasmids were similar to others (MOB-recon; mash distance: 0.000780658–0.00126265) and are circulating among diverse hosts (humans, pig, poultry) and the environment in many different countries, including Portugal (Figure 2); Supplementary Table S5 provides further plasmid details.

Discussion

This study investigated the presence and characteristics of *Salmonella* and other *Enterobacteriaceae* in 55 dog food samples, with a focus on colistin-resistant strains. These samples comprised various types of meat and were obtained from different suppliers and international brands in Portugal. We found *Enterobacteriaceae*, including *Salmonella* and MDR isolates, only in samples from raw pet food, in contrast to a parallel study in the same samples [17], where *Enterococcus* spp. was detected across all sample types, including dry and wet. Current regulations in the EU propose counting *Enterobacteriaceae* (and including *Salmonella* detection) as a hygiene criterion for all categories of pet foods [7]. Numerous studies have demonstrated contamination levels exceeding the

Figure 2

Circular maps of *mcr-1*-harbouring IncX4 plasmids in *Escherichia coli* isolated from Portuguese pet food, September 2019–January 2020 (n = 2) and closely related ones from different sources and geographical regions



Alignment of R26_1_100 of 33789 bp and R52_8 of 32534 bp (Portugal, 2020) against others. Black inner ring: plasmid used as a reference for the alignment; name and size of the reference indicated in the middle of the panel. Genes are represented by black arrows (indicating the position and direction of transcriptional open-reading frames) in the outer circle, with *mcr-1.1* and *pap* genes highlighted in red.

EU limits (i.e. *Salmonella*: absence in 25 g and *Enterobacteriaceae* < 5 × 10^3 CFU/g) in RMBDs [4,5,14,15,27]. Our results strongly suggest that conventionally processed pet food is a safer option, emphasising the critical role of heat treatment in pet food production for effectively mitigating microbiological hazards [1,2].

Although the overall prevalence of *Salmonella* in the RMBDs samples in this study was low (one of 14 batches produced in the EU had unsatisfactory microbiological quality), other studies from Europe also detected *Salmonella*: 4% of raw pet food samples in Switzerland, 20% in the Netherlands and 71% in Italy [14,16,27]. The *Salmonella enterica* detected in this study was of the serotype 1,4,[5],12:i:- and belonged to ST34, which has emerged as the predominant pandemic genotype in recent decades, particularly in food animal production and human infections in the EU [26,28]. Their MDR features (ASSuT+ICE) may have facilitated the adaptation of this serotype to environments with extensive usage of antibiotics and heavy metals, such as pig and poultry farms [26,28,29], whose raw animal by-products are the sources of the pet food industry. Since food animals are asymptomatic carriers of *Salmonella*, these bacteria can spread easily at slaughterhouses through cross-contamination events between flocks or animal by-products or at pet food production plants, in various types of meat, animal species and geographical places of origin [6,19,30]. Notably, we showed genetic similarities between *S.* 1,4,[5],12:i:- from RMBDs and public genomes from human clinical cases from different European countries, suggesting a role of raw pet food as a potential vehicle for the transmission of this serotype considered of human health significance in the EU and carrying a MDR profile. Some studies consistently show a significant difference in *Salmonella* excretion in faeces between dogs fed with RMBDs and those fed with dry food, highlighting the microbiological risk associated with RMBDs [3,31,32]. This risk extends not only to dogs but also to pet owners handling RMBD and dog faeces, as well as to the environment, as documented by recent *Salmonella* outbreaks where WGS confirmed a connection between pets, pet food and human disease [10,11,33,34].

A high percentage of our samples carried MDR *E. coli* isolates, regardless of the raw food types tested, similar to a recent parallel study focused on MDR *Enterococcus* in dog food in Portugal [14]. Resistance to commonly used veterinary antibiotics such as β-lactams, fluoroquinolones, tetracycline and sulphonamides was especially pronounced, mirroring trends seen in other European studies on pet

food samples of diverse origins [14,35]. The use of these antibiotics, particularly in poultry production, has been associated with increased *E. coli* resistance rates [29], suggesting that raw meat-based ingredients might be introducing MDR strains in pet food, which then can persist until they reach humans and their pets [32]. While the percentage of samples containing MDR and *mcr*-carrying *E. coli* isolates was relatively low, in line with findings from other studies [14,16], it underscores the importance of employing antibiotics judiciously within the livestock industry. This is needed to curb the co-selection of genes conferring resistance to colistin, a "highest priority critically important antimicrobial" for human medicine among various bacterial species [20,21]. In fact, *E. coli* ST297 (ExPEC) and ST3997-ST10 Cplx (APEC) identified in this study have been detected worldwide in various animal, food, environmental and human sources, and have been linked to numerous human infections (https://enterobase.warwick.ac.uk/species/index/ecoli), which highlights their capacity to be transmitted to humans through the food chain. In Portugal, the MDR *E. coli* ST297 lineage is predominant in many food sources [20,36] and has now been detected in raw pet food in our study. Meanwhile, in Asia, *E. coli* ST3997-ST10 Cplx isolates found in poultry, the environment and humans also carried *mcr-1* associated with diverse plasmid backgrounds [37]. Notably, both *E. coli* strains obtained in this study from the same pet food brand carried the *mcr-1.1* gene on similar IncX4 plasmids. These findings, along with the similarity of these plasmids to globally distributed ones, suggest possible cross-contamination events and/or diverse origins of pet food contamination arising from ingredients or human handling at the production plant.

In this study, RMBDs were identified as a potential vehicle of MDR zoonotic-related pathogenic bacteria, with some carrying genes such as *mcr-1* conferring resistance to last-line antibiotics. Despite the EU›s efforts to reduce antibiotics such as colistin in livestock production and the successful colistin restrictions on EU farms [20,21], the introduction of colistin-resistant bacteria through imported animal by-products (e.g. from non-EU countries with different antibiotic practices and regulations), raw vegetables (common in most samples) or wildlife (e.g. deer as the main ingredient in one batch with *mcr*-carrying *E. coli*) cannot be excluded. Continuous vigilance is essential to address these potential pathways and mitigate the spread of antibiotic-resistant bacteria. Furthermore, most manufacturers do not provide information on food safety practices (e.g. handwashing, safe handling) for handling raw pet food [38], including on labels found on the raw pet food samples obtained for this study. Appropriate hygiene measures and safe handling practices should be observed when dealing with pets and raw pet food to mitigate the risk of MDR bacterial infections in humans.

Finally, we acknowledge the study's limitations. Firstly, the results should be interpreted considering our convenience-based sampling strategy, which exclusively captured dog food types and brands available on the Portuguese pet food market, primarily in four cities in the Porto metropolitan area. Consequently, the results may not be extrapolated to pet food products available from other suppliers. Secondly, the small sample size and the uneven distribution among suppliers and various canine food items may have introduced unintended selection bias. Moreover, additional studies, encompassing brands available in every region in the world, along with local risk assessment investigations, are required to discern the broader implications of pet food on public health.

Conclusion

This study demonstrates that RMBDs from European brands available in Portugal can be a vehicle for MDR clinically-relevant *Salmonella* and *E. coli* carrying genes encoding resistance to the last-resort antibiotic colistin. Promoting awareness of potential risks linked to RMBDs and providing guidance to pet owners on proper handling and feeding practices are crucial steps in minimising potential health risks. Identifying environmental transmission routes of pathogenic and MDR bacteria to pet food and the continuous microbiological monitoring (pathogens and antibiotic-resistant bacteria/genes) of ingredients and processes used in the fast-growing pet food industry needs to be addressed in future One Health studies to mitigate public health risks.

Ethical statement

Ethical approval was not required for this study because the samples were exclusively from dog food acquired in retail stores.

Funding statement

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Data availability

Raw reads from *E. coli* strains R26_1_100 (Uberstrain number ESC_YA6262AA) and R52_8 (Uberstrain number ESC_YA9779AA), as well as from *Salmonella enterica* Typhimurium monophasic variant 1,4,[5],12:i:- strain R54_S100 (Uberstrain

number SAL_LB4229AA), were deposited at Enterobase (https://enterobase.warwick.ac.uk).

Preprint

A pre-print version of our manuscript, titled "Raw meat-based diet for pets: a neglected source of human exposure to *Salmonella* and *mcr*-carrying *Escherichia coli* in Europe" by Ribeiro-Almeida, Marisa, et al., has been deposited on SSRN, and the corresponding DOI for the preprint is http://dx.doi.org/10.2139/ssrn.4532439, as posted on 9 August 2023.

