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Conference Research Topic: 9th symposium on Antimicrobial Resistance in Animals and the Environment (ARAE 2023)

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Conference Research Topic: 9th symposium on Antimicrobial Resistance in Animals and the Environment (ARAE 2023)

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Editorial: Conference Research Topic: 9th symposium on Antimicrobial Resistance in Animals and the Environment (ARAE 2023)

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Editorial on the Research Topic
Conference Research Topic: 9th symposium on Antimicrobial Resistance in Animals and the Environment (ARAE 2023)

The international symposium on Antimicrobial Resistance in Animals and the Environment (ARAE) is a renowned scientific event gathering many scientists in the field from all over the world every 2 years since 2005. Its 9th edition was held in July 2023 in Tours, France. The aim of the ARAE conference is to present an up-to-date vision of the impact of antibiotic use and resistance in the animal setting, its environment and subsequent impact on human health. All aspects related to epidemiology of antibiotic-resistant strains, genetic spread of antimicrobial resistance genes, emerging resistance mechanisms, resistome in microbiota, and the role of the environment as dissemination route and potential reservoir for resistance genes acquisition are discussed. The 9th edition of ARAE welcomed 170 registered participants from 23 countries (Australia, Belgium, Canada, China, Czech Republic, Denmark, Finland, France, Germany, Ireland, Israel, Italy, Japan, Netherlands, Norway, Philippines, Poland, Portugal, South Korea, Spain, Tunisia, United Kingdom, United States). Early-career scientists represented almost 40% of participants. Five topic sessions gathered 59 oral presentations including keynote lectures and 94 research studies presented as posters. The proceedings of this 9th ARAE symposium can be found at https://hal.inrae.fr/hal-04170355. The present conference Research Topic offered the opportunity to 150 authors worldwide to publish their studies presented during the conference as well as for scientists who were unable to attend the ARAE symposium. It includes one review, one systematic review and 16 original research articles.

First, in an original review, Frederiksen et al. addressed the current knowledge on polyether ionophore resistance and the potential consequences in a One Health perspective. Polyether ionophores are largely used as feed additives in poultry production worldwide, especially monensin, to control avian coccidiosis due to *Eimeria* spp. Beside anti-parasitic activity, polyether ionophores has antibacterial activity but are not used in human medicine due to their toxicity. While Gram-negative bacteria are generally intrinsically resistant to polyether ionophores, Gram-positive bacteria such as

Enterococcus faecium have been reported to harbor plasmid-borne *narAB* resistance genes against narasin with a yet unknown mechanism. Currently, there are sparse evidences of cross-resistance between ionophoric antibiotics and critically-important antibiotics for human medicine. However, the *narAB* resistance genes have been found to co-localize on conjugative plasmids of *E. faecium* with antibiotic resistance genes to macrolides, tetracycline and glycopeptides. This suggests that the use of narasin in broiler production systems could co-select vancomycin-resistant *Enterococci* that can pose a threat to human health.

Salmonella enterica spp. are important zoonotic pathogens related to foodborne diseases worldwide. Multidrug-resistant (MDR) *Salmonella* spp. have been classified by the World Health Organization as high priority pathogens for which new antibiotics are urgently needed. Here, Yan et al. carried out a bibliometric analysis to document trends in past and current researches dealing with horizontal gene transfer implicated in antimicrobial resistance spread in bacterial isolates of *S. enterica*. Since 1999, the number of publications in this field has shown an increasing trend with more than 100 publications per year in the recent years. There has been an evolution of research hotspots from (i) understanding of multidrug resistance in *S. enterica* serovar Typhimurium DT104 and other *S. enterica* serovars, (ii) emergence of plasmid-mediated expanded-spectrum cephalosporin resistance, to (iii) the analysis of whole genome sequences that has significantly enriched our understanding of the population structure, transmission dynamics, and epidemiology of antimicrobial resistance in *S. enterica*. Recent methods such as high-throughput long-read sequencing will provide exciting opportunities to deepen scientific understanding in this research domain.

This conference Research Topic included also three articles focused on recent methodologies for rapid typing of MDR bacterial isolates and to harmonize methodologies in veterinary clinical laboratories. Zendri, Schmidt et al. assessed the potential of the InfraRed spectroscopy using the IR Biotyper for typing of nosocomial outbreaks of MDR pathogens in veterinary hospitals. They compared InfraRed spectroscopy and whole genome analysis of retrospective collections of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated from companion animals (dogs and cats) and horses in two veterinary hospitals. InfraRed spectroscopy revealed a significant discriminatory power for *K. pneumoniae* isolates to identify clonal transmission events within veterinary hospital settings. However, this methodology appears less accurate for typing veterinary *P. aeruginosa* isolates indicating that further optimization is needed before applying in routine veterinary laboratories. In another research article, dealing with bacterial morphology and antibiotic resistance, Ikebe et al. reported morphological differences using light microscopy between laboratory-evolved antibiotic-resistant *E. coli* strains and their susceptible parental ones. They correlated morphological features of resistant strains with phenotypic resistance and gene expression changes in energy metabolism and multidrug efflux systems. They proposed a novel image-based deep learning method for single-cell classification between resistant and susceptible strains. Further improvements of such deep learning algorithm would probably enable the identification of resistant bacterial cells using light microscopy in a near future. Finally, Koritnik et al. reported the results of a European survey about methodologies in veterinary microbiology laboratories (n = 241 labs from 34 European countries). They highlighted a broad diversity of methods for bacterial culture and identification with result reports ranging from 2 to 8 days. In European veterinary microbiological diagnostic laboratories, disc-diffusion method and minimal inhibitory concentration determination were both used for antimicrobial susceptibility testing as well as EUCAST and CLSI clinical breakpoints as interpretative criteria. This large European survey clearly emphasizes that harmonization is needed in bacterial culture and identification and in antimicrobial susceptibility testing for reporting and comparison purposes.

A large set of original research articles (n = 7) concerned molecular epidemiology of antimicrobial-resistant bacteria, mainly *E. coli*, isolated from food-producing animals, food, horses and veterinary hospitals. Antimicrobial resistance (AMR) is an important One Health issue in broiler production worldwide, notably by the carriage of MDR bacteria in the gut of healthy animals that can contaminate meat (Seiffert et al., 2013). Leclercq et al. performed an in depth genomic analysis of MDR *E. coli* isolated in an experimental facility reproducing the entire broiler production pyramid without antibiotic use since more than 10 years. They demonstrated that no transmission of MDR *E. coli* occurred from hens to offspring nor acquisition at the hatchery during three generations, but that the downstream rearing environment may constitute the source of few MDR *E. coli* clonal populations able to colonize young chicks. In addition, they showed a strong association between each major *E. coli* clonal populations and their own MDR IncF plasmid subtypes over chicken generations. Davies et al. reported a study focused on commensal *E. coli* broilers from live bird markets in Bangladesh. They described an extremely high occurrence of genetically-diverse MDR *E. coli* from caecal samples (93%). While ciprofloxacin resistance was very common, other resistances to critically-important antibiotics remained rare or absent (cephalosporins, carbapenems, colistin) with very few plasmid-borne resistance genes [$bla_{\mathrm{CTX-M}}$, *mcr-1*, *fosA*, *tet*(X)]. In another study, Dixit et al. assessed the diversity of resistant bacteria in raw chicken and pork meat samples in Australia. They identified 33 bacterial species belonging to 17 genera. Among a total of 288 isolates, 12% were phenotypically MDR. Chicken meat samples carried more MDR isolates than pork samples. WGS analysis of all isolates revealed a large diversity of AMR genes, few ones being considered of critically importance for human medicine.

Resistance to expanded spectrum cephalosporins in veterinary medicine is a major concern for human health (Seiffert et al., 2013). Leoni et al. investigated the occurrence of Extended-Spectrum β-Lactamase (ESBL)-producing *E. coli* recovered from clams of the Central Adriatic coast in Italy, from 2018 to 2019. They reported a prevalence of 3% genetically-diverse ESBL-producing *E. coli*, the $bla_{\mathrm{CTX-M}}$ resistance genes being the most prevalent. The 13 ESBL/AmpC-producing *Escherichia* spp. recovered from seven out 28 sampling points were of various sequence types and phylogroups suggesting that various microbiological pollution sources can contaminate the surrounding waters of bivalve areas. Zendri, Isgren et al. reported a surveillance pilot study of Gram-negative isolates resistant to expanded spectrum cephalosporins in two veterinary

hospitals. During a 6 months sampling campaign, they highlighted the important occurrence of ESKAPE Gram-negative bacteria in companion animals (equine and small animals) in carriage, clinical and environmental samples. Specific AMR genotypes of *Enterobacter cloacae* complex carrying SHV and or TEM variants and *P. aeruginosa* carrying OXA-50 were prevalent among the equine hospital (horses and environment), while *K. pneumoniae* isolates harboring SHV variants and DHA-1 were only found in the small animal hospital. Although, it remains difficult to conclude on transmission routes, their results strongly suggested that intra-hospital transmission occurred for certain ESC-resistant ESKAPE pathogens. Similarly, in a large epidemiological retrospective study dealing with *K. pneumoniae* in France, Gravey et al. performed a genomic investigation of MDR and hypervirulence of equine isolates between 1996 and 2020. They described a high diversity of *K. pneumoniae* genotypes by MLST and cgMLST including few isolates characterized as MDR and hypervirulent, thus considered as high-risk clones. These STs originate from France but had never been described as MDR-hypervirulent. Plasmid plasticity and horizontal transfer among *K. pneumoniae* populations constitute the risk of emergence of MDR or MDR-hypervirulent high risk clones. Finally, de Lagarde et al. evaluated the impact of a new legislation implemented in 2019 in Québec (Canada) to limit the use of medically-important antibiotics in food-producing animals. They assessed the carriage of ESBL/AmpC-producing *E. coli* in dairy farms before and after the implementation of this new legislation. They described some clonal lineages carrying various AMR genes (bla_{CTX-M}, bla_{SHV}, *qnr*…) that persisted over the 4-year period and are disseminated across different dairy farms.

This conference Research Topic includes also three research articles based on metagenomic approach as tools to analyse microbiota diversity and resistome in various settings. Herman et al. evaluated long-read metagenomic sequencing for the detection of bovine respiratory pathogens (*Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*) from deep nasopharyngeal swab samples of calves. Non-selective bacterial enrichment increased 2–4 orders of magnitude the detection of pathogens as well as their AMR genes to macrolides, sulfonamides and tetracyclines; three antibiotics used for bovine respiratory disease management. Third generation metagenomic sequencing could represent a valuable diagnostic tool for pathogen detection and antimicrobial stewardship. In another study dealing with beef cattle production, Strickland et al. investigated the impact of feed supplementation with tylosin and/or probiotics on the microbial communities within feces, manure and airborne particulate matter. They highlighted different abundances of bacterial populations according to the type of samples and the sampling time, but no change in bacterial community compositions of feces, manure and particulate matter related to treatments, i.e., antibiotics and/or probiotics. Naudin et al. developed an elegant *in vitro* model of biofilm to study the impact of fluoroquinolones on the microbiota composition of sewer biofilms. They demonstrated that biofilm exposure to a low concentration of fluoroquinolones mimicking fluroquinolone concentrations typically found in wastewater had no effect on biofilm diversity nor on the relative abundance of fluoroquinolone mutations in QRDR. While high exposure to fluoroquinolones decreased diversity in *in vitro* biofilms and increased the abundance of ciprofloxacin-resistant bacteria including *E. coli* harboring multiple QRDR mutations. Their results suggested that sewer biofilms may constitute a reservoir of fluoroquinolone-resistant bacteria that can subsequently disseminate in the downstream environment.

Resistance acquisition is based on the horizontal transfer of mobile resistance genes or on mutation acquisitions in chromosomal genes encoding antibiotic targets or regulatory factors (Munita and Arias, 2016). Two research articles investigated the role of the *ramRA* multidrug efflux regulatory locus in acquisition of doxycycline resistance in *K. pneumoniae* and in repression of invasiveness of *S. enterica* serovar Typhimurium. Kenyon et al. described the acquisition of frameshift mutations in the *ramR* gene of *K. pneumoniae* during *in vitro* doxycycline selection and in an *in vivo Galleria mellonella* infection model treated with doxycycline. Frameshift inactivation of *ramR* resulted in 24–48 and over 10-fold increases in doxycycline and ciprofloxacin MICs, respectively. Besides, Giraud et al. investigated the role of *ramRA* regulatory locus in response to bile salts on the *Salmonella* cellular invasiveness. They demonstrated that bile-mediated repression of *S.* Typhimurium invasion is partly regulated by major primary bile salts known to activate *ramA* transcription by interacting with RamR, but also that other unknown pathways likely play a role in bile-mediated repression of invasion independently of the *ramRA* locus.

Finally, Imazaki et al. investigated the minimal selective concentration of oxytetracycline using two isogenic resistant/susceptible strains of *E. coli* in Mueller-Hinton medium and in sterilized intestinal contents of White large pigs. This original comparison showed that the sub-MIC selective window (range between minimal selective concentration and MIC) was lower in Muller-Hinton broth compared to sterilized intestinal contents suggesting that intestinal content composition reduce free oxytetracycline proportion. These findings may have implications in understanding of emergence of AMR in gut as well as for antibiotic administration dosage.

To conclude, this conference Research Topic partly illustrated the diversity of research area related to antimicrobial resistance in the field of animals and the environment. The next edition of ARAE (https://arae2025.de) will be held in Berlin, Germany in July 2025, and chaired by Prof. Stefan Schwarz (Institute of Microbiology & Epizootics of the Free University of Berlin).

Author contributions

BD: Funding acquisition, Project administration, Writing – original draft, Writing – review & editing. SL: Writing – review & editing. MZ: Writing – review & editing. AC: Writing – review & editing.

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Conflict of interest

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

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Doxycycline PEP can induce doxycycline resistance in *Klebsiella pneumoniae* in a *Galleria mellonella* model of PEP

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Background: Four randomized controlled trials have now established that doxycycline post exposure (sex) prophylaxis (PEP) can reduce the incidence of chlamydia and syphilis in men who have sex with men. These studies have concluded that the risk of selecting for antimicrobial resistance is low. We evaluated this risk *in vitro* and *in vivo* using a *Galleria mellonella* infection model.

Methods: We evaluated how long it took for doxycycline resistance to emerge during passage on doxycycline containing agar plates in 4 species – *Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae* and *Neisseria subflava*. We then assessed if *K. pneumoniae* could acquire resistance to doxycycline (and cross resistance to other antimicrobials) during intermittent exposure to doxycycline in a *Galleria mellonella* model of doxycycline PEP.

Results: In our passage experiments, we found that resistance first emerged in *K. pneumoniae*. By day 7 the *K. pneumoniae* MIC had increased from 2 mg/L to a median of 96 mg/L (IQR 64–96). Under various simulations of doxycycline PEP in the G. mellonella model, the doxycycline MIC of *K. pneumoniae* increased from 2 mg/L to 48 mg/L (IQR 48–84). Ceftriaxone and ciprofloxacin MICs increased over ten-fold. Whole genome sequencing revealed acquired mutations in ramR which regulates the expression of the AcrAB-TolC efflux pump.

Conclusion: Doxycycline PEP can select for doxycycline, ceftriaxone and ciprofloxacin resistance in *K. pneumoniae* in a G. mellonella model. The emergent ramR mutations were similar to those seen in circulating strains of *K. pneumoniae*. These findings suggest that we need to assess the effect of doxycycline PEP on resistance induction on a broader range of bacterial species than has hitherto been the case.

KEYWORDS

Neisseria gonorrhoeae*, *Klebsiella pneumoniae*, doxycycline PEP, WGS, *in-vivo* emergence, DoxyPEP, ST220 *Klebsiella pneumoniae

Introduction

Four randomized controlled trials have now established that doxycycline can reduce the incidence of chlamydia and syphilis in men who have sex with men (MSM) (Bolan et al., 2015; Molina et al., 2018; Luetkemeyer et al., 2022; Molina et al., 2023). The Doxycycline Post Exposure Prophylaxis (DoxyPEP) study, for example, found that men randomized to consumption of 200 mg of doxycycline within 24 h of every condomless sex act had an approximately 60% lower incidence of chlamydia, syphilis and gonorrhea (Luetkemeyer et al., 2022). Of concern, however, was that the individuals in the doxycycline arms of these studies consumed between 4 and 16 doses of 200 mg doxycycline per month (Molina et al., 2018; Luetkemeyer et al., 2022). This consumption is 170- to 680-fold higher than the mean population consumption of tetracyclines in European countries in 2021.[1] It is unknown if the consumption of this quantity of doxycycline in an intermittent fashion could result in antimicrobial resistance (AMR) to tetracyclines and other antimicrobials (Kong et al., 2023; Vanbaelen et al., 2023). A recent systematic review of the effects of oral tetracycline on AMR reported increases in tetracycline-resistant *E. coli* in the gastrointestinal tract, Streptococcus strains in the mouth and respiratory tract pathogens (Truong et al., 2022).

Resistance to tetracyclines can emerge via a number of mechanisms (Grossman, 2016; Kong et al., 2023). Decreased entry via mutations in porin proteins or increased activity of efflux pumps are two important mechanisms (Grossman, 2016). The efflux pumps expel a number of antibiotics, including tetracyclines, out of the cell, making them resistant to these drugs (Grossman, 2016). The expression of these pumps is typically regulated by a number of activating and repressing factors, some of which have been found to be inducible by tetracyclines (Grossman, 2016). An important class of these efflux pumps are the RND-type efflux pumps, such as AcrAB-TolC in *E. coli* and Klebsiella, which confer multi-drug resistance to several different antimicrobial classes, including tetracyclines, penicillins, macrolides, fluoroquinolones, phenicols, and rifampicin (Bialek-Davenet et al., 2011; Grossman, 2016).

The ribosomal protection mechanism is another important resistance mechanism coded by specific tet genes such as tet(M) and tet(O) (Grossman, 2016). These homologs of EF-Tu/EF-G GTPase proteins bind to the h34 site on the ribosome, displacing the tetracyclines bound to it (Grossman, 2016).

