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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).

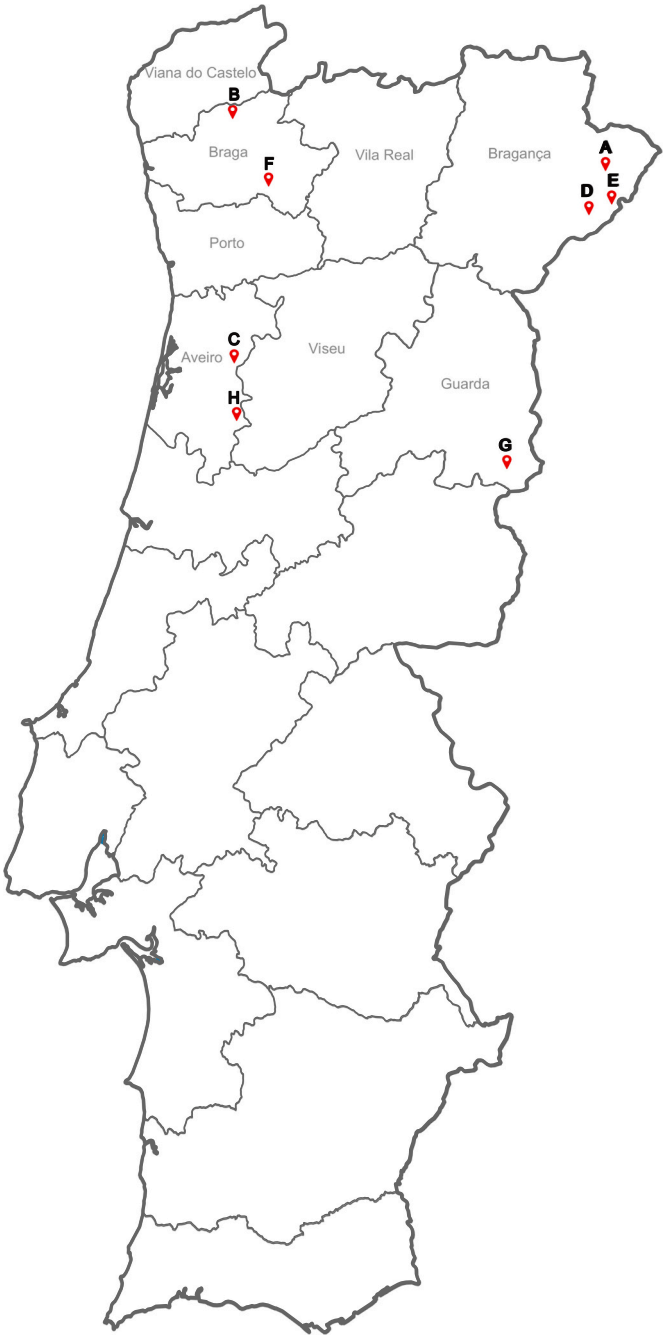

Fig. 1. Geographical localization of the sampled rabbit farms, farm A to H.

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*.