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Conflict of interest

None declared.

Authors' contributions

Marisa Ribeiro-Almeida: Methodology, Software, Formal analysis, Investigation, Visualisation, Writing – original draft, Writing – review & editing. Joana Mourão: Methodology, Software, Formal analysis, Investigation, Visualisation, Writing – review & editing. Mafalda Magalhães: Investigation, Visualisation, Writing – original draft. Ana R Freitas: Formal analysis, Writing – review & editing. Carla Novais: Formal analysis, Writing – review & editing, Funding acquisition. Luísa Peixe: Supervision, Funding acquisition, Writing – review & editing. Patrícia Antunes: Conceptualisation, Methodology, Software, Formal analysis, Investigation, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration.

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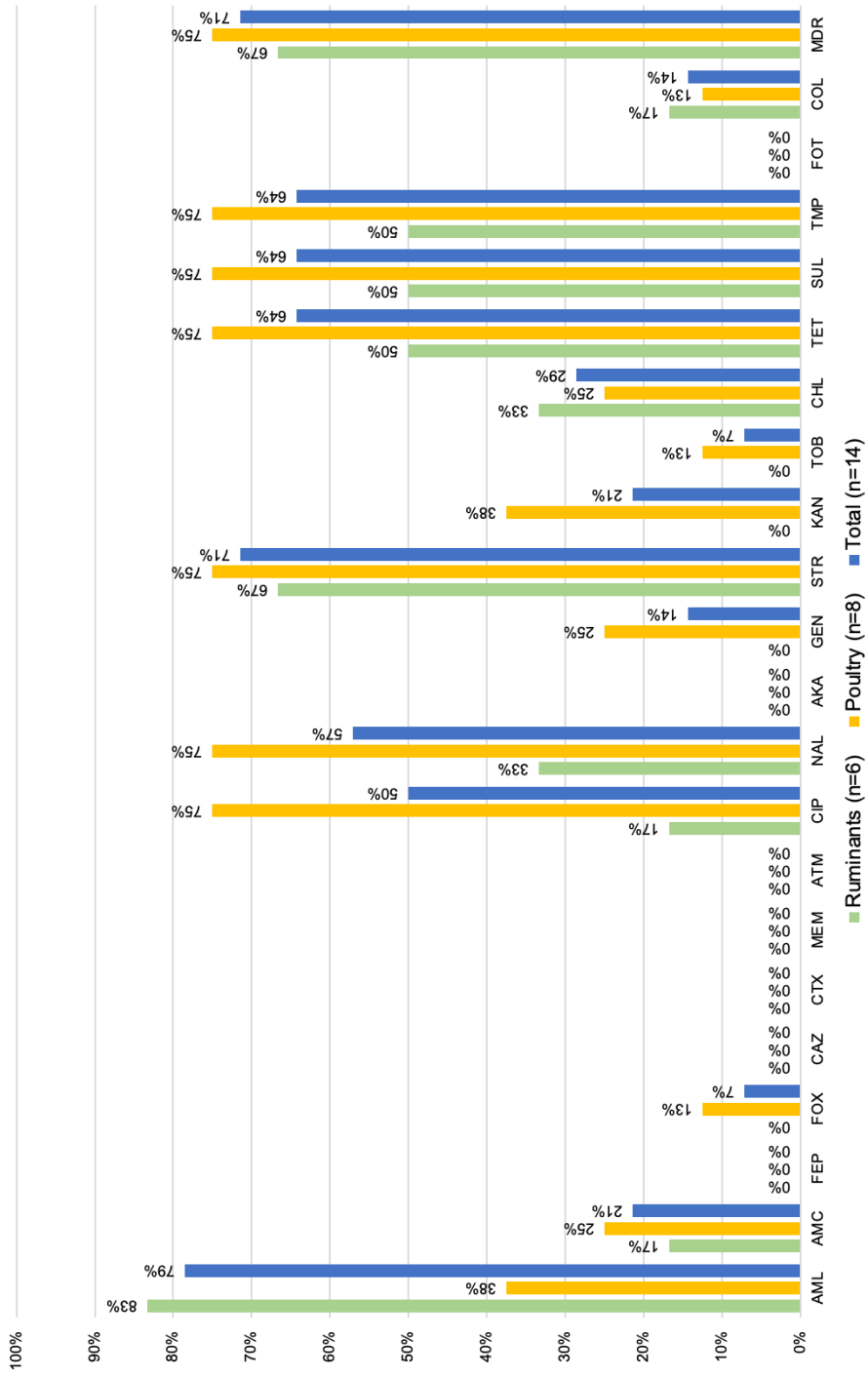

Fig. S1. Occurrence of antibiotic-resistant *E. coli* according to the food type. P>0,05 (Fisher exact test). Abbreviations: AML, amoxicillin; AMC, amoxicillin+clavulanic acid; FEP, cefepime; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; MEM, meropenem; ATM, aztreonam; CIP, ciprofloxacin; NAL, nalidixic acid; AKA, amikacin; GEN, gentamicin; STR, streptomycin; KAN, kanamycin; TOB, tobramycin; CHL, chloramphenicol; TET, tetracycline; SUL, sulphonamides; TMP, trimethoprim; FOT, fosfomycin; COL, colistin; MDR, multidrug resistance.