Mutations at specific sites of the 30S ribosomal subunit and 16S rRNA are additional resistance mechanisms (Grossman, 2016). Finally, enzyme inactivation of tetracyclines can occur in anaerobes like *Bacteroides fragilis* which comprise part of the human intestinal flora. The gene products responsible for enzymatic inactivation include tet(X), tet(34), tet(37) (Grossman, 2016).

Two of the doxycycline PEP studies evaluated the effect of doxycycline on tetracycline resistance in *Neisseria gonorrhoeae*. Both the studies found no statistically significant effect, but the numbers of gonococcal isolates were extremely low [n = 9 (Molina et al., 2018) and n = 47 (Luetkemeyer et al., 2022)]. Neither study has, as yet, published results of the effect of doxycycline PEP on AMR in any other species.

This provided the motivation for the two objectives of this study. Our first objective was to establish the order in which doxycycline resistance emerged in four target species during passage under doxycycline selection pressure – *Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae* and *Neisseria subflava*. We found that resistance first emerged in *K. pneumoniae*, a common colonizer of the gastrointestinal tract that is also a key amplifier and spreader of clinically important AMR genes (Wyres and Holt, 2018). In our second objective, we used a *Galleria mellonella* model of chronic *Klebsiella pneumoniae* infection to interrogate the effect of intermittent exposure to doxycycline on the emergence of doxycycline resistance *in vivo*.

Materials and methods

Bacterial strains and growth conditions

Four bacterial species (*Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae* and *Neisseria subflava*) with doxycycline minimal inhibitory contentrations (MICs) less than 4 μg/mL were selected from our collection of clinical isolates at the Institute of Tropical Medicine, Antwerp. For *N. gonorrhoeae*, three strains were selected - two from the WHO reference panel (WHO-F and -P) and a circulating strain (Unemo et al., 2016). Detailed information on the six bacterial strains used in this study are provided in Table 1.

In vitro induction of doxycycline resistance

The direct colony suspension method was used for inoculum preparation wherein colonies were selected from an 18–24 h (h) BBLTM blood agar (BA) plate. The turbidity of the bacterial suspensions were adjusted to 0.5–1.0 McFarland (McF) standard in phosphate buffer saline (PBS), and replated onto BDTM Chocolate (Choc) agar plates for *K. pneumoniae*/*E. coli* and on BD BBLTM Chocolate II agar (GC II agar with hemoglobin and IsoVitalexTM) for *N. gonorrhoeae*/*N. subflava*.

TABLE 1 Bacterial strains used in this study.

Organism	Isolate Number	Doxycycline MIC	Clinical origin/ Reference
K. pneumoniae	M17125	2	Human clinical isolate from ITM collection
E. coli	ATCC 25922	2	Human clinical isolate from ATCC collection
N. subflava	790/2	1.5	Clinical pharyngeal isolate from an asymptomatic man Laumen et al. (2022)
N. gonorrhoeae	WHO-P	1.5	WHO reference strain [11]
N. gonorrhoeae	WHO-F	0.25	WHO reference strain [11]
N. gonorrhoeae	M22597	3	Clinical urethritis isolate

1 https://www.ecdc.europa.eu/en/antimicrobial-consumption/surveillance-and-disease-data/database

A doxycycline gradient Etest ranging between 0.016 μg/mL and 256 μg/mL (BioMérieux, France) was placed on all the plates. After overnight incubation at 36°C at 5 (v/v)% CO2, the MIC was noted. A standard loopful of culture (5 mm) was taken from the margin of growth from the most resistant colonies, following the protocol of Wadsworth et al. (Balduck et al., 2022; Raisman et al., 2022). This growth was then suspended in PBS and re-inoculated on a fresh BD BBLTM Chocolate II agar plate (GC Choc) and a new doxycycline *E*-test was placed. The above process was repeated every 24 h for each isolate for a total of 7 consecutive days. Control experiments with each isolate were conducted by passaging the isolate according to the above protocol except that no Etest strip was placed. The experiments were conducted in triplicate.

Galleria mellonella infection model of *Klebsiella pneumoniae*

Preparation of live microbial inoculum for infection

The *K. pneumoniae* M17125 isolate was cultured from frozen stocks onto (BA) plate for ≤16 h at 37°C with 5% (v/v) CO2. Single colonies were plated onto fresh Choc agar plates, which were incubated at 37°C with 5% (v/v) CO2 for 6 h. The cultures from the agar plates were suspended in PBS and inoculated into the haemocoel of the *G. mellonella* larvae at a concentration of 104 CFU/larva. This dose of *K. pneumoniae* was determined based on previous experiments that established a dose that enabled the recovery of the bacteria up to 5 days post inoculation with a low mortality rate of the larvae (data not shown).

Galleria mellonella-equivalent dose of doxycycline 200 mg and 100 mg

The doses of doxycycline (Sigma-Aldrich) used were the equivalent of 200 mg (3.333 mg/kg) and 100 mg (1.666 mg/kg) per day used for humans (Wei et al., 2017; Andrea et al., 2019; Khalil et al., 2019). We used larvae with a mean weight of 370 mg (range 300 to 450 mg). This weight was used to calculate the 200 mg-equivalent dose of doxycycline injected into each larva (1.23 ng in 10 μL PBS).

Injection of *Galleria mellonella* larvae

Last larval stage *G. mellonella* (Terramania, Arnhem, NL) were used for the experiments. The larvae were not fed during the experiment. Only macroscopically healthy, non-discolored larvae were selected. The larvae were placed into individual sterile Petri dishes in groups of 10 per Petri dish. The larvae were kept in an incubator at 37°C with a 5% (v/v) CO2 atmosphere for the length of the experiments. Each control and experimental group consisted of at least 30 larvae.

The larvae were injected in the last pro-legs with 10 μL of various doses of doxycycline/bacteria using 0.3 mL U-100 insulin syringes (BD Micro-Fine). One syringe and needle was used for 10 larvae in each Petri dish.

Three test groups were evaluated

Group 1 (DoxyPEP): 104 CFU *K. pneumoniae* inoculum followed 10 min later and every 48 h with human PEP equivalent dose of doxycycline – 1.23 ng in 10 μL PBS.

Group 2 (0.5xDoxyPEP): 104 CFU *K. pneumoniae* followed 10 min later and every 48 h with 50% of a human PEP equivalent dose of doxycycline. 0.615 ng in 10 μL PBS.

Group 3 (Control): 104 CFU *K. pneumoniae* inoculum followed 10 min later by 10 μL PBS.

These experimental groups were designed to evaluate two scenarios. Firstly, could the equivalent of 200 mg doses of doxycycline every 48 h induce doxycycline resistance? Secondly, could 50% of this dose induce resistance? – for example, in individuals who acquired *K. pneumoniae* a few hours after taking the 200 mg doxycycline. In pilot experiments we established that the 200 mg equivalent dose of doxycycline was not toxic to the G. mellonella (data not shown).

Individual- versus network-level induction of AMR

These experimental groups only assess the acquisition of AMR within individuals taking doxycycline PEP. There are a number of population-level mechanisms whereby intense antimicrobial consumption can translate into AMR (Lipsitch and Samore, 2002; Kenyon and Schwartz, 2018). For example, intermittent doxycycline consumption may induce partial resistance in *K. pneumoniae* in one individual. The partial resistance *K. pneumoniae* may then be transmitted (via sex or physical contact) to another individual who is also taking intermittent doxycycline PEP where high level resistance is then induced. To mimic/assess this pathway, 10^4 CFU of *K. pneumoniae* from randomly selected single colonies obtained from each of the above groups on days 2 and 3 were injected into 5 new larvae ensuring that each larva received a single clone of *K. pneumoniae*. These larvae were then all treated with a human PEP equivalent dose of doxycycline (200 mg) 15 min after the receipt of the *K. pneumoniae*. Isolates from these experiments were termed the day 2 and 3 network-level isolates.

Retrieval of *Klebsiella pneumoniae* from *Galleria mellonella*

At 24 h after the injection of the bacteria and 24-hourly intervals thereafter, four larvae from each group of 30 larvae were randomly selected for extraction of hemolymph. This was continued for the duration of the experiments – 4 days. The larvae were immobilized by placing them at −80°C for 60 s. They were then placed on a Petri dish, and an incision was made between the two segments closest to the tail of the larva (Dijokaite et al., 2021). Haemolymph was then extracted by squeezing the haemolymph into 1.5 mL centrifuge tubes containing 50 μL PBS, vortexed and divided onto two plates: Klebsiella ChromoSelect Selective Agar (KCA; Merck [Darmstadt, Germany]) with 4 μg/mL doxycycline and KCA without doxycycline. The plates were then incubated at 37°C with a 5% (v/v) CO_2 atmosphere for 24 h and the number of purple-magenta *K. pneumoniae* colonies were counted. At random, four purple-magenta colonies from the plates with doxycycline per experimental condition were selected for further identification via MALDI-TOF. The method

used for MALDI-TOF-MS-based species identity is detailed elsewhere (Laumen et al., 2022). The doxycycline MIC was determined using Etest. If no purple-magenta colonies emerged on the doxycycline plates per condition, then a random selection of 4 purple-magenta colonies from the plates without doxycycline was subjected to MALDI-TOF and the MIC was determined.

Cross-resistance testing for other antimicrobials was carried out using Etest (BioMérieux, France) for all the colonies ($n = 4$) of *K. pneumoniae* that were obtained from the final day of each experimental condition as well as the parental strains for ceftriaxone, ciprofloxacin and azithromycin antimicrobials. The Etests were performed on BDTM Mueller-Hinton agar plates incubated for 16–18 h at 37°C with a 5% (v/v) CO2 atmosphere. All tests were carried out in compliance with the manufacturer's instructions.

At the end of each experiment both the surviving and dead *G. mellonella*, were kept at −80°C overnight to sedate them. They were then autoclaved at 121°C for 15 min and discarded.

Whole genome sequencing and bioinformatic analyses

Six strains of *K. pneumoniae* were selected for whole genome sequencing (WGS). These were the parental strain as well as a random selection of 5 strains from the final day of the network selection experiment (Supplementary Table 1). The bacterial isolates were outsourced to Eurofins, where total DNA was extracted followed by library preparation with Stranded TruSeq DNA library preparation kit from Illumina. Sequencing of paired-end reads 2 × 150 bp were performed on NextSeq6000, v2 Illumina platform (Illumina Inc., San Diego, CA, United States) The sequencing data from this study is available under BioProject ID PRJNA949453.

Initial quality control (QC) of the raw reads was carried out using FastQC (Andrews, 2015). To assemble the genome, sequences were first trimmed using trimmomatic (v0.39) and then *de novo* assembled using SPAdes v3.14.0 (Bankevich et al., 2012; Bolger et al., 2014). Once assembly was complete, Quast (v5.0.2) was used to assess the quality of the genome assembly. Assembled scaffolds were annotated using Prokka v1.14.6 (Gurevich et al., 2013; Seemann, 2014).

Accurately identifying genetic organization of genes associated with resistance and single nucleotide polymorphisms (SNPs) is key to understanding the emergence of resistance. Using CLC genomics workbench (v20, CLC bio, Denmark), reference mapping was done, and SNPs were extracted.

Data analysis

Statistical analyses were conducted using GraphPad Prism® with the Mann–Whitney test used to compare groups. A p-value <0.05 was considered statistically significant.

Results

In vitro induction of doxycycline resistance

Doxycycline selection led to significant increased doxycycline MICs in *E. coli* and *K. pneumoniae* from day 3 onwards (Figure 1). By day 7 the *K. pneumoniae* MIC had increased from 2 mg/L to a median of 96 mg/L (IQR 64–96; $p < 0.01$), whereas the *E. coli* increased to a somewhat lower median MIC of 24 mg/L (IQR 24–48; $p < 0.01$ at days 3–5). No increases in MIC were evident in the Neisseria spp. isolates (Figure 1).

In vivo induction of doxycycline resistance in *Galleria mellonella*

Individual-level selection

In this experiment, a dose of doxycycline was administered to the larvae at baseline and every 48 h thereafter. No *K. pneumoniae* colonies were observed on the doxycycline plates from the larvae at 24 h (Day 1; Figure 2). The first *K. pneumoniae* colonies to emerge on the plates with doxycycline were from the larvae at day 2/48 h (i.e., before they had received their second dose of doxycycline). In the larvae that were exposed to the equivalent of 200 mg doxycycline (DoxyPEP)/0.5 x DoxyPEP, the doxycycline MIC increased from 2 mg/L to a median of 8 mg/L (IQR 7.5–9 mg/L; $p = 0.013$)/4 mg/L (IQR 3.75–4 mg/L; $p = 0.011$), respectively (Figure 2).

Following the receipt of the second dose of doxycycline at day 2, the MICs increased further in the samples obtained the following day, i.e., day 3, to a median of 24 mg/L (IQR 20–28 mg/L; $p = 0.029$) and 8 mg/L (IQR 7.5–9 mg/L; $p = 0.013$) for the DoxyPEP and 0.5 x DoxyPEP groups, respectively. After this timepoint, *K. pneumoniae* was only cultured from the control group at day 4 when its MIC remained unchanged from baseline (Figure 2). No *K. pneumoniae* from the control group were isolated on the doxycycline plates.

Network-level selection

To assess network-level selection, *K. pneumoniae* isolates obtained from the larvae in the individual-level experiment on days 2 and 3 were injected into new larvae, followed by a 200 mg equivalent dose of doxycycline. No significant increase in doxycycline MIC was evident on day 2 of the experiments. But the day 3 experiments demonstrated an increase in median MIC to 40 mg/L (IQR 30–52; $p = 0.013$) and 64 mg/L (IQR 52–72; $p = 0.014$) for the DoxyPEP and 0.5 x DoxyPEP groups, respectively (Figure 3).

Cross resistance to ceftriaxone, ciprofloxacin, and azithromycin

The four colonies of *K. pneumoniae* that were obtained from the final day of each experimental condition had elevated ceftriaxone, ciprofloxacin and azithromycin MICs compared to the parental strains. There was no difference in the final MICs between the individual and network conditions. Ceftriaxone MICs increased from a median of 0.064 mg/L (IQR 0.064–0.064 mg/L) to a median of 0.38 mg/L (IQR 0.205–0.470 mg/L; $p < 0.0001$). Likewise, ciprofloxacin MICs increased from a median of 0.064 mg/L (IQR 0.064–0.094 mg/L) to a median of 0.38 mg/L (IQR 0.25–0.470 mg/L; $p < 0.0001$). The median azithromycin MIC increased from 96 mg/L (IQR 96–96 mg/L) to 192 mg/L (IQR 144–256 mg/L; $p < 0.0001$).

Increased doxycycline MICs associated with mutations in RamA and Rfr-2

Of the 5 strains with elevated doxycycline MICs that were sequenced, three acquired nonsynonymous mutations in RamR (Table 2). Two strains acquired the Ile26 frameshift mutation, whereas the third strain

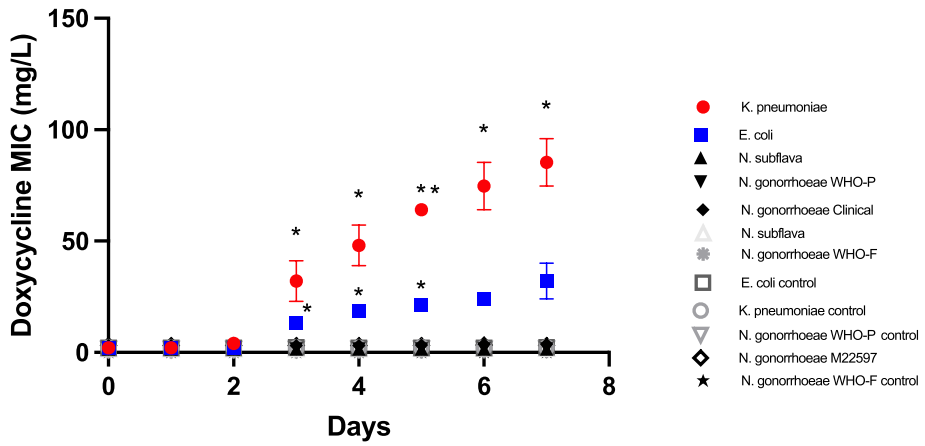

FIGURE 1
Increase in doxycycline MICs in *Klebsiella pneumoniae*, *E coli*, *N. subflava* and two strains of *Neisseria gonorrhoeae* during passage on chocolate agar plates containing a gradient of doxycycline (0.016 μg/mL to 256 μg/mL). Symbols represent the mean MIC at each timepoint, and the error bars show the standard deviation of the mean. Unpaired *t*-test was done to compare the MICs between controls and doxycycline exposed strain at each timepoint. *$p < 0.01$; **$p < 0.001$.

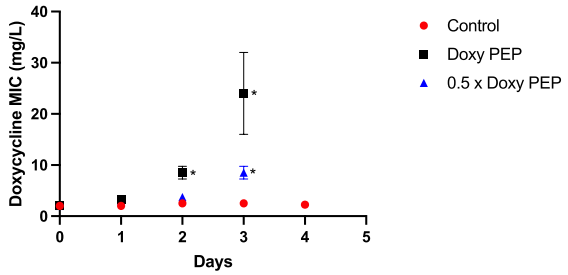

FIGURE 2
Individual-level selection. Increase in doxycycline MICs in *K. pneumoniae* during individual-level selection following PEP equivalent doses of doxycycline (200 mg/day, Doxy PEP) or 50% of this dose (0.5 x Doxy PEP) in a *Galleria mellonella* model of *K. pneumoniae* infection. Symbols represent the mean MIC at each timepoint, and the error bars show the standard deviation of the mean. Unpaired t-tests were done to compare the MICs between controls and doxycycline exposed strains at each timepoint. *$p < 0.01$.