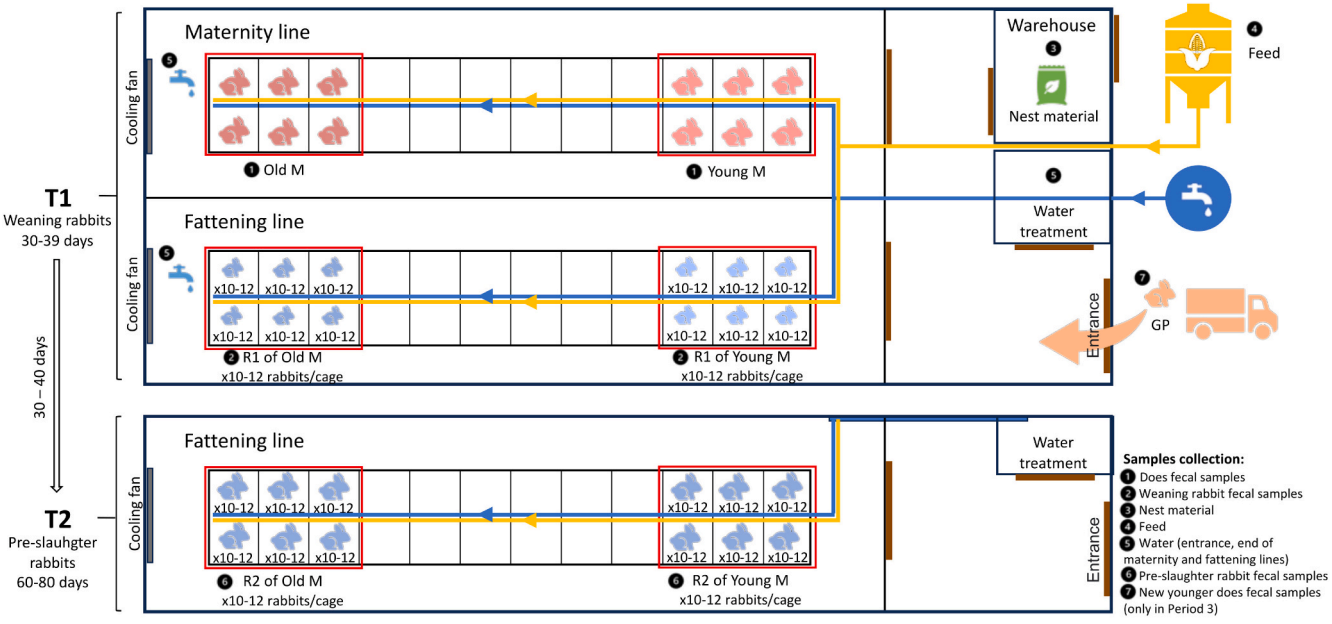

Fig. 2. Sampling strategy at rabbit farm plant. Sample collection points are indicated by numbers. M, does; R1, weaning rabbits; R2, pre-slaughter rabbits; GP, new younger does.