Table S1. Characterization of the 55 dog food samples studied

Sample	Brand	Food type	Main ingredients (% of the main ingredient)	City (store)	Collection Date
1	C	Wet	Quail (>90%); pear	Matosinhos (pet store 1)	Sep/19
2	D	Wet	Meat and animal/vegetal subproducts (4% veal, 4% liver)	Matosinhos (supermarket 1)	Sep/19
3	E	Wet	Meat and animal subproducts (4% rabbit); cereals	Matosinhos (supermarket 2)	Sep/19
4	F	Wet	Meat and animal subproducts (4% chicken); cereals; fish; carrots	Matosinhos (supermarket 3)	Sep/19
5	F	Wet	Meat and animal subproducts (4% chicken); fish; vegetables	Póvoa de Varzim (supermarket)	Oct/19
6	A	Raw-frozen	Veal (59%); salmon (20%); vegetables; salmon oil	Matosinhos (pet store 1)	Sep. 2019 - Jan.2020
7	G	Wet	Chicken (60%); plasma; rice; vegetables	Maia (supermarket 1)	Sep. 2019 - Oct. 2019
8	H	Wet	Meat and derivatives (4% beef); cereals; vegetables; fruit	Póvoa de Varzim (supermarket)	Oct/19
9	I	Wet	Meat and animal subproducts (4% chicken, 4% turkey, 4% lamb)	Póvoa de Varzim (supermarket)	Oct/19
10	D	Wet	Pate with meat and animal subproducts (4% poultry, 4% liver)	Maia (supermarket 1)	Oct/19
11	D	Wet	Meat and animal subproducts (4% chicken); cereals; vegetables	Maia (supermarket 1)	Oct/19
12	R	Dry	Dehydrated proteins of poultry/pork; chicken meat/fat	Matosinhos (supermarket 3)	Oct/19
13	R	Treats	Cereals; oils/fats; meat and animal subproducts	Matosinhos (supermarket 3)	Oct/19
14	F	Treats	Cereals; oils/fats; meat and animal subproducts	Matosinhos (supermarket 3)	Oct/19
15	A	Raw-frozen	Chicken (60%); lamb (19%); vegetables	Matosinhos (pet store 1)	Oct/19
16	A	Raw-frozen	Chicken (60%); veal (19%); vegetables	Matosinhos (pet store 1)	Sep. 2019 - Jan.2020
17	A	Raw-frozen	Turkey (60%); lamb (20%); vegetables	Matosinhos (pet store 1)	Sep. 2019 - Jan.2020
18	A	Raw-frozen	Steer (79%); vegetables; salmon oil	Matosinhos (pet store 1)	Oct. 2019 - Nov. 2019
19	S	Dry	Rice and rice protein; fish oil; hydrolyzed salmon protein; vitamins	Maia (veterinary clinic 1)	Oct/19
20	K	Wet	Stew with chicken (8%) and vegetables (2%)	Maia (veterinary clinic 1)	Oct/19
21	T	Dry	Dehydrated chicken (27%); cereals; oils; dry aromatic plants	Maia (veterinary clinic 1)	Oct/19
22	B	Wet	Beef (>40%); meat broth; tripe; vegetables; rice	Maia (pet store 1)	Oct/19
23	B	Wet	Chicken (>60%); meat broth; tripe; vegetables; rice	Maia (pet store 1)	Oct/19
24	F	Dry	Chicken (20%); dehydrated proteins of poultry; rice; fish oil; corn flour	Maia (veterinary clinic 1)	Oct/19
25	B	Raw-frozen	Duck (80%); vegetables; fruit	Matosinhos (pet store 2)	Nov/19
26	B	Raw-frozen	Deer (80%); vegetables; fruit	Matosinhos (pet store 2)	Nov/19
27	I	Wet	Meat and animal subproducts (4% chicken); cereals; vegetables	Maia (supermarket 2)	Nov/19
28	M	Wet	Meat and derivatives (4% chicken, 4% lamb); cereals; vegetables	Maia (supermarket 2)	Nov/19
29	N	Wet	Meat and derivatives (4% chicken); cereals; vegetables	Maia (supermarket 2)	Nov/19
30	I	Treats	Meat and animal subproducts (4% chicken, 4% cow); cereals; fish	Maia (supermarket 2)	Nov/19
31	U	Dry	Salmon (21%); fish flours; potatoes; oils	Maia (veterinary clinic 1)	Oct/19
32	V	Dry	Dehydrated pork and poultry proteins; rice; bean peel; peas	Maia (veterinary clinic 1)	Oct/19
33	K	Dry	Dehydrated proteins of poultry; corn; corn flour; animal fat; fish oil	Maia (veterinary clinic 1)	Oct/19
34	N	Wet	Meat and derivatives (4% chicken); cereals; vegetables	Maia (supermarket 2)	Nov/19
35	O	Wet	Meat and derivatives (4% cow); fish; vegetable subproducts	Porto (supermarket 1)	Nov/19
36	W	Dry	Meat and animal subproducts; cereals; oils/ fats; fish and subproducts	Porto (supermarket 1)	Nov/19
37	M	Treats	Meat and animal subproducts (4% chicken, 4% cow, 4% lamb)	Porto (supermarket 1)	Nov/19
38	F	Treats	Meat and animal subproducts; cereals; oils/ fats; dairy	Porto (supermarket 1)	Nov/19
39	P	Wet	Chicken (60%); vegetables	Maia (supermarket 3)	Nov/19
40	P	Wet	Meat and derivatives (55%); fish and derivatives (5% salmon)	Maia (supermarket 3)	Nov/19
41	X	Semi-moist	Pork/beef meat; pork heart; cereals	Matosinhos (supermarket 3)	Nov/19
42	Z	Treats	Cereals; vegetables subproducts; meat and animal subproducts; dairy	Maia (supermarket 3)	Nov/19
43	L	Treats	Cereals; meat and animal subproducts; meat flour; oils/fats	Maia (supermarket 3)	Nov/19
44	Q	Wet	Meat and animal subproducts (4% turkey); cereals	Matosinhos (pet store 1)	Nov/19
45	A	Raw-frozen	Steer (79%); vegetables; salmon oil	Matosinhos (pet store 1)	Oct. 2019 - Nov. 2019
46	A	Raw-frozen	Veal (59%); salmon (20%); vegetables; salmon oil	Matosinhos (pet store 1)	Sep. 2019 - Jan.2020
47	Q	Wet	Fish (trout 20%, salmon 14%, sardine 10%); vegetables	Matosinhos (pet store 1)	Jan/20
48	Y	Semi-moist	Meat and animal subproducts (60% cereals; vegetables	Matosinhos (supermarket 1)	Dec/19
49	Y	Semi-moist	Meat and animal subproducts (85% vegetables	Matosinhos (supermarket 1)	Dec/19
50	Y	Semi-moist	Meat and animal subproducts (85% vegetables	Matosinhos (supermarket 1)	Dec/19
51	B	Raw-frozen	Turkey (50%); goose (30%); vegetables; fruit	Matosinhos (pet store 2)	Jan/20
52	B	Raw-frozen	Turkey (40%); salmon (20%); white fish (20%); vegetables	Matosinhos (pet store 2)	Jan/20
53	A	Raw-frozen	Veal (59%); salmon (20%); vegetables; salmon oil	Matosinhos (pet store 1)	Sep. 2019 - Jan.2020
54	A	Raw-frozen	Turkey (60%); lamb (20%); vegetables	Matosinhos (pet store 1)	Sep. 2019 - Jan.2020
55	A	Raw-frozen	Chicken (60%); veal (19%); vegetables	Matosinhos (pet store 1)	Sep. 2019 - Jan.2020

Given the dimension Table S2, S3, S4 and S5 are available at

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2. High diversity of pathogenic *Escherichia coli* clones carrying *mcr-1* among gulls underlines the need for strategies at the environment–livestock–human interface

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RESEARCH ARTICLE

High diversity of pathogenic *Escherichia coli* clones carrying *mcr-1* among gulls underlines the need for strategies at the environment–livestock–human interface

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Abstract

The expansion of *mcr*-carrying bacteria is a well-recognized public health problem. Measures to contain *mcr* spread have mainly been focused on the food–animal production sector. Nevertheless, the spread of MCR producers at the environmental interface particularly driven by the increasing population of gulls in coastal cities has been less explored. Occurrence of *mcr*-carrying *Escherichia coli* in gull's colonies faeces on a Portuguese beach was screened over 7 months. Cultural, molecular and genomic approaches were used to characterize their diversity, *mcr* plasmids and adaptive features. Multidrug-resistant *mcr*-1-carrying *E. coli* were detected for 3 consecutive months. Over time, multiple strains were recovered, including zoonotic-related pathogenic *E. coli* clones (e.g. B2-ST131-H22, A-ST10 and B1-ST162). Diverse *mcr*-1 genetic environments were mainly associated with ST2/ST4-HI2 (ST10, ST131, ST162, ST354 and ST4204) but also IncI2 (ST12990) plasmids or in the chromosome (ST656). Whole-genome sequencing revealed enrichment of these strains on antibiotic resistance, virulence and metal tolerance genes. Our results underscore gulls as important spreaders of high-priority bacteria and genes that may affect the environment, food–animals and/or humans, potentially undermining One-Health strategies to reduce colistin resistance.

Joana Mourão, Luísa Peixe and Patrícia Antunes are active members of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Food and Water-borne Infection Study Group (EFWISG).

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INTRODUCTION

Colistin is a last-resource antibiotic increasingly used to treat severe Gram-negative human infections, classified by the World Health Organization as a *high-priority critically important antimicrobial* (WHO, 2019). Expansion of bacteria carrying mobilized colistin resistance (*mcr*) genes increased the concerns related to the animal–human transmission (Liu et al., 2016), due to their extensive use for decades in livestock (EMA et al., 2016; Poirel & Nordmann, 2016).

The food–animal production sector put efforts in diverse global actions to tackle the expansion of *mcr*-carrying bacteria, mainly focused on restrictions of colistin use (EMA/EFSA, 2017; Olaitan et al., 2021; Usui et al., 2021). In the European Union, colistin restriction actions in food–animal production implemented in the last years (EMA/EFSA, 2017) resulted in a successful decrease of *mcr-1* in animals or meat products (Fournier et al., 2020; Ribeiro et al., 2021). Besides the animal level, the environmental sector (e.g. waterways, sewage, migratory wildlife, etc.) should be involved and contribute to further strengthening a global response to reduce antimicrobial-resistant infectious diseases. However, the role of the environment or non-foodborne animals at the interface of food–animals, agriculture and humans in the dispersion of *mcr* within human communities has been scarcely explored (Anyanwu et al., 2021; Drali et al., 2018; Liakopoulos et al., 2016; Mohsin et al., 2016; Wang et al., 2017), though it would support appropriate measures to contain the spread of colistin-resistant bacteria and *mcr* genes (EFSA, 2021; ec.europa.eu/health/antimicrobial-resistance/eu-action-on-antimicrobial-resistance_en; Cherak et al., 2021).