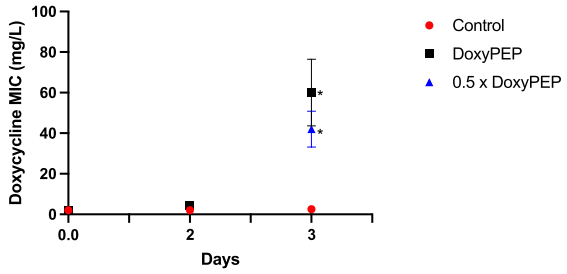

FIGURE 3
Network-level selection. Increase in doxycycline MICs in *K. pneumoniae* during network-level selection following doxycycline PEP equivalent doses of doxycycline in a *Galleria mellonella* model of *K. pneumoniae* infection. Symbols represent the mean MIC at each timepoint, and the error bars show the standard deviation of the mean. Unpaired *t*- tests were done to compare the MICs between controls and doxycycline exposed strains at each timepoint. *$p < 0.05$.

acquired a Tyr47 frameshift mutation. The two other strains acquired Arg5Cys and Ile74Ser mutations in RrF-2 (Table 2; Figure 4).

Discussion

In a novel *in vitro* model of doxycycline PEP, we found that doxycycline use resulted in rapid increase in doxycycline MICs in *E. coli* and *K. pneumoniae* but not in *N. subflava* and *N. gonorrhoeae*. Individual-level selection within G. mellonella resulted in increased doxycycline MICs but to a slightly lesser extent than network-level selection. Of particular concern, these increases in doxycycline MICs were associated with increases in MICs for the other three antimicrobials assessed (ceftriaxone, ciprofloxacin and azithromycin).

These findings could be parsimoniously explained by mutations in ramR and rfr-2 (Figure 4). A number of studies have found that a number of mutations (insertions, deletions and point mutations) in ramR are responsible for tetracycline resistance in both clinical isolates and induced-resistant isolates of *K. pneumoniae*. RamR exerts this effect via increasing the expression of the AcrAB efflux pump (Hentschke et al., 2010; Bialek-Davenet et al., 2011, 2013; Villa et al., 2014; Wang et al., 2015). Rrf-2-transcriptional regulator is a transcriptional regulator immediately upstream of a component of the AcrAB pump – oqxB20-RND-efflux-pump (Yen and Papin, 2017). Importantly, we did not attempt to establish experimentally if these mutations we detected are causally related to the changes in MICs we found.

There are a number of other important limitations to this analysis. In the *in vitro* experiments, we only evaluated four bacterial species, while in the *in vivo* experiments, we examined one species. In humans, the rate of intestinal *K. pneumoniae* colonization has been found to be 5 to 25% (Martin et al., 2016; Gorrie et al., 2017) which may mean it would be placed under less selection pressure than a species such as *E. coli* where the carriage is close to universal (Tenaillon et al., 2010; Martinson and Walk, 2020). Our use of doxycycline PEP every 48 h may also only apply

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TABLE 2 Mutations detected in *Klebsiella pneumoniae* isolates with elevated doxycycline MICs in network experiments.

Strain ID	Doxycycline MIC	Ciprofloxacin MIC	Ceftriaxone MIC	Azithromycin MIC	Gene	Mutations detected	Amino acid change
KPZ_WT	2	0.064	0.064	96	–	–	–
KPZ13_2	64	0.38	0.5	>256	ramR	77_78delTA	Ile26fs
KPZ14_3	64	0.5	0.38	192	rrf-2	C13T	Arg5Cys
KPZ15_2	64	0.38	0.5	>256	ramR	77_78delTA	Ile26fs
KPZ17_2	32	0.5	0.125	96	rrf-2	T221G	Ile74Ser
KPZ18_2	96	0.25	0.38	>256	ramR	132_139delCGCTGTTT	Tyr47fs

fs – frameshift mutation; MIC (mg/L).

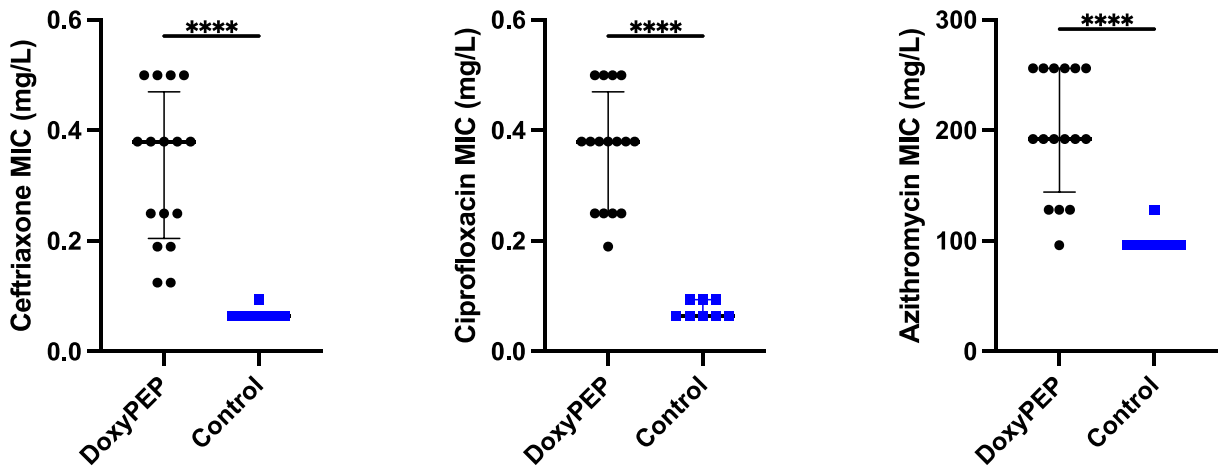

FIGURE 4
Selection of resistance to ceftriaxone, ciprofloxacin, and azithromycin in *Klebsiella pneumoniae* in *Galleria mellonella* exposed to doxycycline PEP (combined individual– and network-level experiments).

to a small proportion of PEP users (Molina et al., 2018, 2023). On the other hand, the experiments were conducted for 4 to 7 days, whereas doxycycline PEP will likely be used in individuals and populations for years to decades. The G. mellonella infection model involves colonization of the hemolymph and not the gastrointestinal tract, which is the typical colonization site for humans. As a consequence, our experimental model likely offers less opportunities for the uptake of resistance-genes from other bacteria via horizontal gene transfer. G. mellonella infection models based on hemolymph infection, including those for *K. pneumoniae* have been shown to provide virulence and therapeutic efficacy results that closely replicate those found in mammals (Wand et al., 2013, 2015; Maguire et al., 2016; Bruchmann et al., 2021). Nonetheless, the large differences between G. mellonella and *Homo sapiens* mean we cannot infer that because doxycycline resistance emerged in the former that it would emerge in the later. We can only conclude that resistance emerged in our model of doxycycline PEP and that this suggests the need for further studies in humans.

We were also unable to evaluate certain indirect pathways whereby doxycycline PEP could select for resistance to tetracyclines and other antimicrobials. Recently, a number of studies have expressed concern that cross-resistance to multiple antimicrobials in various bacterial species may mean that the widespread use of doxycycline PEP will indirectly select for resistance to other antimicrobials (Vanbaelen et al., 2023). Selection of AMR in *N. gonorrhoeae* has frequently been via the selection of clones with resistance to multiple antimicrobials (Sánchez-Busó et al., 2022; Vanbaelen et al., 2023). Gonococcal resistance to tetracyclines is typically caused by the acquisition of the tet (M) gene and/or mutations in rpsJ or porB (Unemo et al., 2016). The intensive use of doxycycline as PEP could provide a selective pressure for the emergence and spread of any or all these mechanisms. This effect would likely be most marked in sexual networks with high rates of partner change and hence a high equilibrium prevalence of *N. gonorrhoeae* (Kenyon and Schwartz, 2018) and intense usage of doxycycline PEP. Intensive consumption of doxycycline in these settings could directly select for these tetracycline resistance associated mechanisms (Vanbaelen et al., 2023). The fact that multidrug resistant clones of *N. gonorrhoeae* are typically resistant to tetracyclines means that doxycycline PEP may inadvertently select for resistance to other antimicrobials (Vanbaelen et al., 2023; Whiley et al., 2023). A similar clustering of resistance to tetracyclines and other antimicrobials has also been shown to pertain to a range of other pathogens such as *K. pneumoniae* and *Staphylococcus aureus* (Gestels et al., 2023). Likewise, a study from France has recently found that 87% of extensively resistant *Shigella sonnei* isolates were doxycycline resistant and likely disproportionately from MSM (Lefèvre et al., 2023). The authors raised the concern that doxycycline PEP may add a further selection advantage to these highly resistant isolates. In a similar vein, we did not evaluate population level selection of AMR. A previous study of minocycline PEP following sexual exposure to *N. gonorrhoeae* found that PEP completely prevented infection with highly susceptible isolates but had no effect on preventing infection with resistant isolates. The authors

concluded that at a population level, the widespread use of minocycline PEP would likely select for AMR and was thus not advisable (Harrison et al., 1979). We did not evaluate this pathway.

Notwithstanding these limitations, our *in vivo* model demonstrated that doxycycline can select for resistance to doxycycline and other classes of antimicrobials. As such, the widespread use of doxycycline PEP could contribute to further emergence and spread of multi-drug resistant (MDR) cases in Gram-negative bacteria such as *Klebsiella pneumoniae*. Our findings thus suggest that clinical studies of doxycycline PEP should evaluate the effect on AMR in a wider array of target bacterial species than those considered up to the present. In particular, the effect on Enterobacteriaceae, such as *E. coli* and *K. pneumoniae* should be included. Finally, the effects should include the induction of cross resistance to other antimicrobials.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, PRJNA949453.

Author contributions

CK, TV, and SM-B conceptualized the study. CK and ZG conducted the experiments. BX was responsible for the bioinformatic analyses. CK, BX, and SM-B were responsible for the statistical analyses. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1208014/full#supplementary-material

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Resistome-based surveillance identifies ESKAPE pathogens as the predominant gram-negative organisms circulating in veterinary hospitals

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Introduction: Healthcare-associated infections (HCAIs) associated with extended-spectrum cephalosporin-resistant gram-negative (ESC-R GN) bacteria are an emerging concern in veterinary hospitals, especially in companion animal intensive care units (ICUs).

Methods: To understand the molecular epidemiology of ESC-R GN isolates in two veterinary hospitals (equine and small animal), a 6-month pilot study was performed during which fecal and environmental samples were obtained twice from selected patients, upon ICU admission and after 48 h of hospitalization. In total, 295 ESC-R GNs were analyzed using the Acuitas Resistome® Test (OpGen, Maryland, US), a PCR-based assay screening for 50 antimicrobial resistance gene families encoding for production of extended-spectrum beta-lactamase (ESBLs), TEM/SHV/OXA or AmpC beta-lactamases and carbapenemases. Combining organism identification and antimicrobial susceptibility data to genotyping results, unique "Acuitas profiles" were generated that can be used for fast typing the isolates and tracking transmission events.

Results: ESKAPE GN pathogens were the most prevalent ESC-R GN isolates circulating in both the small animal and equine hospitals, consisting of *Enterobacter cloacae* complex (21.7%), *Pseudomonas aeruginosa* (20%), *Klebsiella pneumoniae* (15.9%), and *Acinetobacter baumannii* complex (13.6%) followed by *Escherichia coli* (12.2%), most harboring a combination of genes encoding for beta-lactamases and ESBLs. Some ESKAPE genotypes showed likely intra-hospital transmission, including *E. cloacae* (two genotypes, one carrying SHV4, SHV5, and TEM7 and the other TEM1, TEM3, and TEM7 enzymes) in the equine and *K. pneumoniae* (SHV1, SHV5, and DHA1-positive) in the small animal ICUs, respectively. Furthermore, *P. aeruginosa* (carrying OXA-50), *A. baumannii* complex (OXA-51), and *E. coli* (CTX-M-1) genotypes were isolated across both hospitals, suggesting possible transfer mediated via movement of staff and students. Importantly, isolates carrying transmissible resistance to last-resort antimicrobials (i.e. carbapenems) were identified within the hospital environments, consisting of three environmental *Acinetobacter* spp. harboring bla_{OXA-23} and one clinical *E. coli* with bla_{OXA-48}.

Conclusion: We describe the widespread occurrence of ESKAPE gram-negative organisms in veterinary ICU patients and hospital environments. Findings from this project provide baseline data on the epidemiology of ESKAPE pathogens in veterinary settings, which can inform infection control policies to aid in patient management and prevent transmission of nosocomial infections associated with these pathogens.

KEYWORDS

veterinary, infection control, gram-negative, ESKAPE, companion animals, surveillance, veterinary hospitals, intensive care unit (ICU)

1. Introduction

Nosocomial infections, also known as healthcare-associated infections (HCAIs), are either localized or systemic infections that are typically not present at the time of admission but are acquired by patients during their stay in a hospital or other healthcare facility and usually manifest approximately 48 h after admission to the hospital (Monegro et al., 2021). Approximately 4,100,00 new cases of HAI are estimated to occur every year in people in the European Union and European Economic Area (EU/EEA) with the number of deaths occurring as a direct consequence of these infections estimated to be at least 37,000.[1] Of these, the number of HCAIs caused by antimicrobial-resistant (AMR) microorganisms was calculated to be 426,277 occurring in the EU every year (Cassini et al., 2019). In particular, multidrug-resistant (MDR) HCAIs are a major challenge for both human and veterinary medicine as they are associated with increased morbidity and mortality rates as well as increased healthcare costs. Importantly, gram-negative (GN) bacteria within the ESKAPE group of pathogens (***E**nterococcus faecium, **S**taphylococcus aureus, **K**lebsiella pneumoniae, **A**cinetobacter baumannii, **P**seudomonas aeruginosa,* and ***E**nterobacter* spp.) pose a real threat due to their tendency to become MDR and thereby "escape" most antimicrobial agents.

In contrast to human medicine, data on the occurrence of HCAIs in veterinary medicine remain limited although the problem has recently gained increasing awareness (Stull and Weese, 2015). In addition, infection control still remains in its infant stages despite animal HCAIs increasing importance in modern veterinary practice (Walther et al., 2017). This appears to be particularly the case for companion animals, i.e., dogs, cats, and horses, where a growing body of literature has described nosocomial outbreaks of different etiologies which are often associated with, and complicated by, the antimicrobial-resistant and zoonotic nature of the microorganisms involved (Walther et al., 2017). Therefore, their occurrence has great potential to hamper treatment, resulting in poor patient outcomes and extensive outbreaks that can affect not just hospitalized animal patients but also veterinary staff and animal owners.

Environmental contamination of veterinary hospitals and clinics may be an important source of subsequent infection (Murphy et al., 2010) as outlined by studies exploring the colonization burden of patients upon admission or during hospitalization (Gibson et al., 2011; Van den Eede et al., 2012) and studies investigating correlations between clinical infections and environmental detection of targeted pathogens (Weese et al., 2006; Timofte et al., 2016; Bortolami et al., 2017). One important component of nosocomial infection development is the widespread fecal (but also cutaneous or upper respiratory) carriage of multidrug-resistant gram-negative (MDR-GN) pathogens by animals entering veterinary facilities and/or by staff members, with potential direct or indirect dissemination to other patients and, conceivably, seeding, and persistence within the hospital environment (Royden et al., 2019; Soza-Ossandón et al., 2020). Hospital settings are ideal for the development and selection of MDR organisms due to high antibiotic use and selective pressure (Mulvey and Simor, 2009). The development of large and specialized veterinary hospitals providing high-standard animal care involving complex interventions and state-of-the-art intensive care facilities has created similar conditions for the emergence of MDR-GN organisms adapted to the veterinary hospital environment. Studies investigating the risk factors for animal patients becoming carriers of MDR agents (Damborg et al., 2012; Maddox et al., 2012) have shown that environmental contamination with nosocomial pathogens is an important reservoir for subsequent infection (Grönthal et al., 2014; Timofte et al., 2016). Unlike methicillin-resistant *Staphylococcus aureus* (MRSA), which is widely studied in animals and where there is plentiful information regarding shared clones in humans and animals (Harrison et al., 2014; Haenni et al., 2017; Islam et al., 2017), much less is known and understood about the prevalence and epidemiology of MDR-GN pathogens in veterinary hospital environments.

Thus, this study aimed to generate veterinary-specific data on the molecular epidemiology of extended-spectrum cephalosporin-resistant (ESC-R) GN bacteria in small animal and equine veterinary hospitals. Ideally, to be able to implement effective preventative measures, detection of HCAIs infections shall be

1 Healthcare-associated Infections Surveillance Network (HAI-Net). *European Centre for Disease Prevention and Control*. Available online at: https://www.ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/hai-net (accessed January 11, 2022).

performed in real time; for this reason, our data were generated by using a fast bacterial strain-typing tool (Acuitas® Resistome) to investigate the introduction, transmission, and/or persistence patterns of MDR-GN bacteria within small animal and equine veterinary hospital settings, with emphasis on the intensive care units (ICUs).

2. Materials and methods

2.1. Study design

To understand the molecular epidemiology of ESC-R GN bacteria in veterinary environments, we performed a 6-month pilot study (PS) in the ICU of two veterinary referral hospitals (one equine and one small animal) at the University of Liverpool, England. Between January and June 2018, we aimed to recruit two to three patients/week admitted to the hospitals' ICUs. Fecal (F) and environmental (ENV) samples were collected as follows: freshly voided (horses) or passed (dogs) fecal samples were collected upon hospital admission (F1) and again after 48 h of hospitalization (F2) to determine whether intestinal carriage was community- or hospital-acquired. Environmental samples were collected at the same time points as for the fecal samples (ENV1 and ENV2) from high-touch surfaces surrounding the hospitalized patients. Specific bacterial culture protocols were followed to select ESC-R GN pathogens from these samples by using selective media. In addition, any ESC-R GN isolates obtained from clinical (PS-CL) and environmental (PS-ENV) specimens submitted for routine diagnostics from these hospitals during the same time frame were also included in the analysis.

Furthermore, retrospective clinical (RTS-CL) and environmental (RTS-ENV) ESC-R GN isolates obtained between March 2016 and December 2017 from the same hospitals through routine processing of clinical specimens or active hospital environmental surveillance were included in the downstream analysis. Ethics approval was obtained for patients enrolled in the study under the University of Liverpool's Ethical Committee (Reference number: VREC588).