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

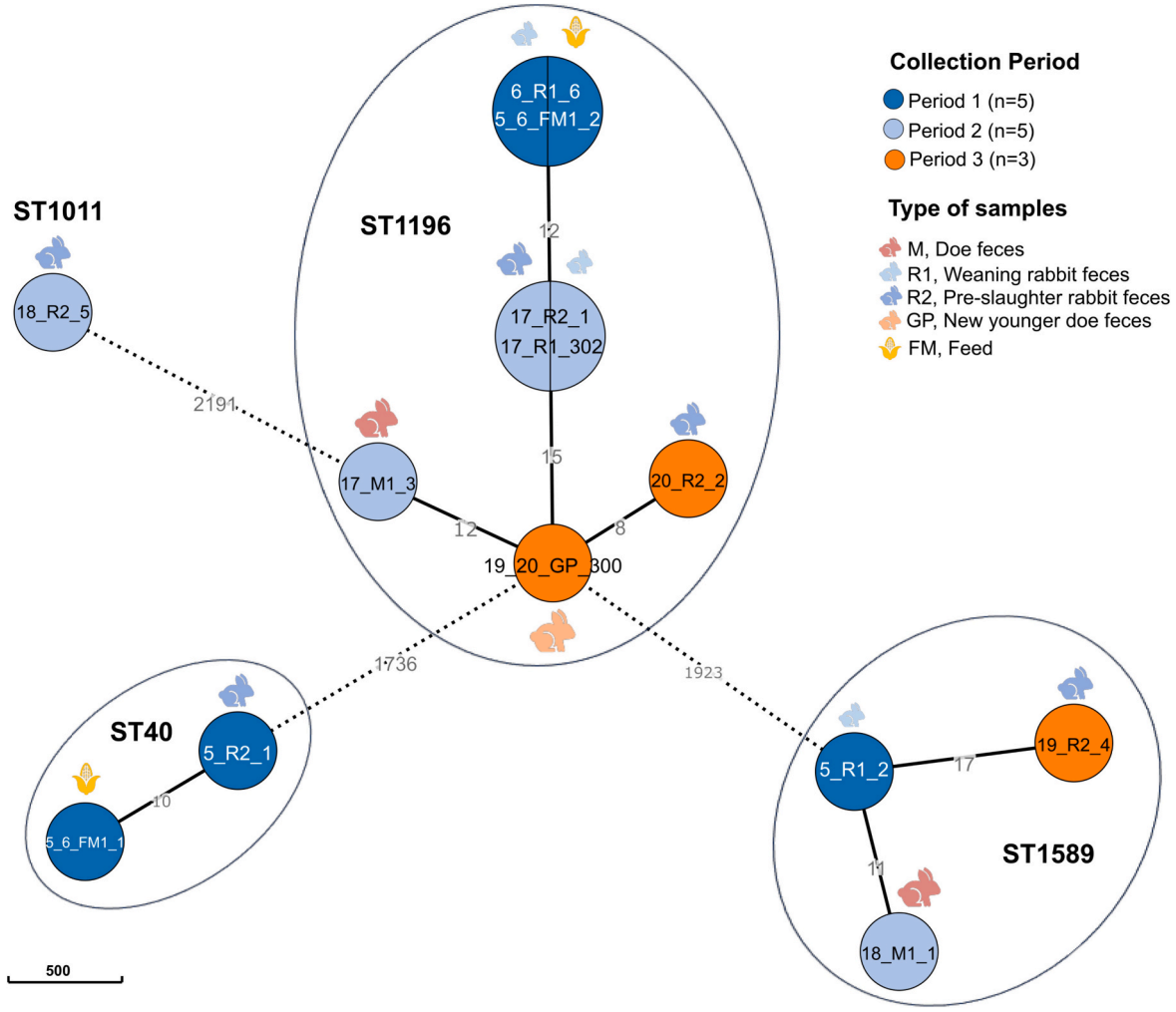

Fig. 3. Grape Tree of the 13 *mcr-1* carrying *E. coli* genomes obtained in farm C, over the three consecutive periods. The three clusters obtained were identified according to the ST (ST1196: HC20-cgST256181, ST40: HC20-cgST256413, ST1589: HC20-cgST256180). The core genome Minimum Spanning Tree (MST) 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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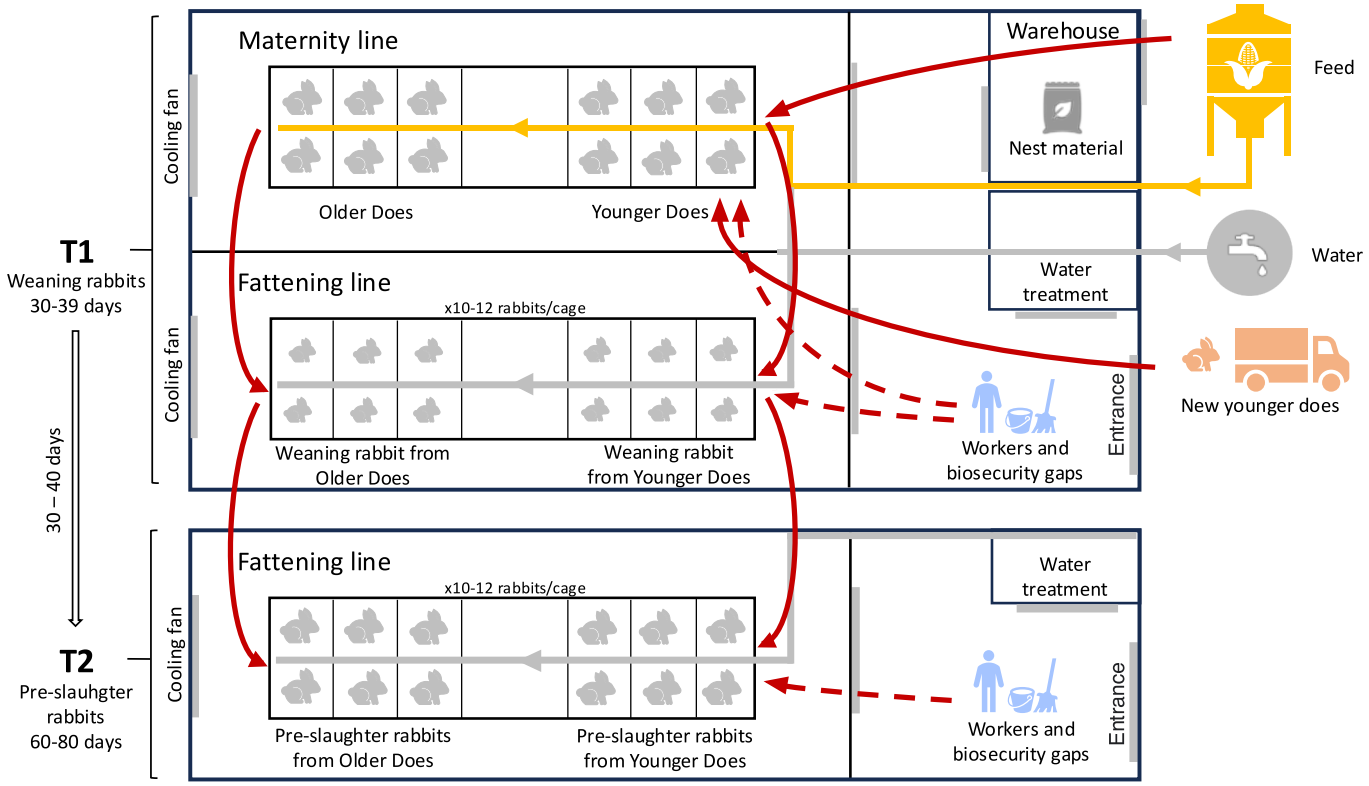

Fig. 5. Potential transmission pathways of *mcr*-positive *E. coli* in rabbit farms across spatial and temporal dimensions. Solid lines represent the transmission routes identified in this study, while dashed lines represent extrapolated transmission pathways.

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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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