Gulls are birds with long migratory behaviour and urban lifestyles due to feeding practices and easy habitat adaptation, increasingly present in big coastal cities of Mediterranean countries (Russo et al., 2021). The higher availability of food in coastal cities (e.g. landfills, surface waters, docks/fish markets, farmlands/aquaculture) led to the development of colonies composed of resident and migratory gulls in urban and surrounding areas (Russo et al., 2021; SPEA, 2018; Zendri et al., 2020). Their foraging behaviour in anthropogenic habitats potentially increases their exposure to antimicrobials and antimicrobial-resistant bacteria, leading to a higher risk of acquisition and dissemination of antimicrobial resistance determinants through their long migratory pathways (Zendri et al., 2020). The role of gulls as vectors of multidrug-resistant (MDR) and *mcr-1*-carrying bacteria is recognized in a few studies (Ahlstrom et al., 2019; Ljubojević et al., 2016). However, the number of comprehensive studies addressing genomic features of colistin-resistant and *mcr*-carrying bacteria in gulls is scarce, especially after implementation of colistin control strategies, impairing the elucidation of *mcr* spreaders in the environment–human interface (Zeballos-Gross et al., 2021).

Our goal was to evaluate, by whole genome sequencing, the occurrence and molecular features of *mcr*-producing *E. coli* identified in gull's faecal samples at extensively used beaches in Porto, Portugal. By representing the environment–human interface, this study will elucidate if gulls might be important *mcr*-spreaders and a further risk factor undermining strategies for reduction of colistin resistance.

EXPERIMENTAL PROCEDURES

Sampling collection and screening of *mcr*-carrying *E. coli*

Fourteen gulls pooled freshly voided faeces samples (corresponding to ±50 birds each) were collected from the Matosinhos beach (Porto, Portugal) over 7 months (September 2018 to March 2019; one pooled sample every 2 weeks). Each pool represented extensively beach areas where colonies composed by resident and migratory gulls' species are present. A predominance of lesser black-backed gulls (*Larus fuscus*) and yellow-legged gulls (*Larus michahellis*) has been reported in the Portuguese northwest region (Simoes et al., 2010; SPEA, 2018). The samples were collected with sterile material and processed on the same day.

The initial processing step consisted of resuscitation of 25 g of tested sample into 225 ml of Buffered Peptone Water (Oxoid, Basingstoke, UK) for 1 h at 37°C. After that, the suspension was spread on Tryptone Bile X-glucuronide agar plates (Biokar Diagnostics, Allonne, Beauvais, France) supplemented with colistin at 3 mg/L (Sigma-Aldrich, Saint Louis, MO, USA) and incubated overnight at 37°C for detection of *Escherichia coli*.

One to 10 typical *E. coli* colonies with different morphotypes were selected from each plate for identification by Matrix-Assisted Laser Desorption-Ionization-Time of Flight Mass Spectrometry (MALDI-TOF VITEK MS, bioMérieux, France). Colistin resistance genes (*mcr-1* to *mcr-5* and *mcr-6* to *mcr-10*) were searched by multiplex PCR, as described (Borowiak et al., 2020; Rebelo et al., 2018; Wang et al., 2020a).

Phenotypic and genotypic characterization of *mcr*-carrying *E. coli*

Relatedness between isolates from different samples was inferred by Fourier-transform infrared (FTIR) spectroscopy with attenuated total reflectance (ATR) using a PerkinElmer Spectrum Two device. After growth in standardized culture conditions (37°C/18 h), a colony was directly deposited on the ATR accessory of the FTIR instrument and air-dried. Spectra were then compared between each other and with those from an in-house spectral database, as previously described (Sousa et al., 2013; Rodrigues, et al., 2020). The FTIR-based assignments were then confirmed using *Xba*I PFGE according to the protocol of the Centres for Disease Control and Prevention (CDC, 2017).

Antibiotic susceptibility profiles of *mcr-1* positive isolates were established for 19 antibiotics (amoxicillin + clavulanic acid—30 μg, ampicillin—10 μg, aztreonam—30 μg, amikacin—30 μg, cefazolin—30 μg, cefotaxime—30 μg, cefoxitin—30 μg, ceftazidime—30 μg, chloramphenicol—30 μg, ciprofloxacin—5 μg, doxycycline—30 μg, gentamicin—10 μg, imipenem—10 μg, levofloxacin—5 μg, nitrofurantoin—300 μg, streptomycin—10 μg, tetracycline—30 μg, tobramycin—10 μg and trimethoprim/sulfamethoxazole-25 μg) by disc diffusion method on cation-adjusted Mueller–Hinton agar (Biokar Diagnostics, Beauvais, France). The interpretation was performed using the Clinical and Laboratory Standards Institute guidelines (CLSI, 2020). The colistin (Sigma-Aldrich) minimal inhibitory concentrations (MICs) were determined by the EUCAST reference broth microdilution method in cation-adjusted Mueller–Hinton broth (Sigma-Aldrich) (EUCAST, 2016).

Plasmids were classified and characterized using the PCR-based replicon typing method (IncHI2, IncI2 and IncX4) (Carattoli et al., 2005; Johnson et al., 2012) and the location of the *mcr-1* gene was detected by S1/I-*Ceu*I-PFGE followed by Southern hybridization experiments with specific probes (*mcr*-1, IncHI2 and IncI2), as described (Campos et al., 2016).

Whole-genome sequencing

One representative isolate of each *mcr*-positive clone was selected for WGS analysis. The DNA was extracted with the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI, USA), with the final concentration measured with Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA) and sequenced with standard HiSeq (2 × 151 bp) Illumina platform (Illumina, San Diego, CA, USA) at Eurofins Genomics (https://eurofinsgenomics.eu/). Raw reads quality control was assessed with FastQC v0.11.9 (Andrews, 2010) using default parameters. Low-quality reads (average quality below 15) were discarded using BBDuk v37.90 (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/). Trimmed reads were then *de novo* assembled using SPAdes v3.15.3 (Bankevich et al., 2012) with 'isolate' and 'cov-cutoff auto' options. The assembly was filtered, retaining contigs of >200 bp, and the quality and completeness were assessed with QUAST v5.0.2 (Gurevich et al., 2013) and BUSCO v5.0.0, respectively (Seppey et al., 2019). The draft genomes were annotated using the RAST server (Aziz et al., 2008). For metal tolerance genes (*pcoGE1ABCDRSE2*, *silESRCFBAGP*, *arsR2CBR1*, *terFD2D1CBAZ* and *merEDACPTR* operons), we used an *in-house* database within the Geneious software v2022.0.1 platform (http://www.geneious.com/).

In silico characterization of *mcr-1*-carrying *E. coli* isolates

Bioinformatic tools from the Centre for Genomic and Epidemiology (http://www.genomicepidemiology.org) were used to evaluate *E. coli* antibiotic resistance genes or known mutations (ResFinder 4.1/PointFinder) (Bortolaia et al., 2020; Zankari et al., 2017), virulence genes (VirulenceFinder 2.0) (Joensen et al., 2015), plasmid replicons (PlasmidFinder 2.1) (Camacho et al., 2009; Carattoli et al., 2014), plasmid typing (pMLST 2.0) (Carattoli et al., 2014) and Multilocus Sequence Typing (MLST 2.0) (Larsen et al., 2012) using the Achtman 7 gene MLST scheme. The serotype and core genome Multilocus Sequence Typing were defined using Enterobase (https://enterobase.warwick.ac.uk). In the case of isolate FG7-302, raw sequencing reads were directly submitted to Enterobase (https://enterobase.warwick.ac.uk) to generate a new MLST allelic profile. *Escherichia coli* phylogenetic groups were defined using ClermontTyper (http://clermontyping.iame-research.center/).

The distribution of virulence-associated, antibiotic resistance, and metal tolerance genes was visualized using a heatmap developed with Morpheus software (https://software.broadinstitute.org/morpheus/). When required, figures were minimally edited manually using Adobe Illustrator v25.3.1. To confirm the presence of the *mcr-1* gene in IncHI2 or IncI2 plasmids and reconstruct them based on draft assemblies, we used the MOB-recon tool v3.0.0 from the MOB-suite package (Robertson et al., 2020; Robertson & Nash, 2018). The *mcr-1* gene was considered part of the plasmid when it was attributed to a specific plasmid by MOB-recon or present on the same contig as the incompatibility replicon sequence.