2.2. Sample collection

2.2.1. ICU pilot study

Between January and June 2018, selected equine and small animal patients admitted to the respective ICUs at the Liverpool Hospitals were enrolled. Informed consent was obtained from owners upon hospital admission. Paired fecal (F) and environmental (ENV) samples were collected on two separate occasions from each patient, specifically upon admission (F1 and ENV1 at *t0*) and after 48 h of hospitalization (F2 and ENV2 a *t1*). Approximately five grams (5 g) of fresh feces were collected in the early hospitalization hours by ICU staff and placed in sterile Universal containers before direct delivery to the on-site microbiology laboratory on the same day. Environmental ICU samples ($n = 4$ or 5 per patient at each timepoint) were collected at the same time of acquiring the fecal samples by trained veterinary technicians or nurses of the infection control team wearing gloves changed between samples. Environmental sites sampled consisted of animal and human high-contact surfaces; for small animals, a total of five ENV samples were collected that included the ICU telephone receiver and computer keyboard, the ICU door handle, the ICU floor, and the patient kennel (walls and floor). For horses, four ENV samples were submitted, consisting of combinations of the following: ICU door handle, water bucket, hay rack, feed bowl, tie ring, and pen window (ledge and bars). Environmental specimens were collected by swabbing ICU surfaces using sterile pre-moistened electrostatic Swiffer® wipes approximatively 5 cm^2 (Procter & Gamble, Ohio, US) to sample the entire object (e.g., for door handles, phone receiver) or a representative surface size (approximatively 0.5 m^2 when possible, e.g., floor, walls). The sampling cloths were then folded and placed in bottles containing 250 ml of buffered peptone water (BPW). Clinical and environmental ESC-R GNs (PS-CL and PS-ENV) obtained during the pilot study from the ICU patients as well as the wider environmental hospital areas were also included in the analysis.

2.2.2. Retrospective clinical and environmental samples

To depict the epidemiology of MDR-GN bacteria, retrospective (RTS) equine and small animal clinical (RTS-CL) obtained through routine diagnostics and environmental (RTS-ENV) GN isolates obtained from routine environmental surveillance between March 2016 and December 2017 were retrieved from the local bacterial strain collection and included in the analysis.

RTS-CL isolates originated from clinical specimens from both sterile and normally contaminated body sites and represented in most cases pure or mixed predominant cultures, respectively. These specimens consisted of infected skin and wound swabs including surgical site infections, urine collected by cystocentesis, feces, bile, orthopedic implants, cutaneous annexes, oropharyngeal swabs, blood, and abdominal fluid.

RTS-ENV isolates were obtained from the active surveillance programme which is an integral part of the local infection control strategy aimed to monitor the occurrence of MDR organisms in the hospital environment. Surfaces from high-risk areas such as surgical theaters, intensive care units, treatment areas, recovery boxes, equipment (endotracheal tubes, anesthetic equipment), and human high-contact surfaces (computer keyboards, door handles, phone receivers), which could represent "hot spots" for cross-contamination between the environment and the patients, between patients, and between patients and medical staff, are generally included in the routine surveillance on a rotating basis. Non-clinical areas are sometimes also included, such as the hospitals' receptions, pharmacies, washrooms, in-house laboratories, and staff and students' dedicated areas. Environmental specimens are collected by the infection control nurse, using the same method as described for the ENV sample collection in the pilot study above.

2.3. Laboratory processing of fecal, environmental, and clinical isolates in the ICU study and retrospective phase

2.3.1. Fecal samples

To screen for ESC-R GNs, fecal samples (1–2 g) were inoculated into 20 ml BPW with overnight incubation at 37°C and sub-cultured onto eosin methylene blue agar (EMBA; Thermo Scientific) containing 1 μg/ml of cefotaxime (Sigma-Aldrich Ltd., UK) and incubated for 24 h at 37°C aerobically. All ESC-R isolates were sub-cultured onto 5% sheep blood agar (SBA, Oxoid, Basingstoke, UK) for bacterial identification.

2.3.2. Environmental samples

For the environmental samples, targeted screening for ESC-R GNs was carried out beginning with an enrichment stage incubating the Swiffer in BPW at 37°C overnight, followed by sub-culture (10 μl) onto EMBA with cefotaxime (1 μg/ml) and Pseudomonas Selective Agar (all from Oxoid, Basingstoke, UK) incubated aerobically at 37°C for 18–24 h. If colonies were phenotypically different, each colony morphotype (including both EMBA positive and negative ones) was sub-cultured onto 5% SBA for bacterial identification.

2.3.3. Clinical samples

Pilot (PS-CL) and retrospective clinical (RTS-CL) isolates were obtained through the Liverpool Veterinary Microbiology Diagnostic Service. Clinical specimens were processed according to the local diagnostic protocols for pathogen detection and antimicrobial susceptibility testing from different sample types, which in most cases included plating out on a non-selective media such as 5% SBA (Oxoid, Basingstoke, UK) and Fastidious Anaerobe Agar (FAA; E&O Laboratories Ltd., Bonnybridge, UK) cultured aerobically and anaerobically. Clinical isolates included in this study were selected based on their resistance to extended-spectrum cephalosporins, determined as part of routine antimicrobial susceptibility testing for clinical isolates. Cefpodoxime (10 μg) was used as the screening agent and testing was performed on Mueller-Hinton agar (MHA) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for processing and interpretation (CLSI, 2018a).

2.3.4. Bacterial species identification

All clinical and environmental isolates obtained before 2018 were identified using the Analytical Profile Index (API) system and APIWEB Software (bioMérieux, Marcy-l'Étoile, France), and their identification was later confirmed via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Isolates acquired from 2018 onwards were directly identified by MALDI-TOF MS (MALDI Biotyper 4.1.100 Software, Bruker Daltonics, Bremen, Germany) with a score >2.0.

Following identification, the isolates selected for this study (PS and RTS) were batched, collected using Amies gel-based charcoal swabs, and sent to the OpGen Clinical Services Laboratory in the United States for the performance of the OpGen Acuitas® Resistome Test (OpGen Inc., Gaithersburg, MD).

2.4. Acuitas resistome

The Acuitas® Resistome Test can detect a large number of antimicrobial resistance genes in GN bacteria in a single run, providing comprehensive and rapid phenotypic/genotypic typing results. The methodology consists of two tests run in parallel: (i) the Acuitas Test screening for antibiotic resistance determinants and (ii) the MDR-GN culture screen with species identification and antimicrobial susceptibility testing (ID/AST) by VITEK2 (bioMérieux, Durham, NC). The Acuitas Test is a real-time polymerase chain reaction (qPCR) microfluidic array assay which screens for 50 antibiotic resistance beta-lactamase gene families, including those encoding production of extended-spectrum beta-lactamases (ESBLs) (CTX-M, TEM, SHV, BEL, BES, TLA, PER, VEB, GES, OXA-2, OXA-10, OXA-18), AmpC beta-lactamases (ACC, ACT, CMY, DHA, FOX, MIR, MOX), carbapenemases (GIM, IMI, IMP, NDM, SIM, SPM, VIM, KPC, SFC, NMC-A, SME, OXA-23, OXA-24, OXA-45, OXA-48, OXA-50, OXA-51, OXA-54, OXA-55, OXA-60, OXA-62), and non-ESBL beta-lactamases of the SHV/TEM/OXA types. The Acuitas Resistome Test qPCR methodology was illustrated by Reuben et al. (2017) and Voulgari et al. (2020). The antimicrobial panel comprised ampicillin/sulbactam, piperacillin/tazobactam, cefazolin, ceftriaxone, ceftazidime, cefepime, aztreonam, imipenem, meropenem, ertapenem, amikacin, gentamycin, tobramycin, ciprofloxacin, levofloxacin, trimethoprim/sulphomethoxazole, and tigecycline. The results were interpreted according to the CLSI human clinical breakpoints (CLSI, 2018b). Another important feature of the Acuitas Resistome is the ability to indicate possible strain relatedness of isolates. For this, phenotypic and genotypic results are combined to generate unique "Acuitas profiles" that can be used for typing, cluster identification and tracking transmission events, as clonal isolates share the same Acuitas profiles. Each profile comprises codes that identify (1) organism genus and species, (2) phenotype code determined by AST results, (3) listing of up to 3 AMR gene codes determined by the Acuitas Resistome Test, (4) a unique numerical code representing the pattern of all positive and negative assays from the Acuitas Resistome Test results, and (5) the AST profile code linked to the unique pattern of non-susceptible AST results (Supplementary Figure 1). Genetically similar types, which could represent related isolates, were established by combining the organism's name and the code for the Acuitas Resistome Test results. These types were further divided into subtypes using the profile's AST code. All results were uploaded on the Acuitas Lighthouse MDRO Management System (OpGen) website portal for real-time access.

TABLE 1 Summary of all pilot study (*PS*) and retrospective phase (*RTS*) companion animal ESC-R GN isolates included in the study (March 2016–June 2018).

Small animal hospital		Equine hospital	
Pilot study (PS)			
F1	2	F1	12
F2	3	F2	15
ENV1	26	ENV1	45
ENV2	15	ENV2	52
CL	24	CL	9
ENV	4	ENV	0
Retrospective phase (RTS)			
CL	6	CL	3
ENV	46	ENV	33

F1 and ENV1, fecal and environmental samples collected upon ICU admission; F2 and ENV2, fecal and environmental samples collected after 48 h from Intensive Care Unit (ICU) admission; CL, clinical isolates obtained from hospital routine diagnostic samples; and ENV, environmental isolates obtained from hospital routine environmental surveillance.

3. Results

3.1. Samples and bacterial isolates from the ICU pilot study and retrospective phase

Overall, 279 samples (n= 49 fecal and n= 230 environmental) were collected during the ICU pilot study from 28 selected patients (equine, n= 20 and small animal, n= 8) admitted to the ICUs and their surroundings (Supplementary Table 1). The majority of samples originated from the equine hospital (199/279) and the remainder from the small animal hospital (80/279). In general, fecal samples were relatively equally distributed across the first (F1 = 26/49) and second (F2 = 23/49) sampling time points. However, F1 and/or F2 fecal samples could not be obtained for some patients, for which only one fecal sample at either time point was processed (Supplementary Table 1). Environmental ICU PS samples (n = 230, of which ENV1 = 120 and ENV2 = 110) comprised n = 160 equine ICU and n = 70 small animal ICU specimens. ENV2 sample sets could not be obtained for two patients (Supplementary Table 1). Additionally, clinical (PS-CL) and hospital environmental (PS-ENV) ESC-R GN isolates obtained from routine diagnostics during the same period were included amongst the PS isolates (Supplementary Table 2). In addition to the 28 patients enrolled in the pilot study, n = 42 ESC-R GN clinical isolates (PS-CL and RTS-CL) from diagnostic submissions of 36 other patients were included.

Table 1 summarizes all PS (n = 207) and RTS (n = 88) bacterial isolates included in this study (total of n = 295 ESC-R GNs).

3.1.1. Overall ESC-R gram-negative organisms' prevalence

The overall prevalence of companion animal ICU samples (F1&2 and ENV1&2) positive for ESC-R GNs during the pilot study (PS) was 51.3% (143/279) (Table 2). Twenty-three bacterial species were detected overall, with the most prevalent ESC-R GN organisms circulating in the equine and small animal hospitals between March 2016 and June 2018 being members of the ESKAPE group of pathogens, namely *Enterobacter cloacae complex* (64/295 = 21.7%), *Pseudomonas aeruginosa* (59/295 = 20.0%), *Klebsiella pneumoniae* (47/295 = 15.9%), and *Acinetobacter baumannii complex* (40/295 = 13.6%), followed by *Escherichia coli* (36/295 = 12.2%). Other organisms detected at significantly lower rates (≤5/295) are indicated in Figure 1.

The top ESC-R GNs isolated from the ICUs during the pilot study (January–June 2018) corresponded to the same organisms showing the highest prevalence across both hospitals during the entire study period (March 2016–June 2018). The prevalence rates of the five most commonly isolated ESC-R organisms and that of all other bacterial isolates collectively per hospital ICU are shown in Figure 2. With the exception of *K. pneumoniae* subsp. *pneumoniae*, predominantly isolated from small animal ICU, all other ESC-R GN pathogens were largely retrieved from the equine ICU.

3.1.2. Prevalence of ESC-R GNs circulating in the equine hospital

Within the equine ICU, 50% (100/199) of PS-F1&2 and PS-ENV1&2 samples collected between January and June 2018 yielded ESC-R GN organisms (Tables 1, 2). The distribution of ESC-R GN organisms per ICU PS sample type is shown in Figure 3A. Overall, *E. cloacae* complex and *P. aeruginosa* were most commonly recovered from all four sample types of equine origin. ESC-R *E. cloacae* complex (4/9) and *E. coli* (3/9) were cultured from equine clinical specimens during the PS.

Of the retrospective ESC-R GNs from 2016 to 2017, most represented environmental surveillance isolates (33/36) dominated by *E. cloacae* complex (Table 3). For ease, individual environmental sites detailed in Supplementary Table 2 have been grouped within unit areas from where isolated in Table 3. For example, the equine stables group comprises stable walls, floors, drains, pen window ledge and colic recovery boxes located next to the stable; the ICU unit includes keyboards, door handles, and other items such as bandage trolley or stocks located inside the ICU. Samples from non-clinical areas included students' hot desks and keyboards, in-house laboratories' worktops and equipment, washrooms, pharmacies, and reception/waiting room areas. Of all RTS environmental isolates collectively, the greatest proportion was isolated from the horse stables (18/33), followed by non-clinical areas (11/33) and ICU (4/33).

3.1.3. Prevalence of ESC-R GNs circulating in the small animal hospital

Of the small animal ICU PS samples (PS-F1&2 and PS-ENV1&2, January–June 2018), 65% (52/80) were positive for ESC-R GNs (Tables 1, 2). *P. aeruginosa* was commonly detected amongst small animal ENV1 samples, whilst *K. pneumoniae*

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TABLE 2 Prevalence data for samples collected from the Intensive Care Units (ICUs) during the pilot study (PS, January–June 2018).

Small animal hospital			Equine hospital				
ICU pilot study							
	Samples	Positive		Samples	Positive	Tot samples	Tot positive
F1	6	2	F1	20	11	26	13
F2	4	3	F2	19	13	23	16
ENV1	40	23	ENV1	80	35	120	58
ENV2	30	15	ENV2	80	42	110	57
Tot	80	52		199	100	279	143

Please note that PS-CL and PS-ENV isolates obtained through routine diagnostics during the same time frame were not included. F1 and ENV1, fecal and environmental samples collected upon ICU admission; F2 and ENV2, fecal and environmental samples collected after 48 h from ICU admission.

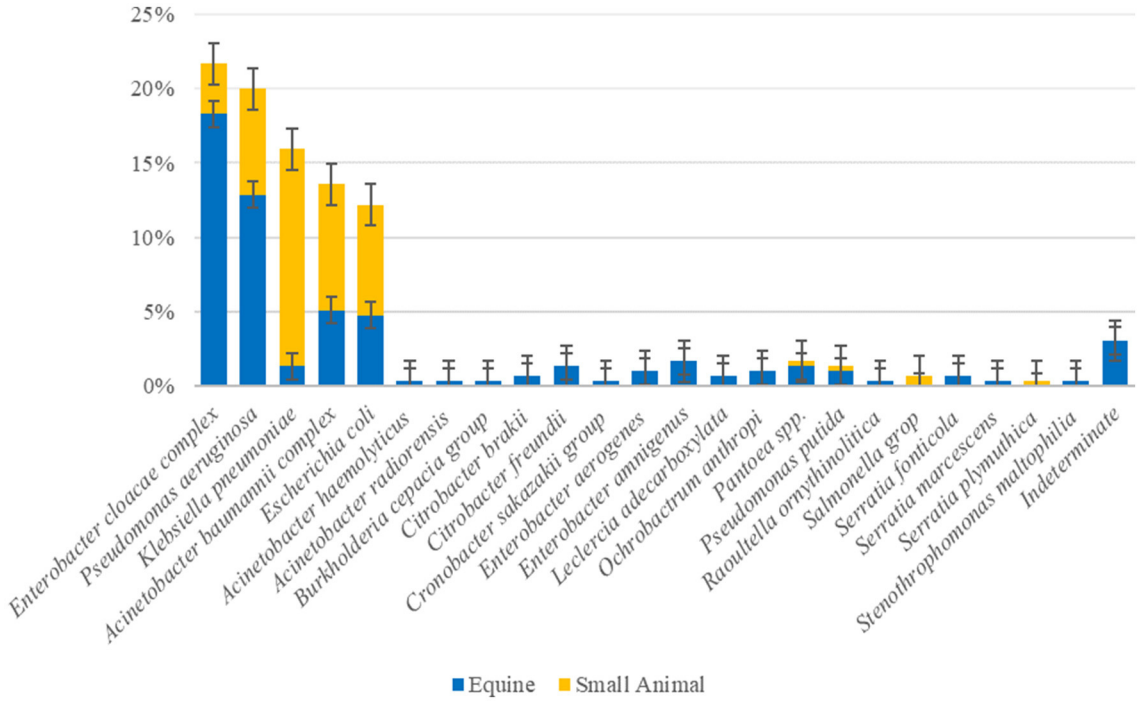

FIGURE 1
Overall prevalence of the ESC-R GN organisms ($n = 295$) circulating in the equine and small animal hospitals between March 2016 and June 2018. Error bars represent 95% confidence intervals.

subsp. *pneumoniae* accounted for the most encountered pathogen across all four sample types in dogs at both time points. Figure 3B illustrates the distribution of ESC-R GN organisms per ICU PS sample type. The majority of PS-CL isolates detected from the small animal hospital during the same time were *E. coli* (11/24), *K. pneumoniae*, and *A. baumannii* complex (each 4/24).