TABLE 1 Characterization of *mcr*-positive *E. coli* isolates recovered from gulls' faeces in Matosinhos beach (Porto, Portugal)

Sample collection month[a]	Isolate name	FTIR (PFGE-type)[b], no. isolates	PhG/MLST/cgST[c]	Serotype	*mcr-1* location[d] Chr/PL-size (kb)/pMLST	Colistin MIC (mg/L)	Antibiotic resistance phenotype other than colistin[e]
FG6—Oct 2018	FG6_310	E-I (A), $n = 1$	B1/ST162/cgST190769	O9:H9	PL-HI2-260/ST4	4	AMC, AMP, CLO, CIP, DOX, LEV, SXT, TET
	FG6_311_1	E-II (B), $n = 1$	A/ST10/cgST190770	O71:H27	PL-HI2-260/ST4	4	AMC, AMP, ATM, CAZ, CTX, CFZ, TET
	FG6_312	E-III (C), $n = 1$	A/ST10/cgST190771	O16:H48	PL-HI2-330/ST2	4	AMC, AMP, CLO, DOX, GEN, LEV, TET, TOB, SXT
FG7—Nov 2018	FG7_300	E-IV (D), $n = 2$	B2/ST131/cgST190772	O25:H4	PL-HI2-220/ST4	4	AMP
	FG7_301						
	FG7_302	E-V (E), $n = 1$	A/ST12990/cgST190773	O149:H38	PL-I2-70	4	AMP, TET
FG8—Nov 2018	FG8_300	E-VI (NT), $n = 1$	A/ST4204/cgST190774	O6:H10	PL-HI2-240/ST4	4	AMC, AMP, CLO, CIP, DOX, FOX, LEV, TET
FG9—Dec 2018	FG9_301	E-VII (F), $n = 1$	A/ST656/cgST190775	O141:H4	Chr	4	AMC, AMP, TET
FG10—Dec 2018	FG10_301_1	E-VIII (G), $n = 1$	F/ST354/cgST190777	O153/O16:H34	PL-HI2-260/ST2	4	AMP, CIP, GEN, LEV, SXT, TET, TOB

[a]Samples collected every 2 weeks between September 2018 and March 2019.
[b]FTIR types, *Escherichia coli*: E-I to E-VIII; PFGE-types, *Escherichia coli*: A to G; NT, non-typeable.
[c]PhG, phylogenetic group; MLST, multilocus sequence typing; cgST, core genome sequence typing.
[d]Chromosome (Chr) and/or plasmid (PL) location of *mcr-1* gene. pMLST, plasmid multilocus sequence typing.
[e]AMC, Amoxicillin + clavulanic acid; AMP, Ampicillin; ATM, Aztreonam; CLO, Chloramphenicol; CFZ, Cefazolin; CAZ, Ceftazidime; CIP, Ciprofloxacin; CTX, Cefotaxime; DOX, Doxycycline; FOX, Cefoxitin; GEN, Gentamicin; LEV, Levofloxacin; TET, Tetracycline; TOB, Tobramycin; SXT, trimethoprim/sulfamethoxazole.

FIGURE 1 Heatmap generated using Morpheus online web-server (see methods for more details) representing the distribution of virulence-associated genes (VirulenceFinder), antibiotic resistance (ResFinder) and metal tolerance genes (in-house database) from the sequenced *Escherichia coli* genomes. Only known mutations conferring fluoroquinolone resistance are presented. Coloured squares indicate the presence of genes sorted by category. The virulence-associated gene content associated with ExPEC is indicated in bold. All isolates were positive for the presence of *mcr1.1* variant by ResFinder, although when using AMRFinderPlus, isolate FG9_301 was positive for *mcr1.32*. Abbreviations: ND, not determined; NA, not applicable

RESULTS AND DISCUSSION

High occurrence of MDR *mcr-1*-carrying *E. coli* among gull colonies

This study detected a high rate of gull pooled faecal samples (36%, five out of 14 sampling periods) carrying MDR *E. coli* with *mcr-1* gene over 7 collection months. In fact, *mcr-1* is the most widespread *mcr* gene variant circulating in humans, animals and environmental sources in Portugal (Ahlstrom et al., 2019; Campos et al., 2016; Mendes et al., 2018) and worldwide (Ling et al., 2020). Studies varying in sample type or the experimental approach (most screened collections of isolates versus samples) in diverse EU countries (e.g. Lithuania, Spain, Poland) have reported *mcr-1* sporadically in gulls (Ahlstrom et al., 2019; Ruzauskas & Vaskeviciute, 2016; Skarżyńska et al., 2021). In low and middle-income countries-LMICs (e.g. African countries) that are part of the gulls' migratory routes from/to Europe (Shamoun-Baranes et al., 2017; SPEA, 2018), *mcr*-carrying bacteria are widely spread in different niches (e.g. food-producing environments, soil, water and humans), including among gulls (as high as 20%; e.g. Egypt) (Ahmed et al., 2019; Anyanwu et al., 2021), probably reflecting colistin usage still largely unregulated and widespread in livestock (e.g. most LMICs still use colistin as a growth promoter). Additionally, the possible contact of gulls with intensive aquacultures, especially in integrated systems (fishes fed with the faeces of animals reared with extensive antibiotic usage) with high prevalence of colistin-resistant bacteria/*mcr* (Shen et al., 2019) alerts for other potential sources of *mcr*. These studies suggest a role for gulls (capable of migrating 3000 km per week across several countries) as vectors for *mcr* spread from regions with high levels of antibiotic resistance to less affected areas (Zeballos-Gross et al., 2021). Short and long-distance *mcr* environmental transmission is likely to occur by faecal contamination of bathing and irrigation water as well as farmlands, which suggest that environmental sector should act together with food-producing animal in order to reduce colistin resistance spread. Regardless of recent results suggesting that banning colistin in food-producing animals can limit *mcr* spread (Luo et al., 2020; Ribeiro et al., 2021; Wang et al., 2020b), the role of wide-ranging movements of migratory birds with urban lifestyle as gulls on colistin resistance spread should also be considered in global coordinated actions against antibiotic resistance (Skarżyńska et al., 2021).

Interestingly, we found *E. coli* carrying the *mcr-1* gene over 3 consecutive winter months, which may be related to gull's increased migratory behaviour to south countries during this period and/or urban lifestyle (feeding habits and habitat use), as recently proposed in a metagenomic study comparing gulls with other birds (Jarma et al., 2021). In fact, migratory gulls wintering in Southern European and African countries are converging from different breeding areas (e.g. Northern Europe), leading to complex antibiotic-resistant (ABR) bacteria/genes dissemination between colonies of gulls' and the environment of migratory path countries, like Portugal and Spain (Jarma et al., 2021; Tarabai et al., 2019). A possible source of *mcr-1* acquisition during migratory routes might be related to gull's feeding sites since they contact with terrestrial and aquatic environments such as landfills, food–animals, aquacultures and other aquatic ecosystems, other birds, and urban areas from different regions, where last-resource ABR bacteria/genes might be present, making them vectors for *mcr* (inter)continental dispersal (Jarma et al., 2021; Shamoun-Baranes et al., 2017).

On the other hand, the continuous introduction of *mcr-1*-carrying bacteria or mobile genetic elements in the studied colonies composed by resident and migratory gulls in later autumn may have led to their persistence throughout the 3 winter months. A recent study showed that ring-billed gulls could be colonized and shed *mcr-1*-carrying *E. coli* for an extended period (e.g. 16 days). Also, those strains can be detected in the environment for even longer (e.g. 29 days), highlighting the importance of environmental pathways (through gull's faecal contamination of water, public areas and livestock/agriculture) for continuous *mcr* dissemination (Franklin et al., 2020). The possibilities of *mcr* transmission are aggravated due to the proximity and interactions between coastal gulls and human urbanized areas. Gulls foraging for habitat and food (e.g. 20–30 km per day) in cities also contact with livestock/agriculture areas, aquacultures and fish markets, and human wastes/landfills (Garthe et al., 2016). Also, colonies' fixation in the same beaches where recreational activities occurred constitutes a human health risk for exposure and acquisition of antimicrobial-resistance bacteria (Franklin et al., 2020; Zendri et al., 2020).

Detection of diverse emerging zoonotic *mcr-1*-carrying *E. coli* clonal lineages over time

We recovered nine colistin-resistant (MIC = 4 mg/L) and *mcr-1*-carrying *E. coli* isolates among the gull beach colonies. They were discriminated in eight clones by both FTIR and pulsed-field gel electrophoresis (PFGE) (Table 1), demonstrating a similar discriminatory potential between both typing methods, as reported (Silva et al., 2020; Sousa et al., 2013). Besides, FTIR spectroscopy proved to be a useful tool for accurate, fast and inexpensive evaluation of isolates' clonal relationship from the same or different samples. Previous applications have been focused on outbreak detection and surveillance in the clinical setting (Novais et al., 2019; Silva et al., 2020; Sousa

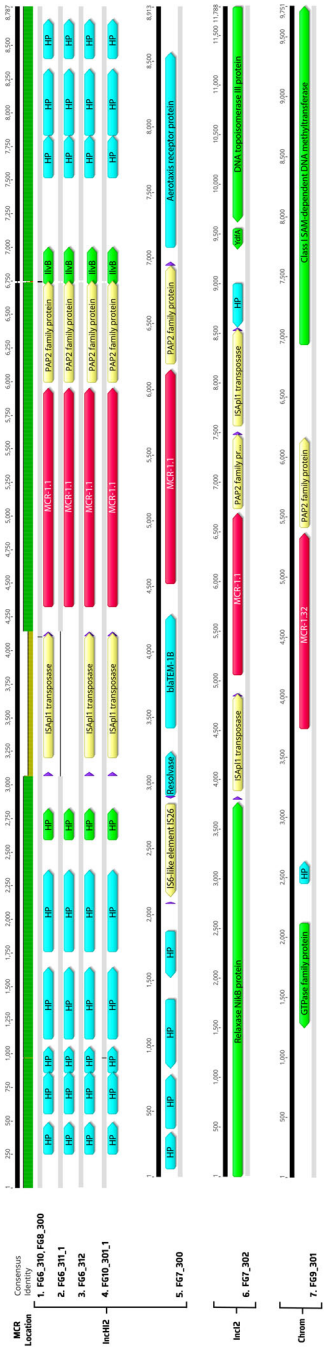

FIGURE 2 Gene synteny of the environment surrounding the conserved *mcr*-containing cassette. The black and green bars in the upper panel represent consensus region and nucleotide sequence identity, respectively, between isolates FG6_310, FG8_300, FG6_311_1, FG6_312 and FG10_301_1 (IncHI2 plasmids). Unique *mcr-1* environments are represented in the second (FG7_300, IncHI2 plasmid), third (FG7_302, IncI2 plasmid) and fourth (FG9_301, chromosome) panels. All isolates were positive for the presence of *mcr1.1* variant by ResFinder, although when using AMRFinderPlus, isolate FG9_301 was positive for *mcr1.32*. Filled arrows indicate open reading frames' position and transcriptional direction (red—*mcr-1* gene, yellow—PAP2 family protein and insertion sequence (IS), green—genes immediately flanking the conserved *mcr*-containing cassette, purple—inverted repeat (IR) and blue—other genes)

et al., 2013). However, this study and others (Ribeiro et al., 2021; Ribeiro-Almeida et al., 2021) demonstrate that this methodology can be extended to food, veterinary or environmental contexts, being the isolate classification dependent on the availability of a representative clonal database and appropriate machine-learning models.

The eight clones corresponded to phylogenetic groups (PhG) A, B1, B2 and F and seven Sequence Types-STs (ST10, ST131, ST162, ST354, ST4204, ST656 and ST12990) (Table 1). According to EnteroBase (https://enterobase.warwick.ac.uk/species/index/ecoli), all but one of the STs are recognized as globally dispersed, being identified in the environment and diverse food production, wild and companion animals, as well as associated with human infections (e.g. urinary tract infections—UTIs, septicemia and meningitis), supporting diverse potential origins. Interestingly three detected clones (B2-ST131-H22 sublineage, A-ST10 and B1-ST162) have been recognized as zoonotic-related pathogenic clones (Denamur et al., 2021; Zhuge et al., 2019). WGS analysis revealed that all strains were enriched in diverse genes coding for adhesins, capsules, siderophores, toxins, protectins, bacteriocins and various other putative virulence and/or colonization-associated markers, including ExPEC virulence genes [e.g. F-ST354 (*iutA*, *kpsMII*), B2-ST131-H22 (*yfcV*, *chuA*, *fyuA*)] (Denamur et al., 2021; Tetzschner et al., 2020; Zhuge et al., 2019) (Figure 1).

The *E. coli* O25:H4 ST131-H22 sublineage (ST131 clade B with *fimH22*) is an important etiologic agent for community-acquired UTIs with a food animal origin, which became established in poultry production due to their ability for avian colonization (Liu et al., 2018). To our knowledge, this is the first report of *mcr*-1 in this zoonotic-related pathogenic clone recovered from gulls, reinforcing their potential role as hotspots and transmission routes between wildlife–animals–foods–humans interface. The intruder behaviour of those birds in urban areas worldwide and the easy access to food–animal wastes, food-producing animals, wastewater treatment plants and sewage effluents may justify these results, which constitute a public health concern. Urgent environmental containment strategies for managing food wastes or sewages and the increasing gull populations are needed to control the dissemination of *mcr* genes associated with successful clones.

Diversity in *E. coli* clonal backgrounds from gull faecal samples is in line with data from other studies in Portugal and other EU countries (Ahlstrom et al., 2019; Simoes et al., 2010; Skarżyńska et al., 2021; Varela et al., 2015; Wang et al., 2017), suggesting diverse sources of bird colonization, as discussed previously. Nevertheless, in this study, by using pooled environmental sampling (each pool representing extensive areas used by the beach gull's colonies) we were able to detect a higher *mcr*-1-carrying *E. coli* clonal diversity

(eight clones recovered from five sampling periods over 3 consecutive winter months) than in studies sampling individual birds (Ahlstrom et al., 2019; Ruzauskas & Vaskeviciute, 2016; Skarżyńska et al., 2021). In two cases, co-occurrence of several clones in the same sample was detected, but none of the colistin-resistant *E. coli* clones persisted over time (Table 1). In fact, in the absence of colistin, the persistence rates of *mcr*-carrying bacteria could vary depending on their genomic background and host features (e.g. between gulls), as recently reported (Franklin et al., 2020; Li et al., 2020).

Plasmid dynamics of *mcr-1*-carrying *E. coli*

The *mcr-1* was either located in the chromosome ($n = 1$; ST656), IncI2 ($n = 1$; ST12990) or IncHI2 ST2/ST4 ($n = 6$ from four samples; ST10, ST131, ST162, ST354, ST4204) plasmid types dispersed in different samples and clones (Table 1). These results agree with the global distribution of *mcr*-associated plasmids, where IncHI2-type plasmid predominates in *mcr*-carrying Enterobacterales from diverse sources in Portugal (Campos et al., 2016; Freitas-Silva et al., 2018; Gilrane et al., 2017; Ribeiro et al., 2021), other European countries (Matamoros et al., 2017), or some LMIC countries (Anyanwu et al., 2020).

Concerning the environment surrounding the conserved *mcr*-containing cassette (Poirel et al., 2016), the WGS analysis revealed three different genetic contexts (Sun et al., 2018). In six isolates carrying *mcr*-containing cassette in IncHI2 plasmids (ST2/ST4), four of them had only one copy of IS*Apl1* upstream *mcr-1*, and in two, neither IS*Apl1* nor the corresponding inverted repeat (IR) right and left were present (Figure 2), suggesting a possible fixation of the *mcr* colistin resistance platform (Li et al., 2017). Despite these slight differences in the genetic environments, in five of these isolates, the *mcr-1-pap2* element was always found in the same location, between two hypothetical proteins, with only one of them (IlvB) belonging to a known putative superfamily. The only exception was one isolate (FG7-300) where IS*Apl1* was absent, and both the hypothetical proteins were located together at 48.160 bp upstream *pap2* gene. Curiously, in this isolate, an IS*15DII* (IS*26*-like) transposase, member of IS*6* family, was located upstream of the *mcr-1* and seemed to be responsible for the $bla_{\text{TEM-1B}}$ mobilization and integration. The IS*6* family transposases play a key role in the dissemination of multiple antibiotic resistance genes in Gram-negative bacteria (Varani et al., 2021) and were recently associated with *mcr-3*-IncFII/IncHI2/IncP1 (Du et al., 2020; Kalová et al., 2021; Li et al., 2021; Wang et al., 2018b), *mcr-1*-IncX4- (Du et al., 2020) and *mcr-9*-HI2-carrying plasmids (Faccone et al., 2020; Kieffer et al., 2019). In one isolate (FG7-302) carrying *mcr-1.1* in an IncI2 plasmid, we observed the presence of a common Tn*6330* element with two IS*Apl1* flanking the *mcr*-containing cassette and inserted between the *top* (encoding a DNA topoisomerase III) and *nikB* (relaxase) genes (Figure 2). Although this genetic environment is relatively uncommon, it was recently described in an *mcr-1.5*-IncI2-carrying *E. coli* from Argentina (Tijet et al., 2017). In the present study, only one isolate carried *mcr-1-pap2* on the chromosome, with an absence of IS*Apl1* and its IRs, inserted between *yeeP* (encoding a GTPase family protein) and *yeeA* (class I SAM-dependent DNA methyltransferase) genes (Figure 2). Over time, loss of IS*Apl1* elements has been associated with the stabilization of the *mcr-1*-containing cassette in a diverse range of genomic backgrounds, particularly plasmids (Li et al., 2021; Snesrud et al., 2018; Wang et al., 2018a), highlighting the importance of this event for *mcr-1* maintenance. A similar genetic environment, although with one or two copies of IS*Apl1* upstream *mcr-1-pap2*, was already described in the chromosome of *E. coli* isolates (Shen et al., 2020). In fact the occurrence of the *mcr-1* gene in diverse genetic contexts (e.g. different clones, plasmid types and/or genetic environments) may reflect their dissemination among gull's colonies by diverse transmission events either between birds, the environment, or within gull's gut microbiome (e.g. IncHI2-ST4 in two clones from the same sample).