Of the 52 small animal RTS GN isolates from 2016 to 2017, RTS-CL isolates were mostly *E. coli*-associated urinary tract infections (4/6). *A. baumannii* complex isolates predominated in the environment (18/46), followed by *K. pneumoniae* (8/46), *P. aeruginosa*, and *E. cloacae* complex (each 7/46) (Table 3). A broad range of units are found in the small animal hospital, of which the ICU held the highest proportion of ESC-R GNs (13/46) during the period 2016–2017.

3.2. Acuitas resistome test results

Phenotypic AST results are presented within the Supplementary Figures 2, 3 and Supplementary Table 3 as this section's focus is on genotypic findings. All 295 ESC-R GNs isolates tested generated Acuitas Resistome Test results (Supplementary Table 4); overall, extended-spectrum beta-lactamase (ESBLs), TEM/SHV/OXA and AmpC beta-lactamase or carbapenemase genes were identified in 82% (242/295) of isolates whilst 18% (53/295) of ESC-R GNs had no detectable gene amongst

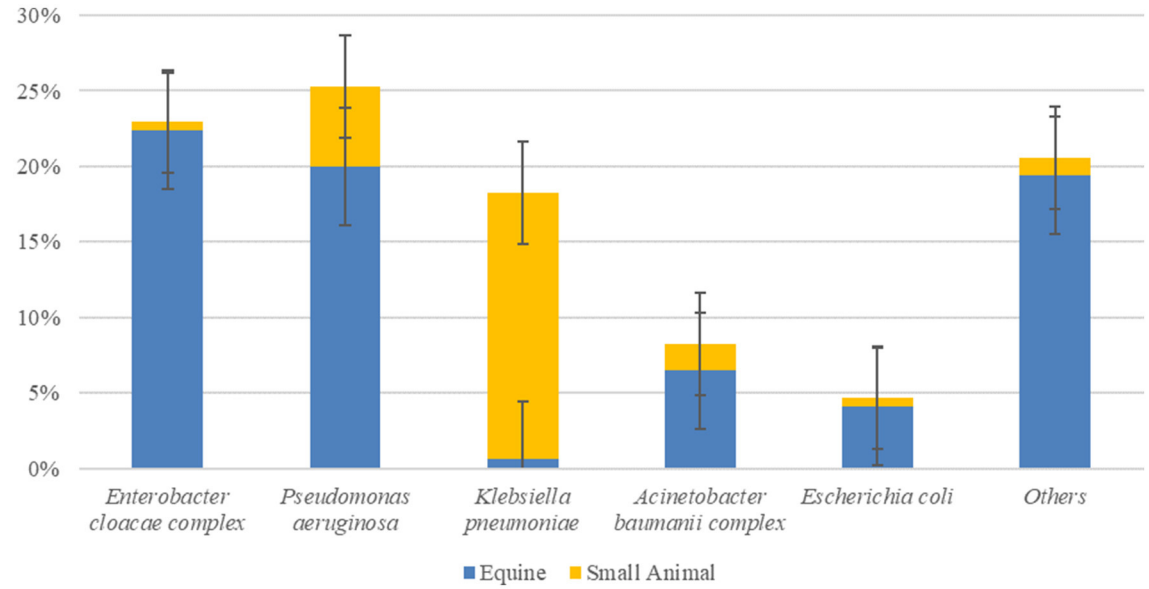

FIGURE 2
Prevalence of ESKAPE organisms, *E. coli*, and all other pathogens circulating in the equine and small animal Intensive Care Units (ICUs) between January and June 2018 during the pilot study ($n = 170$, consisting of PS-F1 and 2 and PS-ENV1 and 2 isolates). Error bars represent 95% confidence intervals.

the 67 tested belonging to 50 antibiotic resistance gene families. With regard to the overall resistome prevalence (Figure 4A), antimicrobial resistance genes harbored by ESC-R GNs varied more between hospitals than they did between PS and RTS isolates within the respective hospital. In addition, genetic makeup was frequently consistent within species of ESKAPE organisms (and *E. coli*). Figure 4B illustrates the resistome distribution across ESC-R ESKAPE and *E. coli* organisms.

Altogether, beta-lactamase enzymes of the TEM (29.2–0.3%) and SHV (28.8–12.9%) families were the most commonly detected, followed by ACT-5 beta-lactamase (21.4%). TEM and ACT-5 genes were common amongst *E. cloacae complex* isolates and, to a lesser extent, amongst *K. pneumoniae* and *E. coli*, whilst SHV enzymes were amongst *K. pneumoniae* and, secondarily, *E. cloacae complex*. Simultaneous carriage of multiple TEM and/or SHV subtypes was a feature of most positive isolates. Gene families mediating resistance to extended-spectrum cephalosporins were mostly restricted to pAmpC enzymes of the DHA (DHA-1 prevalence of 15.3%) and CMY (CMY-2 and CMY-70 prevalence of 2.4 and 0.3%, respectively) families. DHA-type enzyme was predominant amongst *K. pneumoniae* whilst CMY amongst *Citrobacter* species. CTX-M class enzymes were detected to a lesser extent than pAmpC and identified exclusively with *E. coli* CTX-M-1 (7.8%) and CTX-M-9 (0.3%). Oxacillinase enzymes of the OXA-50 (prevalence of 19%) and OXA-51 (prevalence of 9.5%) types were also detected amongst the majority of *P. aeruginosa* (53/59) and *A. baumanni complex* (27/40), respectively. One important finding is the identification of ESC- R-GN isolates carrying transmissible resistance to last-resort antimicrobials (i.e., carbapenems) within the veterinary hospitals, represented by three (1%) *Acinetobacter* spp. harboring *bla*OXA-23 and one (0.3%) *E. coli* with *bla*OXA-48. No AMR genes were identified belonging to other families (Supplementary Table 4). The proportion of ESC-R GNs carrying beta-lactamase resistance determinants amongst PS and RTS sets of isolates was 79.7% (165/207) and 87.5% (77/88), respectively.

3.3. Likely strain relatedness of ESC-R GNs

Genetically related types were proposed on the basis of identical bacterial species and Acuitas Resistome Test's code, whilst the Acuitas profile's AST code was used to determine subtypes. Overall, 58 Resistome types were detected across all isolates ($n = 295$; Supplementary Table 4).

There was overall heterogeneity amongst profiles of typed ESC-R ESKAPE and *E. coli* organisms; nonetheless, recurrent Acuitas patterns were detected within but also across the two hospitals and especially amongst ICU pilot isolates (Figure 5). Some cluster examples of possible genetically related ESKAPE and *E. coli* pathogens are provided in the paragraphs below.

3.3.1. *Enterobacter cloacae* complex

Fifty-four *E. cloacae complex* isolates from the equine hospital were grouped into nine Acuitas Resistome types and 14 subtypes (Supplementary Table 4). Of these, two major and one minor patterns were identified, consisting of 20 (Group 1: E10:CephR-GNB:SHV4_SHV5_TEM7), 19 (Group 2: E10:S-GNB:TEM7_TEM3_TEM1), and 6 (Group 3: E10:CephR-GNB:TEM7_TEM3_TEM1) isolates, respectively. Group 1 isolates harbored SHV4 & 5 and TEM7 enzymes, and this was made up

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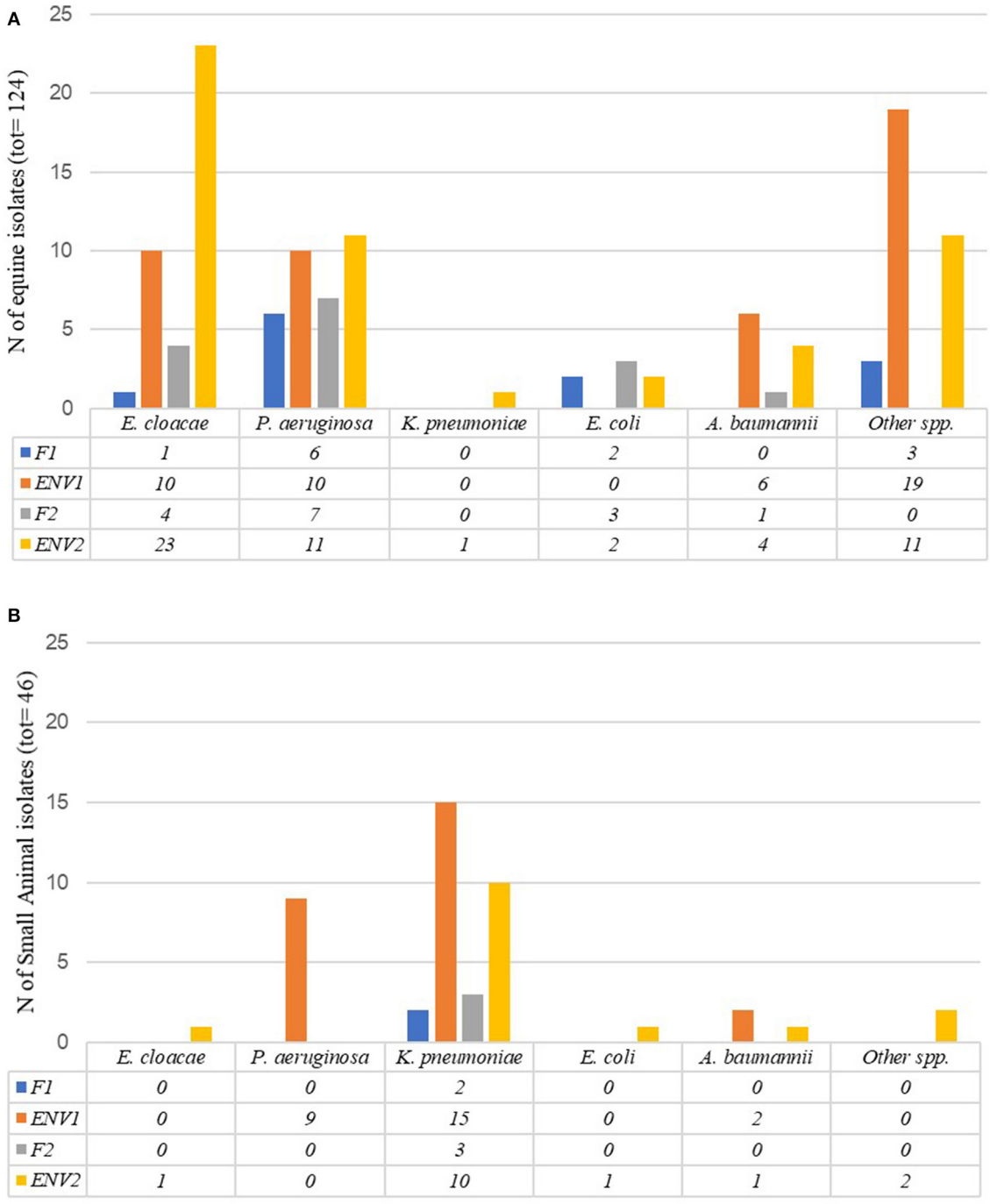

	E. cloacae	P. aeruginosa	K. pneumoniae	E. coli	A. baumannii	Other spp.
F1	1	6	0	2	0	3
ENV1	10	10	0	0	6	19
F2	4	7	0	3	1	0
ENV2	23	11	1	2	4	11

	E. cloacae	P. aeruginosa	K. pneumoniae	E. coli	A. baumannii	Other spp.
F1	0	0	2	0	0	0
ENV1	0	9	15	0	2	0
F2	0	0	3	0	0	0
ENV2	1	0	10	1	1	2

FIGURE 3
Number of ESC-R GN isolates obtained per Intensive Care Unit sample type (PS-F1 and 2 and PS-ENV1 and 2) during the pilot study (January–June 2018) in the equine **(A)** and small animal **(B)** hospital Intensive Care Units.

of both PS ($n = 12$) and RTS ($n = 8$) isolates collected over a 2-year time period. Most PS isolates (9/12) were recovered from fecal and environmental samples connected to five horses admitted to the ICU over 24 days. *E. cloacae* complex from Group 2 carried TEM1, 3, and 7 enzymes and the vast majority (17/19) circulated during the pilot phase of the study (PS) in the equine ICU; they were associated to a total of 10 horses between March and May 2018 and were mostly found on various environmental surfaces ($n = 14$ samples) and in one fecal sample. Similarly, Group 3 consisted of PS isolates from ICU surfaces ($n = 6$) relative to four horses' pens over a month. Groups 2 and 3 isolates only differed by detection of phenotypic ESC-R in Group 3 but not in Group 2. Furthermore, all isolates from Groups 2 and 3 were of the same subtype within their respective group. In all three groups, over 50% of environmental PS isolates were collected at the second time point (ENV2 samples) and, in the case of some patients, a crossover of *E. cloacae* complex

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TABLE 3 Retrospective clinical (RTS-CL) and environmental surveillance (RTS-ENV) ESC-R GN isolates (March 2016–December 2017) and isolation sources within the equine and small animal hospitals.

	Retrospective phase			
	Small animal hospital		Equine hospital	
	Organism species (no)	Site	Organism species (no)	Site
Clinical (RTS-CL)	*E. coli* (4)	Urine	*E. coli* (1)	Skin and wound infection
$n = 9$	*P. aeruginosa* (1)	Skin and wound infection	*P. aeruginosa* (1)	Skin and wound infection
	Salmonella spp. (1)	Skin and wound infection	*A. baumanni* complex (1)	Skin and wound infection
Environmental (RTS-ENV)	*E. cloacae* complex (7)	ICU (3), non-clinical areas (2), chemotherapy (1), dermatology (1)	*E. cloacae* complex (12)	Stables (5), non-clinical areas (5), ICU (2)
$n = 79$	*P. aeruginosa* (7)	Wards (4), ICU (1), chemotherapy (1), dermatology (1)	*P. aeruginosa* (3)	Stables (1), non-clinical areas (2)
	E. coli (5)	Chemotherapy (1), dermatology (1), imaging (1), cardiology (1), anesthesia (1)	*E. coli* (3)	Stables
	K. pneumoniae (8)	ICU (3), non-clinical areas (3), dermatology (1), anesthesia (1)	*K. pneumoniae* (3)	Stables (1), non-clinical areas (2)
	A. baumanni complex (18)	ICU (6), non-clinical areas (7), cardiology (2), dermatology (1), wards (1), theater (1)	*A. baumanni* complex (2)	Non-clinical areas (2)
	Serratia plymuthica (1)	Anesthesia	*Citrobacter freundii* (4)	Stables (3), ICU (1)
			Enterobacter aerogenes (3)	Stables
			Citrobacter braakii (2)	Stables
			Serratia marcescens (1)	ICU

isolates from the three groups was observed in their surrounding ICU sites.

3.3.2. *Pseudomonas aeruginosa*

Fifty-one out of 59 total *P. aeruginosa* isolates were typed to the same Acuitas Resistome profile P1:CephR-GNB:OXA50, consistent with OXA-50-producing organisms (Supplementary Table 4). Five subtypes were identified overall, although 44 out of 51 isolates belonged to the same AST subtype ([R70]96-[A17]181). Equine vs. small animal (35 and 16, respectively) and PS vs. RTS (41 and 10, respectively) isolates constituted the broad majority of this large *P. aeruginosa* single group. In the equine hospital, 31 *P. aeruginosa* were collected during the PS from 10 ICU patients over a 2-month period. Four horses shed this type in their feces at both time points whilst the others at either time point but predominantly with F2 samples. It was not uncommon for some horses to yield this *P. aeruginosa* type across multiple ICU samples (both fecal and environmental) with up to 80% positive samples from a single patient at both time points. At the same time, isolates of this *P. aeruginosa* type were also isolated from small animal ICU sites ($n = 6$, keyboard, kennels, floor and phone receiver) in connection to three hospitalized dogs. The majority of small animal *P. aeruginosa* of this pattern ($n = 10$) were, however, detected amongst retrospective isolates beginning in 2016. This was also the case for the equine hospital, with the first *P. aeruginosa* isolate of this type recorded in 2016.