mcr-1-carrying *E. coli* are enriched in antibiotic resistance and metal tolerance genes

All the *mcr-1*-carrying colistin-resistant *E. coli* isolates presented co-resistance to other antibiotics belonging to several classes, including ampicillin (100%), tetracycline (78%), amoxicillin + clavulanic acid (44%) as well as the clinically relevant ciprofloxacin (33%), cefoxitin (11%) and ceftazidime (11%) (Table 1). Those MDR phenotypes rates were consistently reported in *mcr*-carrying *E. coli* isolates from human and animal settings, especially poultry in different countries (Anyanwu et al., 2021; EFSA/EMA, 2020; Ribeiro et al., 2021). Genes encoding resistance to aminoglycosides [*aadA*/*aph*/*aac(3)*], beta-lactams ($bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-32}}$), phenicols (*catA*/*cmlA*/*floR*), tetracyclines [*tet(A)*/*tet(B)*/*tet(M)*], sulfonamides (*sul1*/*sul2*/*sul3*) and trimethoprim (*dfrA*) were identified in diverse clones and were consistent with the observed phenotypes (Table 1 and Figure 1). Also, we identified chromosomal mutations in the quinolone resistance determining region of topoisomerase genes *gyrA*/*parC*/*parE* in three clones (B1-ST162, A-ST4204 and F-ST354; B2-ST131-H22 sublineage) and the plasmid-mediated quinolone resistance genes (PMQR) genes *qnrB19* or *qnrS1* in other clones (A-ST10, A-ST656 and F-ST354) (Figure 1). In the zoonotic-related pathogenic ST10 clone (E-II) we also detected the $bla_{\text{CTX-M-32}}$ gene encoding

resistance to extended-spectrum cephalosporins. In fact, CTX-M-32 is an extended-spectrum beta-lactamase (ESBL) widely spread in Southern European countries, particularly in clinical and food–animal production (e.g. pig farming) (Rodríguez et al., 2014), but also described in migratory gulls studied in Sweden, a country with low ESBL prevalence (Atterby et al., 2017). In addition, diverse metal tolerance gene operons encoding for copper/silver (*cus*/*pco*/*sil*), arsenic (*ars*), mercury (*mer*) and/or tellurite (*ter*) were detected in all the genomes (Figure 1). These data suggest a gull's colonization with bacteria both enriched in antibiotic resistance and metal tolerance genes, possibly due to contact with antimicrobial/heavy metal polluted environments where those bacteria might persist. This migratory birds/gulls faecal MDR microbiota (here including with *mcr-1*), also observed in other studies (Jarma et al., 2021; Ngaiganam et al., 2019; Wang et al., 2017; Zendri et al., 2020), is the reflection of their mobility and urban lifestyle. In fact, Jarma et al. (2021) found a higher abundance of potentially pathogenic bacteria and several ABR genes (including *mcr-1*) in birds with anthropogenic habitats like gulls compared with other bird species. Gulls are exposed to diverse human-influenced environments contaminated with antimicrobials and ABR bacteria (e.g. animal-based food residues, food-producing settings, rivers and beaches with a high human density, industry/hospital/urban wastes, and wastewaters/landfills). Therefore, they could indirectly contribute to the transmission and persistence of *mcr-1*, which suggest that environmental sectors should also be involved in strategies for reduction of colistin resistance together as the food–animal producing sector (EFSA, 2021; EMA/EFSA, 2017). Thus, tackling ABR not only requires coordinated Global Health interventions focused on impairing the selection of ABR bacteria (either due to antibiotics or by other co-selecting agents like heavy metals and biocides) in specific niches but should also include measures targeted to break transmission routes between different ecosystems through vectors of AMR bacteria/genes like gulls (EFSA, 2021; Hernando-Amado et al., 2019; Hernando-Amado et al., 2020). A recent study showed positive outcomes of urban waste management/collection policy on control of the yellow-legged gulls' breeding population (Coccon et al., 2021). This opens the possibility for environmental strategies containing urban gull's population to act as a complement to colistin restrictions on both food-producing animals and healthcare sectors.

Conclusions and future perspectives

Our study revealed gulls as carriers of a high diversity of global pathogenic MDR *E. coli* clones, including the zoonotic relevant B2-ST131-H22, ST10 or ST162, sharing diverse *mcr-1*-carrying plasmids, ESBL, PMQR and/or metal determinants. Our results underscore gulls as important spreaders of high-priority bacteria and genes between wildlife, the environment, humans and potentially food–animals. Thus, they could represent a further risk factor undermining the reduction of MCR producers already achieved through colistin restrictions measures at the livestock sector. Further studies at the environment–livestock–human interface linked to international strategies to combat AMR (e.g. EU Farm to Fork strategy within the EU Green Deal) are highly recommended (EFSA, 2021).

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The raw reads of *Escherichia coli* FG6_310 (Uberstrain number ESC_YA6250AA), FG6_311_1 (ESC_YA6252AA), FG6_312 (ESC_YA6253AA), FG7_300 (ESC_YA6254AA), FG7_302 (ESC_YA6255AA), FG8_300 (ESC_YA6256AA), FG9_301 (ESC_YA6261AA) and FG10_301_1 (ESC_YA6262AA) were deposited at the Enterobase.

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CHAPTER 4 – Conclusions

This thesis (*Beyond Colistin Withdrawal: Tracking Colistin-Resistant Enterobacterales within a One Health Context from Local to Global Drivers*), provides an integrated assessment of colistin resistance dynamics with the overall results pointing to the following general conclusion:

Through comprehensive tracking of colistin-resistant Enterobacterales across food-animal production systems and associated ecological compartments, following the long-term ban on colistin use, a general reduction - and in some cases elimination - of *mcr*-carrying Enterobacterales was observed in intensive poultry and rabbit production systems. Nevertheless, persistence occurred in specific contexts, sustained by co-selection pressures, including the use of other antibiotics and metals, environmental reservoirs, and management practices. In addition, animal by-products and wildlife emerged as overlooked sources of colistin resistance. These findings reinforce the need for continuous surveillance and integrated One Health interventions to curb the spread of colistin resistance and safeguard the efficacy of last-resort antibiotics.

Further conclusions are summarized as follows:

1) **Persistence of multidrug-resistant and colistin-resistant *E. coli* and *K. pneumoniae* across poultry and rabbit production chains after long colistin withdrawal.**

This thesis shows that the ban of colistin in food-animal production contributed to a marked reduction in *mcr*-mediated resistance, with the detection of bacteria carrying these genes at low frequency in a single rabbit farm and absence in all poultry farms. These findings confirm the effectiveness of colistin withdrawal as a key policy measure to limit the dissemination of *mcr* genes. Nevertheless, despite the reduction achieved, colistin withdrawal was ineffective in eliminating colistin-resistant *K. pneumoniae* from poultry farms and *mcr*-carrying *E. coli* from rabbit farms. Moreover, high rates of resistance to multiple antibiotics - including clinically important agents such ciprofloxacin - were observed in *K. pneumoniae* isolates from poultry and *mcr*-carrying *E. coli* from rabbits. These finding underscore the complexity of antimicrobial resistance dynamics and highlight the role of potential co-selection pressures sustaining resistance even after colistin withdrawal.