3.3.3. *Klebsiella pneumoniae*

Forty-seven *K. pneumoniae* isolates were typed in total, belonging to seven Resistome types and nine AST subtypes altogether. The vast majority (40/47) were of Resistome type K1:CephR-GNB:SHV1_SHV5_DHA1 (Supplementary Table 4); within this main group, 85% of the isolates (34/40) also shared the same AST subtype ([R70]147-[A17]180). All 40 *K. pneumoniae* were from the small animal hospital; of these, 85% were PS isolates and the remainder were RTS isolates. All but three PS isolates were linked to four dogs admitted to the ICU over a 17-day time interval in May 2018. Samples collected from these dogs displayed high K1:CephR-GNB:SHV1_SHV5_DHA1 prevalence rates at both time points (42–75% of ICU PS samples/patient). Fecal samples and multiple ICU sites (e.g., keyboard, phone receiver, door handle and individual dog kennels) were repeatedly found positive. Of note, the same *K. pneumoniae* isolate was also obtained from two clinical specimens received by the diagnostic laboratory during the same 17-day period, namely one abdominal fluid sample and one catheter-urine sample from dogs in post-surgical recovery. The peritonitis case was a patient admitted to the ICU and enrolled in the PS who yielded fecal *K. pneumoniae* at both sampling

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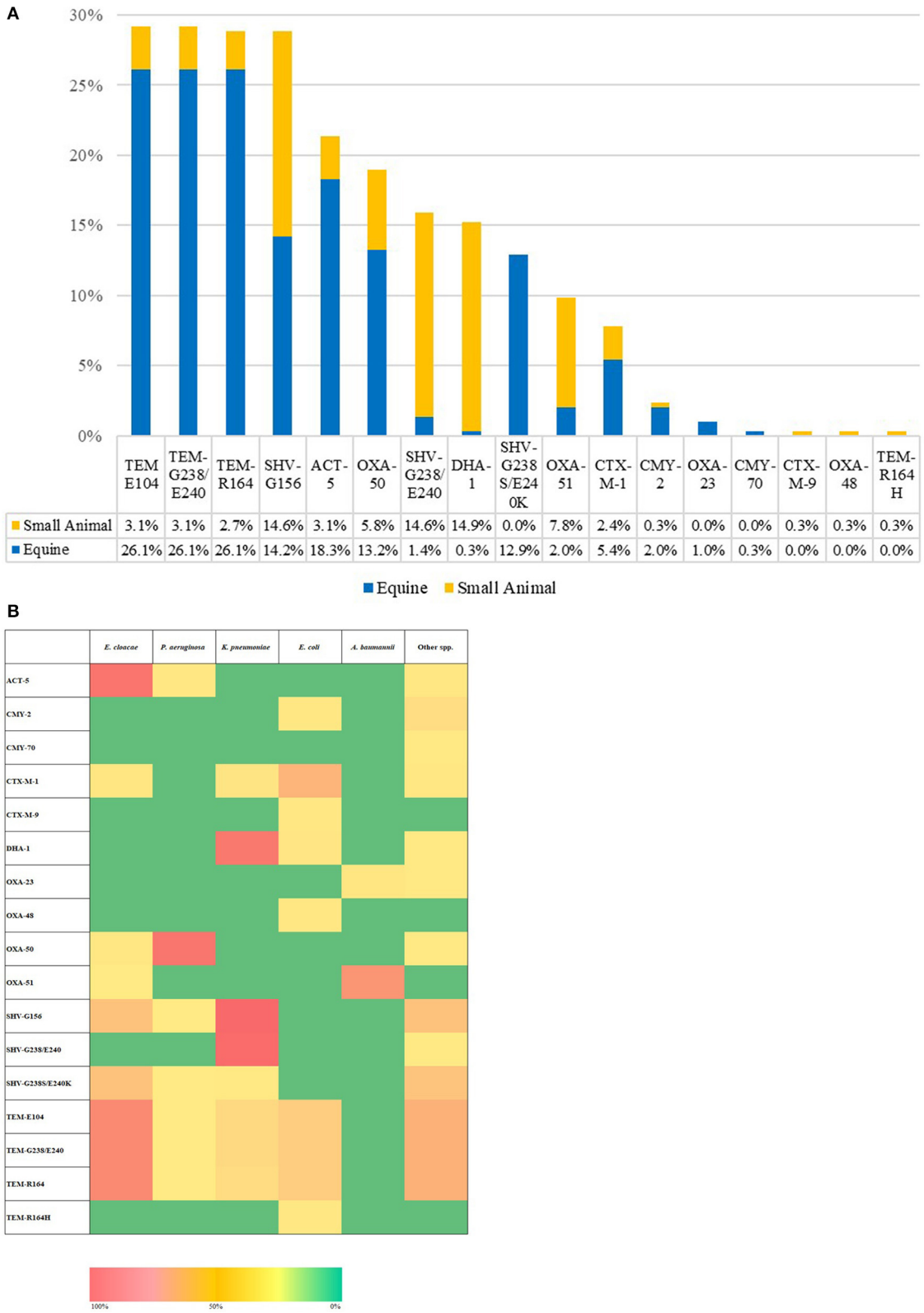

	TEM E104	TEM-G238/E240	TEM-R164	SHV-G156	ACT-5	OXA-50	SHV-G238/E240	DHA-1	SHV-G238S/E240K	OXA-51	CTX-M-1	CMY-2	OXA-23	CMY-70	CTX-M-9	OXA-48	TEM-R164H
Small Animal	3.1%	3.1%	2.7%	14.6%	3.1%	5.8%	14.6%	14.9%	0.0%	7.8%	2.4%	0.3%	0.0%	0.0%	0.3%	0.3%	0.3%
Equine	26.1%	26.1%	26.1%	14.2%	18.3%	13.2%	1.4%	0.3%	12.9%	2.0%	5.4%	2.0%	1.0%	0.3%	0.0%	0.0%	0.0%

FIGURE 4
Overall extended-spectrum beta-lactamase (ESBLs), TEM/SHV/OXA, and AmpC beta-lactamase or carbapenemase genes per hospital **(A)**. Heatmap of the distribution of extended-spectrum beta-lactamase (ESBLs), TEM/SHV/OXA and AmpC beta-lactamase or carbapenemase encoding genes **(B)**; [*n* = 295, March 2016–June 2018].

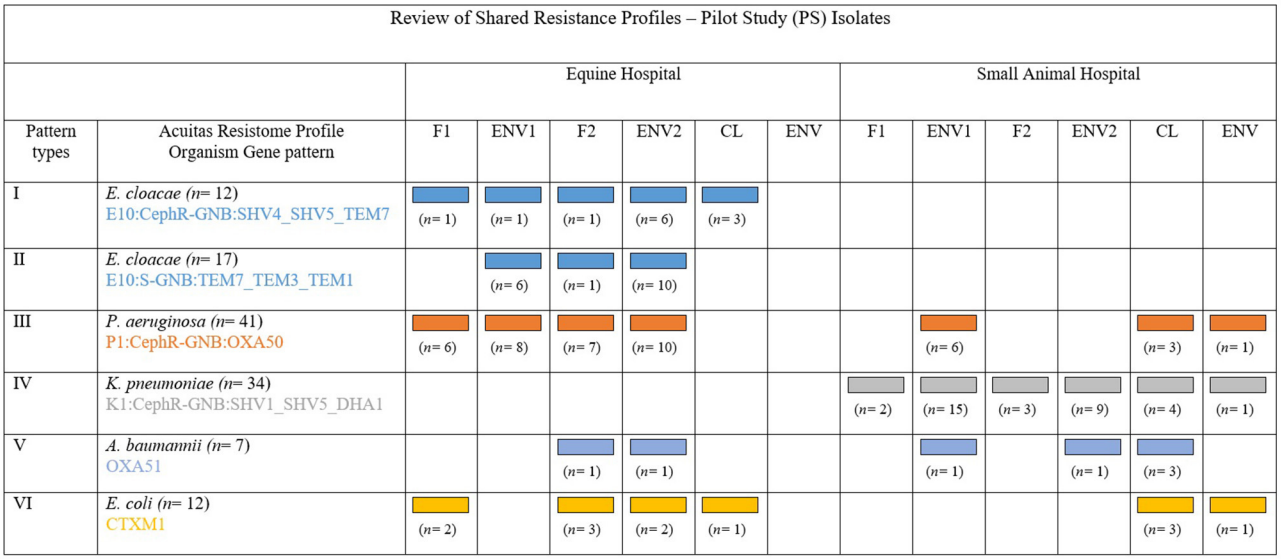

FIGURE 5
Adapted and with permission from Reuben et al. (2017). Review of the principal shared resistance profiles. Representation of five groups of MDR-GN organisms identified by Acuitas profiles as genetically related and their distribution amongst the two hospitals and sample type from where isolated during the pilot (PS). Patterns I, II, and IV show possible transmission within veterinary facilities and Patterns III, V, and VI show possible inter-facility transmission.

points. Other *K. pneumoniae* Resistome types carrying various combinations of SHV, TEM, and/or CTX-M-1 enzymes were identified in low numbers and random fashion.

3.3.4. *Acinetobacter baumannii* complex

Forty *A. baumannii complex* isolates fell into an overall of four types and 11 subtypes with a major group containing 26 isolates and two subtypes (Supplementary Table 4). The main Resistome type A4:CephR-GNB:OXA51 consisted of OXA-51-producing *A. baumannii* identified in both hospitals ($n = 21$ small animal and $n = 5$ equine), largely being RTS isolates ($n = 19$). OXA-51 small animal isolates were cultured from a variety of hospital sites over time (including the ICU, non-clinical areas, and hospitals' departments) and from fewer clinical specimens ($n = 3$). Eleven ESC-R *A. baumanni* isolates tested negative for all AMR genes with differences in phenotypic codes reported as ESC-S, ESC-R, MDR, or CR *A. baumannii*. Importantly, Two OXA-23-producing PS isolates were identified from the water bucket and feed bowl of one horse; both were recovered at the first sampling point without repeated isolation at the second sampling. These isolates retained *in vitro* susceptibility to a number of agents, including ampicillin/sulbactam, piperacillin/tazobactam, ceftazidime, ciprofloxacin, levofloxacin, gentamicin, tobramycin, imipenem, and meropenem. A single OXA-23-producing *Acinetobacter radioresistens* isolate was obtained from another horse's hay rack in the ICU 3 days after the OXA-23-positive *A. baumannii*.

3.3.5. *Escherichia coli*

ESC-R *E. coli* showed a proportionally higher degree of genetic heterogenicity when compared to the other organisms, with 36 isolates falling into eight Resistome types and 24 AST subtypes (Supplementary Table 4). One main *E. coli* group was identified ($n = 15$, Resistome profile E1:CephR-GNB:CTXM1) that contained CTX-M-1 and was further divided into eight AST subtypes; 10/15 isolates were equine and the 5/15 small animal. A small ICU cluster of seven ($n = 7$) equine PS isolates was identified from five ICU patients over 37 days. Four horses eliminated CTX-M-1-producing *E. coli* in their feces (at the second [$n = 2$], first or both [each $n = 1$] time points), and 2/5 had positive ICU surroundings. No epidemiological correlation was identified between small animal CTX-M-1-positive *E. coli* ($n = 5$, mostly PS-CL) of this Resistome type. The second largest *E. coli* group shared a lack of any AMR gene tested. Scattered *E. coli* isolates harboring combinations of TEM, CTX-M, and other beta-lactamase enzymes were identified; of note, one OXA-48 positive RTS isolate co-harboring CTX-M-9 was cultured from a dog surgical wound. This isolate retained *in vitro* susceptibility only to aminoglycosides, carbapenems and tigecycline.

4. Discussion

To the best of our knowledge, this is the first study to identify ESKAPE pathogens as the most prevalent ESC-R gram-negative bacteria circulating within veterinary ICUs and other hospital environments. Rapid identification of potential contamination reservoirs and understanding of the transmission dynamics of these pathogens are key to a successful infection control programme and prevention of HCAIs in both human and veterinary hospitals. Several technological advances have been implemented to guide patient management and support antimicrobial stewardship and

infection control programmes in human healthcare settings; however, these are slow to be adopted within veterinary facilities.

Our study highlighted an overall high ESC-R GNs prevalence within the veterinary ICU (50% of equine and 65% of canine colonization and environmental samples altogether) and hospital environments. ESKAPE pathogens were more prevalent than the fecal contamination biomarker *E. coli* bacteria (Poirel et al., 2018), a common colonizer encountered in other veterinary hospital studies (Rubin and Pitout, 2014; Walther et al., 2014; Zogg et al., 2018). *E. cloacae* complex, *P. aeruginosa*, and *A. baumanni* complex followed by *E. coli* were more common in the equine, whilst *K. pneumoniae* predominated in the small animal hospital environment. Animal-associated gram-negative ESKAPE organisms have been reported in clinical infections (Singh, 2018) and environmental contamination of slaughterhouses (Savin et al., 2020), although no study focussed on companion animal clinics. Reports of some ESKAPE bacteria associated with companion animal ICUs and veterinary HCAIs are on the rise in recent times, particularly for MDR *A. baumannii* (van der Kolk, 2015) but are limited for other ESKAPE GN species. *Klebsiella* spp. have been described in pet nosocomial infections (Seliškar et al., 2007; Haenni et al., 2012; Ewers et al., 2014); however, considerably less data are available for *E. cloacae* (Gibson et al., 2008; Wilberger et al., 2012) and *P. aeruginosa* (Bernal-Rosas et al., 2015; Hassan et al., 2021; Soonthornsit et al., 2023) veterinary hospital dissemination and HCAIs occurrence.

Various contaminated environmental sites were identified at our hospitals (March 2016–June 2018) including ICUs (door handles, keyboards, floor, phone receivers, etc.), wards/stables (walls, floor, windows, pump holders, etc.), and non-clinical areas' high-contact surfaces (student keyboards, washroom tables, reception, etc.) amongst others. This is relevant because an increased risk of HCAIs in human patients has been demonstrated when hospital surface surroundings are contaminated (Weber et al., 2013; Nutman et al., 2016). In addition, key nosocomial pathogens have been shown to persist in the hospital environment for variable lengths of time (from days to months) acting as reservoirs of infection leading to further contamination, via staff hands or patient-to-patient transmission (Kramer et al., 2006).

Although the definition of the transmission patterns (introduction, transmission, and/or persistence) of ESC-R organisms isolated from our hospitals appears difficult, generally higher ESKAPE prevalence rates were recorded for ICU pilot samples collected at the second time point than at the first. A few possible Acuitas® Resistome transmission events were recognized through the pilot study via the identification of a common Resistome profile in multiple sampling points. These included (i) *E. cloacae* complex Group 1 (E10:CephR-GNB:SHV4_SHV5_TEM7) and Group 2 (E10:S-GNB:TEM7_TEM3_TEM1) in the equine ICU; (ii) *K. pneumoniae* (K1:CephR-GNB:SHV1_SHV5_DHA1) in the canine ICU; and (iii) *E. coli* CTX-M-1 in the equine ICU, all of which circulated over a relatively short time including environmental, colonization, and clinical isolates. Furthermore, *E. coli*, *P. aeruginosa,* and *A. baumanii* main patterns occurred in both hospitals during the study period, suggesting possible inter-hospital spread. Staff and students may conceivably mediate cross-contamination via hands or footwear between large and small animal university hospitals located nearby (Singaravelu et al., 2023). Not infrequently, however, isolates with shared Acuitas patterns were collected at broad time intervals (months or years), also pointing toward possible pathogen persistence within hospital environments and/or to limited typing resolution.

The overall resistome trends identified TEM, SHV, and ACT-type enzymes as the most prevalent amongst companion animal ESC-R GNs in our hospitals, with lower molecular detection of ESBL (CTX-M-1 and−9), pAmpC (DHA-1, CMY-2 and−70) type enzymes and carbapenemases (OXA-23 and−48). Concerningly, the occurrence of carbapenem-resistance in critically important human gram-negative bacteria has been acknowledged in companion animals although yet at low prevalence (Rincón-Real and Suárez-Alfonso, 2022). Resistant organisms are known to spread amongst companion animals and staff in veterinary healthcare settings (Boerlin et al., 2001), and this may soon be the inauspicious case also for carbapenem-resistant bacteria, since their detection in veterinary hospital environments has been described, ranging from pet carriage to hospital outbreak (Gentilini et al., 2018; Nigg et al., 2019; Lavigne et al., 2021; Cole et al., 2022). Therefore, the implementation of routine hospital screening appears crucial to improve the surveillance of these unexpected phenotypes in veterinary settings.

The Acuitas® Resistome has been employed in human hospitals for the rapid determination of carbapenemase-producing organisms (CPOs) prevalence in colonized and infected patients, for hospital and regional surveillance (Reuben et al., 2017; Lapp et al., 2021), and for the rapid information on empiric antimicrobial use (Evans et al., 2019). Its routine diagnostic applications have been trialed for fast CPO detection with promising results (Vanstone et al., 2018; Voulgari et al., 2020). Previous evaluation of the Acuitas® Resistome analytical performance highlighted several benefits of this molecular approach, including simultaneous detection of a wide range of carbapenemase types with the ability to distinguish between different genotypes, the high diagnostic accuracy, the rapid turnaround time (24 h) from laboratory receipt, and high (87–100%) agreement rates with phenotypic AST (Vanstone et al., 2018; Walker et al., 2019; Voulgari et al., 2020). Limitations described include the inability to detect novel AMR genotypes and resistance to newer βlactam/inhibitor combinations in isolates of *P. aeruginosa* (Evans et al., 2019).

In our study, this novel bacterial typing method was investigated as a potential tool for conducting routine veterinary infection control and hospital surveillance as a possible alternative to conventional typing methods. Importantly, typing nosocomial MDR pathogens in "real time" has the potential to improve the cost–benefit relationship for surveillance and infection control measures through early identification and swift implementation of control procedures. The comprehensive isolate characterization offered by the Acuitas® Resistome test provides an effective tool for guiding antimicrobial selection and aid in patient management, as shown in human hospitals for MDR and carbapenem-resistant *Enterobacterales, P. aeruginosa,* and *A. baumannii* (Reuben et al., 2017; Evans et al., 2019; Voulgari et al., 2020). To the best of our knowledge, this is the first application of this test to veterinary infection control; our results using veterinary ESC-R GNs suggest that this technology has great potential to provide full bacterial pheno- and genotyping in a very short timeframe.

It would be beneficial to compare our findings to those obtained from larger veterinary hospital populations as we acknowledge sample size (i.e., number of patients) is a limitation of the present study. However, one limitation of this tool applied in the veterinary setting may lie in the fact that it is designed to accurately cluster clonally related pathogens carrying multiple resistance genes (Lin et al., 2015; Walker et al., 2019; Voulgari et al., 2020). Therefore, this technique may lack resolution for the detection of clonal dissemination of pathogenic bacteria harboring a reduced arsenal of AMR genes, such as OXA-50 and OXA-51-producing *P. aeruginosa* and *A. baumannii*, respectively. Also, dissemination of bacterial clones which may be virulent but not associated with known resistance genes remains undetected (Petrova et al., 2019). Furthermore, this technology is currently not cost-effective or available to perform on-site in the routine diagnostic laboratory; testing is only performed at centralized facilities in the United States and, despite the rapid test turnaround times (as early as 24 h from sample receipt) and easy online access to results in real time, there may be the delay in results generation due to sample shipping.

In conclusion, we report a high prevalence of ESC-R GN organisms, particularly of the ESKAPE group of pathogens, amongst clinical, colonization and environmental samples collected at two UK veterinary hospitals with emphasis on their ICUs (equine and small animal). This included the detection of resistance to last-resort antimicrobials (i.e., carbapenems) carried by four isolates. The Acuitas® Resistome test is a useful technology for veterinary infection control purposes, allowing to track intra-hospital dissemination of specific genotypes as suspected here for *E. cloacae* and *K. pneumoniae* in the equine and small animal hospital ICUs, respectively. Possible inter-hospital spread of certain ESKAPE genotypes was also detected, which may be consistent with staff or student movement across hospitals. Nevertheless, further typing is necessary to confirm the spread of genetic types, especially for those carrying comparably less AMR genes than their human nosocomial counterparts; for this reason, the Acuitas® Resistome test may be more beneficial in human rather veterinary hospital settings at present. Further research is warranted to investigate the occurrence and molecular epidemiology of ESKAPE GNs within veterinary hospitals and the correlations these have with veterinary HCAIs, as such pathogens may be more widespread in veterinary settings than currently acknowledged, in similar but less alarming trends than seen in human hospitals.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by University of Liverpool Veterinary Research Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

CI and JD supported sample collection in the equine hospital, whilst VS and RR in the small animal hospital. FZ processed the hospital samples and wrote the manuscript together with DT. All authors read and approved the manuscript for submission.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1252216/full#supplementary-material

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Occurrence and temporal distribution of extended-spectrum β-lactamase-producing *Escherichia coli* in clams from the Central Adriatic, Italy

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The spread of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* is a major public health issue. Bivalves are filter-feeder animals capable of bioaccumulating the microorganisms present in water. This physiological characteristic makes them both good indicators of environmental contamination and possible carriers of pathogenic bacteria, including those resistant to antimicrobials. The aim of this study was to investigate the occurrence of ESBL-producing *E. coli* in clams (n=308) collected from harvesting areas of the Central Adriatic Sea between 2018 and 2019. ESBL- /class C β-lactamase (AmpC)- producing *E. coli* and *Escherichia* spp. were isolated by streaking over the surface of MacConkey agar plates supplemented with cefotaxime enriched broths of the initial shellfish suspension. *E. coli* and *Escherichia* spp. resistant to cefotaxime were screened for ESBL production by using the double disk synergy test. Susceptibility to different antimicrobials and confirmation of ESBL-production were determined by the minimum inhibitory concentration (MIC) test. Isolates were further characterized by whole genome sequencing (WGS) and bioinformatic analysis of genomes with different tools. Overall, ESBL-producing *E. coli* were isolated from 3% of the samples. Of 13 ESBL- and ESBL–/AmpC-producing *Escherichia* spp. (n=11 *E. coli*, n=1 *E. marmotae*, n=1 *E. ruysiae*) isolates, 13 were resistant to ampicillin and cefotaxime, 9 to sulfamethoxazole, 6 to tetracycline and nalidixic acid, 4 to trimethoprim, and 3 to ceftazidime, cefoxitin, ciprofloxacin, and chloramphenicol. Moreover, the majority (8/11) of the ESBL-producing *E. coli* isolates were multidrug-resistant. WGS showed that the isolates predominantly carried the $bla_{\text{CTX-M-15}}$ gene (3/11) and $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-1}}$ (2/11 each). The AmpC β-lactamase CMY-2 was found in two isolates. Phylogroup A was the most prevalent (5/11), followed by phylogroups D (4/11), F (1/11), and B2 (1/11). Ten different sequence types (STs) were identified. Occurrence at sampling sites ranged between 0 and 27%. To identify associations between the occurrence of ESBL-producing *E. coli* and *E. coli* levels, samples were divided into two groups, with *E. coli* at >230 MPN/100g and *E. coli* at ≤230 MPN/100g. ESBL-producing *E. coli* isolates were significantly more commonly recovered in samples with higher *E. coli* levels (14%) than in those with lower levels of *E. coli* (2%). Moreover, the majority (3/4) of the potentially pathogenic

strains were isolated in samples with higher *E. coli* levels. These findings provided evidence for the bacterial indicator of fecal contamination, *E. coli*, as an index organism for ESBL-producing *E. coli* isolates in bivalves.

KEYWORDS

bivalves, *Escherichia coli*, ESBL, antimicrobial resistance, CTX-M, clam, *Escherichia ruysiae*, *Escherichia marmotae*

Introduction

Antimicrobial resistance (AMR) is one of the most significant public health threats (World Health Organization, 2021), responsible for hundreds of thousands of estimated deaths annually worldwide (O'Neill, 2016).

Third/fourth/fifth-generation cephalosporins are the highest priority critically important antimicrobials (HPCIAs) in human medicine (World Health Organization, 2019), and Enterobacteriaceae producers of extended-spectrum β-lactamases (ESBLs) are on the critical-priority WHO list of antibiotic-resistant bacteria for research and development of new antibiotics (Tacconelli et al., 2018). An 8-fold increase in the intestinal carriage rate of ESBL *Escherichia coli* in the community has occurred globally over the past two decades (Bezabih et al., 2021), and the global and regional human intestinal carriage of ESBL *E. coli* is increasing in both community and healthcare settings (Bezabih et al., 2022). The spread of ESBL poses a serious threat to public health; therefore, it is important to investigate sources and transmission routes and encourage studies contributing to the "One-Health" approach.

Human- and animal-gut bacteria, including those resistant to antimicrobials, can reach marine environments through various routes (e.g., runoff from land, sewage systems, and feces from birds and wild animals), with the potential to contaminate seafood products. Bivalves are invertebrate filter-feeder animals capable of bioaccumulating microorganisms present in the surrounding waters. Thus, they are good indicators of environmental contamination and may act as possible carriers of bacteria derived from fecal pollution (Lee and Silk, 2013), including those that are resistant to antimicrobials (Albini et al., 2022).

In the European Union, regulation concerning the sanitary safety of live bivalve mollusks (Anonymous, 2004, 2019) stipulates that classified production areas shall be periodically monitored to check the microbiological quality of shellfish by using the bacteriological indicator of fecal contamination, *E. coli*. The latter is a genetically diverse species that comprises non-pathogenic gut commensals and strains responsible for intestinal and extra-intestinal disease. Enterotoxigenic *E. coli* (ETEC) strains are able to bind and colonize the intestinal epithelium and also produce various enterotoxins, of which heat-labile and heat-stable toxins and/or enteroaggregative heat-stable toxin 1 (EAST1) lead to diarrhea. Extra-intestinal pathogenic *E. coli* (ExPEC) are non-commensal *E. coli* isolates capable of causing extra-intestinal disease due to the possession of pathogenic virulence factors (Russo and Johnson, 2000). *Escherichia coli* isolates containing at least two genes coding virulence factors (*papA* and/or *papC*, *sfa*/*foc*, *afa*/d*raBC*, *kpsM II*, and *iutA*) are defined as ExPEC (Peirano et al., 2013).

Antimicrobial resistance monitoring programs in the EU are focused on terrestrial animals (Aerts et al., 2019). Studies on the occurrence of ESBL-producing *E. coli* in bivalves are limited. The prevalence of ESBL-producing *E. coli* has been investigated in retail bivalves in studies conducted in European or North African countries (Boss et al., 2016; Vu et al., 2018; Sola et al., 2022). In European studies, ESBL- or AmpC-producing *E. coli* isolates were not recovered in retail oysters sampled in Switzerland (Boss et al., 2016), and ESBL-producing Enterobacteriaceae were isolated in 20% of bivalve samples collected at retail in Berlin (Vu et al., 2018). In another study conducted in Tunisia, bivalves were sampled in unrelated markets in different regions, and ESBL-producing Enterobacterales (mostly *E. coli*) were cultured from 1.6% of clam pools (Sola et al., 2022). Other studies investigated the occurrence of ESBL-producing *E. coli* isolates in bivalves from production areas (Rees et al., 2015; Bueris et al., 2022). Hence, there are relatively few studies assessing the occurrence of ESBL-producing *E. coli* in bivalves from production areas over different seasons, and none of them were carried out in Italy. Furthermore, to our knowledge, previous studies have not investigated the relationship between levels of the bacterial indicator of fecal contamination, *E. coli*, and ESBL-producing *E. coli* presence in bivalve mollusks.

Clam is a major commercial species in Italy, and among EU countries, Italy is the main producer, accounting for 77% of farmed clams in the EU, reaching 24,452 tons in 2020 (European Commission, 2022). As clams are grown in coastal waters, they can also represent a sentinel species in determining AMR in the marine environment.

The aim of this study was to investigate the occurrence of ESBL-producing *E. coli* in clams collected from harvesting areas of the Central Adriatic Sea between 2018 and 2019 and study the correlation between ESBL-producing *E. coli* and levels of *E. coli*, the bacterial indicator of fecal contamination of bivalve mollusks. Moreover, EBSL- and ESBL-/AmpC-producing *E. coli* isolates from bivalves were characterized phenotypically, for susceptibility to antimicrobials, and genotypically, by whole-genome sequencing (WGS), to assign them to a serotype, phylogroup, sequence type (ST), and identify the presence of resistance and virulence genes and mutations that confer antimicrobial resistance.

Materials and methods

Sampling

A total of 308 samples of clams (*Venus gallina*), collected from 28 sampling sites of harvesting areas located along the coast of the region of Marche (Supplementary Appendixes 1, 2), were analyzed for the presence of *E. coli*-producing ESBL and/or class C β-lactamase (AmpC). Of these, 127 were from bivalve mollusk harvesting areas classified as B, which requires a post-harvest treatment before being placed on the market to meet *E. coli* health parameters. The remaining 181 samples were from areas classified as A.

Monitoring for the presence of *E. coli* ESBL/AmpC was performed approximately each month between 2018 and 2019 for the majority (25) of the areas.

Bivalve mollusks were externally cleaned with running potable water; then, the flesh and liquor of the bivalve mollusks were aseptically collected, diluted, homogenized, and further diluted in a 0.1% sterile peptone water to achieve a final suspension of 1:10. Subsequent decimal dilutions were prepared in a 0.1% sterile peptone solution. *E. coli* enumeration on bivalve mollusks was performed by a most probable number (MPN) method according to ISO 16649-3 (Anonymous, 2015).

ESBL-/AmpC-producing *E. coli* were isolated by streaking over the surface of MacConkey (MC) agar plates supplemented with 1 μg/mL of cefotaxime (Sigma Aldrich-Merck KGaA, Darmstadt, Germany) enriched broths of the initial shellfish suspension in double-strength mineral-modified glutamate (MMGB) broth from the *E. coli* enumeration method. Inoculated MC agar plates with cefotaxime were incubated at 37° C ± 1° C for 24 h ± 2 h in aerobic conditions. Two colonies showing typical characteristics of *E. coli* were randomly selected from each sample and isolated in trypticase soy agar (TSA, Biolife, Italy).

Escherichia coli identification

Presumptive colonies of *E. coli* were analyzed by PCR for the *uidA* gene with primers uidA-277F and uidA-934R[1] and by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS Biotyper, Bruker Daltonics) analysis.

Antimicrobial susceptibility testing and ESBL/AmpC phenotype

For the isolates identified as *E. coli*, disk diffusion susceptibility tests (EUCAST, 2017a) were conducted for nine antibiotics (Supplementary Appendix 2). Inhibition diameter sizes were interpreted by using the EUCAST breakpoint tables (EUCAST, 2018), except for nalidixic acid and tetracycline, for which CLSI breakpoint values were used (CLSI, 2019).

E. coli isolates resistant to cefotaxime were screened for ESBL production by using the double disk synergy test (DDST) (EUCAST, 2017b).

For each sample, according to antimicrobial resistance screening results, one *E. coli* isolate was selected and further investigated for the determination of the minimum inhibitory concentration (MIC) for different antimicrobial classes and for the confirmation of ESBL production. If differences were observed in the antimicrobial susceptibility profiles of *E. coli* isolates from the same sample, each isolate underwent MIC tests. MIC tests were performed with Sensititre EU Surveillance *Salmonella*/*E. coli* EUVSEC Plates and Sensititre EU Surveillance ESBL EUVSEC2 Plates (Thermo Fisher Scientific), according to the Thermo Scientific Sensititre Plate Guide for Antimicrobial Susceptibility Testing (Thermo Fisher Scientific).

Clinical breakpoints provided by the Clinical and Laboratory Standards Institute (CLSI, 2021) were used for the interpretation of MICs (S: susceptible, I: intermediate, SDD: susceptible-dose dependent, and R: resistant) of the following antimicrobials: ampicillin (AMP), cefepime (FEP), cefotaxime (FOT), cefoxitin (FOX), ceftazidime (TAZ), ertapenem (ETP), imipenem (IMI), meropenem (MER), colistin (COL), gentamicin (GEN), ciprofloxacin (CIP), trimethoprim (TMP), tetracycline (TET), chloramphenicol (CHL), sulfamethoxazole (SXT), and nalidixic acid (NAL). For tigecycline (TGC) and temocillin (TRM), for which no clinical breakpoints were available from CLSI, EUCAST clinical breakpoint tables (EUCAST, 2023) were used for MIC interpretation (S: susceptible and R: resistant). In the case of azithromycin (AZI), for which no clinical breakpoint was defined, the epidemiological cutoff (ECOFF) value of 16 mg/L (EUCAST, 2023) was used for the classification of *E. coli* isolates as susceptible/non-susceptible. Isolates were considered ESBL if ≥8-fold reduction was observed in the MIC of any of the cephalosporins (cefotaxime or ceftazidime) combined with clavulanic acid compared with the MIC of that cephalosporin alone (EUCAST, 2017b). Isolates resistant to cefoxitin and cefepime, negative to the synergy test, were characterized as ESBL based on genetic characterization.

Multidrug resistance (MDR) was considered when isolates were resistant to three or more antimicrobial classes (Magiorakos et al., 2012).

DNA extraction and whole-genome sequencing

Genomic DNAs were extracted from 1 mL of logarithmic phase broth cultures from pure *E. coli* cultures by using the QIAamp DNA Mini Kit (Qiagen Inc., Hilden, Germany) following the manufacturer's protocol for Gram-negative bacterial organisms. DNA was quantified with the Qubit fluorometer (QubitTM DNA HS Assay, Life Technologies, Thermo Fisher Scientific Inc.). DNA libraries were prepared by using the Nextera DNA Flex Library Prep Kit (Illumina Inc., San Diego, CA), according to the manufacturer's manual, loaded onto NextSeq 500/550 Mid Output Reagent Cartridge v2, 300 cycles kit (Illumina Inc., San Diego, CA) and then sequenced on an Illumina NextSeq 500 platform, to generate 150 bp paired-end reads.

Sequence analysis

Raw data were checked for quality, trimmed using Trimmomatic v0.36 (Bolger et al., 2014), and assembled using SPAdes genome assembler v3.11.1 (Bankevich et al., 2012). Quality checks of raw data and assembled genomes are reported in the Supplementary Appendix 3, 4.

The assembled genomes were analyzed by online tools available at the Center for Genomic Epidemiology (CGE), Technical University of Denmark.[2] Briefly, the FASTA files were analyzed using the following CGE databases: ResFinder (v.4.1) for antimicrobial resistance genes (ARGs) and chromosomal point mutations associated with resistance (Camacho et al., 2009; Zankari et al., 2017; Bortolaia et al., 2020), multilocus sequence typing (MLST v.2.0.9) for defining the ST (Lemee et al., 2004; Bartual et al., 2005; Wirth et al., 2006; Jaureguy et al., 2008; Camacho et al., 2009; Griffiths et al., 2010; Larsen

1 STEC Reference Center, http://www.shigatox.net/new/tools/ecmlst.html.

2 DTU, https://www.genomicepidemiology.org/services/.

et al., 2012), PlasmidFinder (2.0.1) for plasmid replicons (Camacho et al., 2009; Carattoli et al., 2014), VirulenceFinder (2.0.3) for virulence determinants (Camacho et al., 2009; Joensen et al., 2015; Malberg Tetzschner et al., 2020), and SeroTypeFinder (2.0) for serotyping (Joensen et al., 2015). Ribosomal multilocus sequence typing (rMLST, last updated 13 September 2022) at the Public databases for molecular typing and microbial genome diversity (PubMLST) was used for species identification (Jolley et al., 2012).

The presence of chromosomal mutations was evaluated based on the criteria that one single chromosomal mutation in the *gyrA* gene confers low-level resistance to quinolones, and several mutations in DNA gyrase genes (*gyrA* and *gyrB*) and topoisomerase IV genes (*parC* and *parE*) are required to increase the level of quinolone resistance in Enterobacteriaceae (Correia et al., 2017). ARG or plasmid replicons were considered present if length coverage and identity to the reference sequence were 100% and ≥ 95%, respectively. Virulence genes were considered present if length coverage and identity to the reference sequence were 100% and ≥ 90%, respectively.

Escherichia coli phylogroup and *Escherichia* clade assignment was performed *in silico* (Beghain et al., 2018) with ClermonTyping 21.03.[3]

To gain insight into the chromosomal or plasmid location of ESBL-/AmpC-encoding genes, assembled genomes were analyzed by MOB-suite (Robertson and Nash, 2018; v.3.0.3) to predict plasmid- and chromosome-derived sequences. Contigs harboring ESBL/AmpC-encoding genes were analyzed with ResFinder (v.4.1), PlasmidFinder (v2.0.1), and MobileElementFinder (v1.0.3; Johansson et al., 2021).

The raw sequencing data have been submitted to NCBI's Sequence Read Archive (SRA) repository (BioProject: PRJNA882336, BioSample accessions SAMN30930934 to SAMN30930946).

Statistical analysis

Quantitative *E. coli* results were divided into two groups based on the level of fecal contamination (*E. coli* ≤ 230 MPN/100 g and *E. coli* > 230 MPN/100 g). Statistical analysis was performed with Fisher's test (Stata 16.1®), and values of $p < 0.05$ were considered statistically significant.

To study the seasonality of ESBL *E. coli* in clams, samples were categorized as summer (21st of June to 22nd of September)–autumn (23rd of September to 20th of December), and winter (21st of December to 20th of March)–spring (21st of March to 20th of June), according to the season of collection.

Results

Occurrence of ESBL-and ESBL/AmpC-producing *Escherichia coli* and other ESBL-producing *Escherichia* species isolates in clam samples

Overall, ESBL-producing *E. coli* isolates were cultured from 10 (3%, C.I.: 2–6%) of the 308 clam samples collected between July 2018 and November 2019 from the 28 sampling points. Of these, six (2%, C.I.: 0.7–4%) and three (1%, C.I.: 0.2–3%) samples harbored ESBL- or ESBL-/AmpC-producing *E. coli* isolates, respectively, while both types of isolates were recovered from one sample. Of note, other ESBL-producing *Escherichia* species were isolated, specifically *E. ruysiae* from one sample, and *E. marmotae*, from another sample, which also harbored an isolate of ESBL-/AmpC-producing *E. coli*. The latter species were presumably identified as *E. coli*, by PCR for the *uidA* gene and MALDI-TOF, and subsequently as *E. marmotae* and *E. ruysiae* by rMLST (100% with 53 exact matches) of WGS data.