2) **Species-specific patterns and genetic determinants of colistin-resistant Enterobacterales in intensive poultry and rabbit production systems.**

Colistin resistance was detected in both poultry and rabbit production systems, but with notable species-specific differences. In poultry, *mcr* genes disappeared following the colistin ban however, colistin-resistant *K. pneumoniae* strains exhibited a high diversity of chromosomal mutations, including some imposing a low biological cost, which may explain the persistence of colistin-resistant *mcr*-negative *K. pneumoniae* isolates after long colistin withdrawal. In contrast, *mcr*-positive *E. coli* from rabbits persisted, suggesting sustained circulation within this production system, associated with recurrent contamination and reintroduction from external sources. Most of these isolates harboured *mcr* genes on IncHI2 plasmids, highlighting a plasmid-mediated mechanism capable of horizontal transmission and long-term maintenance of MDR clones over three years. Together, these findings indicate the circulation of different colistin-resistant genotypes in poultry and rabbit production, responding differently to colistin withdrawal. The identification of MDR *mcr-1*-carrying *E. coli* STs in rabbits and humans (ST1011, ST1196, ST1589), together with the similarity of *K. pneumoniae* lineages (ST15-KL19, ST15-KL146, and ST392-KL27) between poultry and human, underscores the potential for cross-species circulation of clinically relevant lineages. Moreover, the high similarity of IncHI2 and IncF plasmids to those reported in human and animal collections from diverse regions, highlights their potential for interspecies and interregional dissemination, linking local farm-level events to broader global AMR dissemination.

3) **Co-selection and environmental factors sustaining colistin resistance in farm environments.**

Results from poultry and rabbit farms indicate maintenance of colistin-resistance even after colistin ban, associated with co-selection pressures from metals - particularly copper - and other antimicrobials, together with environmental reservoirs and farm management gaps. In rabbit production, *mcr-1* genes were consistently located on multireplicon IncHI2 plasmids, which also carried copper and silver decreased susceptibility determinants (*sil±pco*) alongside multiple antibiotic resistance genes. In poultry farms, colistin-resistant *K. pneumoniae* co-harboured antibiotic resistance genes and copper decreased susceptibility *sil±pco* gene clusters in multireplicon F-type plasmids. The continued use of antimicrobials in food-animal production and the widespread presence of these genes may contributed to changes in poultry and rabbit microbiota, providing strong evidence of co-selection of strains with copper-

decreased susceptibility and MDR, including those resistant to colistin, even after long-term colistin ban. These findings reinforce the role of co-selection as a key driver shaping antimicrobial resistance dynamics. Interestingly, despite assumptions that organic trace mineral diets could reduce co-selection risk, our results show that in poultry no difference was observed compared with inorganic trace mineral diets, suggesting that farm environment and management practices play a more decisive role than feed type.

The farm environment, combined with external inputs, acts as a reservoir and continuous contamination source. This leads to the maintenance of colistin-resistant Enterobacterales and *mcr* genes, highlighting gaps in farm management and biosecurity practices. Differences between poultry and rabbit production systems, linked to species biology and management, contribute to distinct AMR profiles. Examples of management gaps include insufficient vacancy periods, incomplete cleaning and disinfection, contaminated feed or water, and cross-contamination via equipment or personnel. The recovery of closely related MDR *K. pneumoniae* isolates across poultry production stages, and the detection of genetically similar *mcr-1*-carrying *E. coli* and IncHI2 plasmids in rabbit faeces and farm environments, indicate diverse contamination events along the production chain and suggest farm environments as persistent sources of dissemination. Tracking these bacteria highlights the complex interplay of local drivers and environmental reservoirs in sustaining AMR.

4) Commercial raw meat-based pet food and wild animals are underestimated reservoirs of *mcr* genes, undermining colistin resistance reduction strategies.

This thesis demonstrates that pet food, particularly raw meat-based diets (RMBDs) for pets, are potential vehicles of MDR zoonotic-related pathogenic bacteria. In this study, *mcr*-1-carrying *E. coli* strains, including widespread ST297 (ExPEC) and ST3997-ST10 Cplx (APEC), were detected in RMBDs, with *mcr-1* genes located on IncX4 plasmids, known for high transferability. These findings highlight the role of local-to-global pathways, linking farm-level contamination to wider dissemination through food and by-products trade and handling. Similarly, MDR *mcr-1*-positive *E. coli* strains were consistently found in gulls, over three consecutive months. Clinically relevant strains identified included B2-ST131-H22, ST10 and ST162, all harbouring *mcr*-carrying plasmids (IncI2 and IncHI2) and metal decreased susceptibility gene clusters (*pco/sil*, *ars*, *mer*, *ter*). These wildlife vectors are exposed to diverse environments, including landfills, food-animal facilities, and urban areas, and their migratory behaviour can facilitate the dissemination of colistin-resistant bacteria across regions and continents. Tracking these vectors highlights their role in connecting local farm

and urban environments to global AMR dissemination, emphasizing the need to consider migratory wildlife in One Health strategies.

5) **Integrative interventions within a One Health framework are essential to curb colistin resistance through cross-sector transmission and shared drivers.**

Taken together, the findings highlight the complexity of colistin resistance persistence and dissemination, sustained by cross-sectoral transmission and common selective pressures. Effective mitigation strategies require coordinated interventions across human, animal, and environmental sectors, addressing not only antibiotic use but also metal exposure, waste management, and farm biosecurity. Robust tracking and microbiological surveillance systems across species and ecosystems are essential to detect AMR spread and implement timely interventions. Education and awareness campaigns for farmers, veterinarians, pet owners, and the public are critical for fostering responsible practices and shared accountability. Ultimately, only through a One Health framework that recognizes the interdependence of humans, animals, and the environment can colistin's efficacy and broader AMR mitigation be preserved.

Closing Remarks

This thesis provides a comprehensive investigation of colistin resistance and *mcr* genes across food-animal production, commercial pet food, and wildlife, tracking colistin-resistant Enterobacterales over time and across ecological compartments. The findings reveal that colistin resistance can persist and re-emerge through mechanisms that transcend direct antibiotic use, including co-selection by metals and reinforcement through on-farm management practices and external reservoirs such as contaminated feed and wildlife. These often-overlooked drivers provide pathways for introduction and spread, underscoring the ecological complexity of colistin resistance.

This work demonstrates that interventions focused only on human and veterinary sectors are insufficient. Understanding the persistence of *mcr* genes and tracking their movement across local-to-global ecological networks is essential for effective mitigation. Future efforts should build on this approach to strengthen environmental surveillance, understand persistence mechanisms, and support evidence-based risk assessment and policy, prioritizing integrated One Health strategies. Only sustained, collaborative and cross-sectoral actions can safeguard colistin's efficacy and curb antimicrobial resistance globally.

Success isn't about how much money you make,

it's about the difference you make in people's lives.

Michelle Obama, 2018

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RESEARCH ARTICLE

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High diversity of pathogenic *Escherichia coli* clones carrying *mcr-1* among gulls underlines the need for strategies at the environment–livestock–human interface

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Abstract

The expansion of *mcr*-carrying bacteria is a well-recognized public health problem. Measures to contain *mcr* spread have mainly been focused on the food–animal production sector. Nevertheless, the spread of MCR producers at the environmental interface particularly driven by the increasing population of gulls in coastal cities has been less explored. Occurrence of *mcr*-carrying *Escherichia coli* in gull's colonies faeces on a Portuguese beach was screened over 7 months. Cultural, molecular and genomic approaches were used to characterize their diversity, *mcr* plasmids and adaptive features. Multidrug-resistant *mcr*-1-carrying *E. coli* were detected for 3 consecutive months. Over time, multiple strains were recovered, including zoonotic-related pathogenic *E. coli* clones (e.g. B2-ST131-H22, A-ST10 and B1-ST162). Diverse *mcr*-1 genetic environments were mainly associated with ST2/ST4-HI2 (ST10, ST131, ST162, ST354 and ST4204) but also IncI2 (ST12990) plasmids or in the chromosome (ST656). Whole-genome sequencing revealed enrichment of these strains on antibiotic resistance, virulence and metal tolerance genes. Our results underscore gulls as important spreaders of high-priority bacteria and genes that may affect the environment, food–animals and/or humans, potentially undermining One-Health strategies to reduce colistin resistance.

Joana Mourão, Luísa Peixe and Patrícia Antunes are active members of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Food and Water-borne Infection Study Group (EFWISG).

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Antimicrobial resistance: a concise update

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