Antimicrobial resistance phenotype of ESBL- and ESBL-/AmpC-producing *Escherichia coli* and other ESBL-producing *Escherichia* spp.

Distribution of MIC values among the 13 *Escherichia* spp. isolates is reported in Table 1. Overall, all 13 ESBL- and ESBL-/AmpC-producing *Escherichia* spp. ($n = 11$ *E. coli*, $n = 1$ *E. marmotae*, $n = 1$ *E. ruysiae*) isolates showed resistance to ampicillin and cefotaxime, while 2 and 3 isolates were resistant to cefepime and ceftazidime, respectively, and 4 isolates had intermediate susceptibility to ceftazidime. Resistance and intermediate susceptibility to cefoxitin were found in 3 and 1 of the 13 isolates, respectively. Moreover, resistance to non-beta-lactam antibiotics was also observed to nalidixic acid (6/13), tetracycline (6/13), chloramphenicol (3/13), trimethoprim (4/13), sulfamethoxazole (9/13), gentamicin (2/13), and azithromycin (2/13). Of note, resistance to ciprofloxacin was found in three of the isolates. All (13 out of 13) isolates showed susceptibility to carbapenems (ertapenem, imipenem, and meropenem). Additionally, all isolates were susceptible to tigecycline, colistin, and temocillin. The majority (8 out of 11) of the ESBL- or ESBL-/AmpC-producing *E. coli* isolates were MDR (Table 2). The ESBL-producing *E. marmotae* was resistant to ampicillin and cefotaxime (Table 2), whereas the *E. ruysiae* was resistant to ampicillin, cefotaxime, and sulfamethoxazole and intermediate- and susceptible-dose dependent to ceftazidime and cefoperazone (Table 2).

Genomic analysis of the ESBL- and ESBL-/AmpC-producing *Escherichia coli*

Among the 11 sequenced *E. coli* isolates, ARGs were detected for beta-lactams ($n = 11$), fluoroquinolones ($n = 5$), tetracyclines ($n = 6$), aminoglycosides ($n = 8$), sulphonamides ($n = 6$), phenicols ($n = 4$), trimethoprim ($n = 4$), spectinomycin ($n = 4$), macrolides ($n = 3$), and lincosamide ($n = 1$; Table 2).

ESBLs were encoded in 3 out of 11 *E. coli* isolates from clams by the $bla_{\text{CTX-M-15}}$ gene; other common CTX-M variants were $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-1}}$ (2 out of 11 isolates each), whereas $bla_{\text{CTX-M27}}$ and $bla_{\text{CTX-M55}}$ were each present in 1 of the 11 isolates (Table 2). Other ESBL-producing genes found in the *E. coli* isolates from clams were the bla_{SHV12} gene (1 out of 11 isolates) and one bla_{TEM} gene that had a sequence identity of 99.8% to $bla_{\text{TEM-106}}$ (859/861 bp) and $bla_{\text{TEM-126}}$

3 http://clermontyping.iame-research.center

TABLE 1 Distribution of MIC (minimum inhibitory concentration) values among the 13 ESBL- or ESBL-/AmpC-producing *Escherichia coli* (11 isolates) and ESBL-producing *E. marmotae* (1 isolate) and *E. ruysiae* (1 isolate) from clams.

Antibiotic molecule	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024
Ampicillin													13 (100)				
Cefoxitin								1 (8)	3 (23)	5 (38)	1 (8)	2 (15)	1 (8)				
Ceftazidime							2 (15)	4 (31)		4 (31)	2(15)	1 (8)					
Cefotaxime									1 (8)	2 (15)	5 (38)	1 (8)	4 (31)				
Cefepime						1 (8)	1 (8)	2 (15)	4 (31)	3 (23)	1 (8)	1 (8)					
Tetracycline								7 (54)					6 (46)				
Tigecycline					11 (85)	2 (15)											
Meropenem		13 (100)															
Imipenem				6 (46)	7 (54)												
Ertapenem	6 (46)	4 (31)	2 (15)		1 (8)												
Ciprofloxacin	3 (23)	1 (8)		1 (8)	5 (38)					3 (23)							
Nalidixic acid									5 (38)	1 (8)	1 (8)		2 (15)	4 (31)			
Colistin							9 (69)	4 (31)									
Trimethoprim					6 (46)	3 (23)						4 (31)					
Chloramphenicol										10 (77)			1 (8)	2 (15)			
Gentamicin						4 (31)	7 (54)				1 (8)	1 (8)					
Sulfamethoxazole										4 (31)							9 (69)
Azithromycin								1 (8)	5 (38)	4 (31)	1 (8)		2 (15)				
Temocillin									1 (8)	9 (69)	3 (23)						

Percentages are shown in brackets. The shaded areas show the range of values tested for each antibiotic.

(859/861 bp), respectively. The combination of an ESBL (CTX-M-14 or a TEM enzyme with a gene sequence identity of 99.8% to $bla_{TEM-106}$ and $bla_{TEM-126}$) with a plasmidic AmpC β-lactamase (CMY-2) was found in two out of four of the isolates (Table 2). The remaining AmpC-producing *E. coli* isolates had mutations in the AmpC promoter (p.L9R, p.R8C, and p.R11Q; g.-28G>A, Table 2). The identified fluoroquinolone resistance genes were *aac(6′)-Ib-cr* and *qnrS1* in three and two isolates, respectively. Moreover, seven isolates had at least one point mutation known to mediate quinolone resistances in the chromosomal *gyrA*, while three isolates also possessed at least one additional mutation in the genes *parC*/*parE*. Tetracycline resistance genes *tetA*, *tetB*, and *tetM* were found in 6, 1, and 1 of the 11 isolates, respectively.

By the MOB-suite analysis of assembled genomes, 9 of the 11 contigs harboring ESBL-encoding genes and the two contigs harboring AmpC-encoding genes were classified as plasmid-derived sequences (Table 3; Supplementary Appendix 5). The presence of mobile elements and other antibiotic resistance genes in the same contigs harboring ESBL-/AmpC-encoding genes was also investigated and is reported in Table 3.

ESBL- and ESBL/AmpC-producing *E. coli* strains were diverse in serotype and fimbriae (Table 4). Most (9 out of 11) of the *E. coli* isolates were included in 6 different clonal complexes (Table 4); of these, CC10 and CC69 were present in 3 and 2 of the 11 ESBL and ESBL/AmpC *E. coli* isolates, respectively. Notably, the pandemic extra-intestinal pathogenic *E. coli* (ExPEC) ST131 clone (clonal complex CC131) was detected. Four phylogenetic groups (Table 5) were identified, of which phylogroup A was the most prevalent (45%), followed by phylogroup D (36%).

Most strains harbored a broad virulence repertoire; moreover, in 3 out of 11 of the ESBL- and EBSL-/AmpC-producing *E. coli* isolates, at least two genes encoding for ExPEC virulence factors (*papA* and/or *papC*, *kpsM II*, *iutA*, *afa*/*draBC*, and *sfa*/*foc*) were identified (Table 4).

Genomic analysis of the ESBL-producing *Escherichia* spp.

Among the 13 sequenced *Escherichia* spp., two isolates of ESBL producers were identified by rMLST (100% with 53 exact matches each) as *E. marmotae* and *E. ruysiae* and assigned by phylogroup analysis to clades V and III, respectively. The *E. marmotae* strain harbored the *astA* gene for the heat-stable enterotoxin 1 and other virulence genes (Table 4), whereas the ESBL was encoded by the $bla_{CTX-M-1}$ (Table 2). The latter was predicted to be located in a chromosome-derived sequence (Table 3, AN5). A new ST (14425) was identified in the *E. marmotae* strain (Table 4). The *E. ruysiae* strain was assigned to ST 3568 by MLST analysis. Several putative virulence genes were predicted from the genome sequence of the *E. ruysiae* strain, including the enterotoxin *astA* gene (Table 4). The ESBL was encoded by the $bla_{CTX-M-15}$ gene (Table 2), which was located in a contig classified as a plasmid-derived sequence (Table 3, AN9).

Occurrence of ESBL- and ESBL-/AmpC-producing *Escherichia coli* isolates and seasonality at sampling points

Over the studied period, the prevalence of ESBL- (including ESBL- and AmpC-)-producing *E. coli* isolates (Figure 1) ranged from 0% (no isolation) at 21 (75%) of the 28 sampling sites to 27% (3 out of 11 samples) in an area that was not suitable for the direct human consumption of bivalve mollusks.

Considering the seasonality, ESBL-producing *E. coli* were isolated in 3 of 85 (4%, C.I.95 1–10%), 3 of 62 (5%, C.I.95 1–14%), and 4 of 54 (7%, C.I.95 2–18%) of the samples collected in autumn, winter, and spring, respectively. Furthermore, ESBL-producing *E. coli* were not isolated from 107 samples of clams collected during the summer season.

TABLE 2 Phenotypic antimicrobial resistance (AMR) profile, resistance genes, and gene mutations for AMR of ESBL- and ESBL-/AmpC-producing *Escherichia* spp. isolates from clams.

Isolate No.	β-lactamase profile	Phenotypic AMR	Resistance genes and/or mutations							
			β-lactams	Quinolone and Fluoroquinolone	Tetracycline	Aminoglycoside	Sulf	Phenicol and trimethoprim	Spec	MLS
AN2	ESBL	AMP AZI (NS) FOT NAL TET SMX TMP (MDR)	***bla*$_{CTX-M-27}$**	*gyrA* (p.S83L)	***tet(A)***	***aph (3'')-Ib aph(6)-Id aadA5***	***sul1 sul2***	***dfrA17***	***aadA5***	***mph(A)***
AN9	ESBL	AMP FOT FEP (SSD) TAZ (I) SMX	***bla*$_{CTX-M-15}$**							
AN1	ESBL	AMP FOT TAZ CHL NAL TET SMX (MDR)	***bla*$_{SHV12}$**	*gyrA* (p.D87N)	*tet(A)* ***tet(B)***	***aadA1*** *aadA2b*	***sul3***	*cmlA1*	***aadA1*** *aadA2b*	
AN 5	ESBL	AMP FOT	***bla*$_{CTX-M-1}$**							
AN6	ESBL/AmpC	AMP FOT TAZ NAL TET FOX CIP (MDR)	***bla*$_{CMY-2}$ *bla*$_{OXA-1}$** *bla*$_{TEM-126}$/*bla*$_{TEM-106}$ *bla*$_{TEM-1B}$	***aac(6')-Ib-cr*** *parC (p.S80I)* gyrA (p.D87N) *gyrA* (p.S83L)	***tet(A)***	***aac(6')-Ib-cr***				
AN8	ESBL	AMP AZI (NS) FOT FEP (SSD)	***bla*$_{CTX-M-14}$**	*aac(6')-Ib-cr*		*aac(6')-Ib-cr aac(6')-Ib3*		*cmlA1*		***mph(A)***
AN3	ESBL	AMP FOT TMP TET SMX FEP (SSD) (MDR)	***bla*$_{CTX-M-1}$ *bla*$_{TEM-1B}$**	***qnrS1***	***tet(A)***	***aph(3'')-Ib aph(6)-Id*** *aadA2b*	***sul2***	***dfrA5***	*aadA2b*	***mph(A)***
AN4	ESBL/AmpC	AMP FOT TAZ (I), NAL FOX FEP (SSD)	***bla*$_{CTX-M-14}$ *bla*$_{CMY-2}$ *bla*$_{TEM-1B}$**	*gyrA* (p.S83L)						
AN13	ESBL/AmpC*	AMP FOT SMX FOX FEP (SSD) (MDR)	*bla*$_{CTX-M-1}$				***sul2***			
AN7	ESBL	AMP FOT CIP TAZ (I) CHL NAL TMP TET SMX FEP (SSD) (MDR)	***bla* $_{CTX-M-55}$ *bla*$_{TEM-1B}$**	*gyrA* (p.D87Y) g*yrA* (p.S83L) *parE* (p.S458A) *parC* (p.S80I)	*tet(A)*	***aph(3')-Ia aph(3'')-Ib aph(6)-Id***	***sul2***	*catA2* ***dfrA14***		
AN10	ESBL	AMP FOT TAZ (I) FEP SMX	***bla*$_{CTX-M-15}$ *bla*$_{TEM-35}$**	*gyrA* (p.S83A)						
AN11	ESBL/AmpC**	AMP FOT CIP TAZ NAL GEN SMX FOX (I) FEP (MDR)	***bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$ *bla*$_{TEM-1B}$**	***aac(6')-Ib-cr*** *gyrA* (p.D87N) *gyrA* (p.S83L) *parC* (p.S80I) *parC* (p.E84V) *parE* (p.I529L)		***aac(6')-Ib-cr*** *aac(3)-IIa*				
AN12	ESBL	AMP FOT CHL TMP TET GEN SMX FEP (SSD) (MDR)	***bla*$_{CTX-M-15}$ *bla*$_{TEM-1B}$**	***qnrS1***	*tet(M) tet(A)*	***aph(3')-Ia aph(3'')-Ib*** *aph(6)-Id* ***aadA1*** *aac(3)-IId*	***sul2 sul3***	*cmlA1* ***dfrA14 dfrA12***	***aadA1***	***lnu(F)***

*Mutations in the ampC-promoter: ampC-promoter: p.L9R, ampC-promoter:p.R8C, ampC-promoter:p.R11Q. **Mutation in the ampC-promoter: ampC-promoter:g.-28G > A. Genes indicated in black and bold were identified with 100% length coverage and 100% sequence identity. Genes indicated in black were identified with 100% length coverage and identity ≥ 95%. In brackets: SDD, susceptible dose-dependent; I, intermediate; NS, non-susceptible; Sulf, Sulfonamide; Spec, spectinomycin; MLS, macrolides–lincosamides–streptogramins.

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TABLE 3 Predicted genomic location (plasmid or chromosome) of ESBL- and AmpC-encoding genes using the MOB-Suite and presence of mobile genetic elements (MGEs) and other antimicrobial resistance genes in the same contig.

Isolate No.	ESBL-/AmpC-genes	ESBL/AmpC Contig ID and length	ESBL-/AmpC-gene position in Contig	MOB-Suite Contig classification	MGE in Contig*	Other genes
AN1	bla_{SHV12}	NODE 108; 3,167 bp	2157.3017	Plasmid		
AN2	$bla_{CTX-M-27}$	NODE 61; 1,513 bp	248.1123	Plasmid		
AN3	$bla_{CTX-M-1}$	NODE 42; 3,843 bp	2598.3473	Plasmid		
AN4	$bla_{CTX-M-14}$	NODE 149; 1,558 bp	564.1439	Plasmid		
	bla_{CMY-2}	NODE 84; 9,387 bp	6989.8134	Plasmid	ISEc9	
AN 5	$bla_{CTX-M-1}$	NODE 15; 101,323 bp	84738.85613	Chromosome	ISEc9	
AN6	bla_{CMY-2}	NODE 44; 32,775 bp	17810.18955	Plasmid	ISEc9, IncI1	
	$bla_{TEM-126}$/ $bla_{TEM-106}$**	NODE 52; 19,233 bp	14392.15252	Plasmid	Tn2, IncX1	
AN7	$bla_{CTX-M-55}$	NODE 1; 567,011 bp	152290.153165	Chromosome		
AN8	$bla_{CTX-M-14}$	NODE 39; 13,443 bp	11952.12827	Plasmid	IS6100	*mphA, cmlA1, aac(6′)-Ib-cr aac(6′)-Ib3*
AN9	$bla_{CTX-M-15}$	NODE 33; 5,012 bp	1898.2773	Plasmid	ISEc9	
AN10	$bla_{CTX-M-15}$	NODE 9; 166,240	3287.4162	Chromosome	ISEc9	
AN11	$bla_{CTX-M-15}$	NODE 43; 4,019 bp	432.1307	Plasmid		
AN12	$bla_{CTX-M-15}$	NODE 69; 14,059 bp	1920.2795	Plasmid		
AN13	$bla_{CTX-M-1}$	NODE 125; 4,978 bp	1226.2101	Plasmid		

*ISEc9: 100% (1656/1656 bp) sequence identity to GenBank accession number AJ242809; Tn2: 99.8% (4949/4950 bp) sequence identity to GenBank accession number HM749967; IncI1: 100% sequence identity to GenBank accession number AP005147; IncX1: 98.4% sequence identity to GenBank accession number EU370913; IS6100: 100% (880/880 bp) sequence identity to GenBank accession number X53635. **bla_{TEM} gene with a sequence identity of 99.8% to $bla_{TEM-106}$ (859/861 bp) and $bla_{TEM-126}$ (859/861 bp).

TABLE 4 Phylogroup, sequence type (ST), clonal complex (CC), replicon type, serotype, and virulence genes of ESBL- and ESBL-/AmpC-producing *Escherichia* spp. isolates from clams.

Isolate No.	β-lactamase profile	Phylogroup	ST No. (CC No.)	Replicon Type	Serotype	Virulence genes
AN2	ESBL	D	7401 (CC 69)	**IncFIA IncX4 IncFII(pRSB107)** IncFIB(AP001918) IncI2(Delta)	**H18**:O15	***chuA eilA fyuA gad irp2 iss*** *terC* ***ipfA ompT kpsE*** *kpsMII*
AN9	ESBL	Clade III	3568	**IncI1-I(Alpha) IncI2** IncFII(pHN7A8)	H56:*O36*	***astA iss traT*** *ompT sitA chuA gad terC kpsE kpsMII_K5*
AN1	ESBL	A	398 (CC 398) ETEC	IncFIB(AP001918) IncFII IncX1 **IncI1-I(Gamma)**	**H20**:O8	***astA cmA gad hlyF iss iroN sitA traT*** *ompT terC*
AN 5	ESBL	V	14425	IncFIB (AP001918) IncFII(29)	H56:*O103*	***astA*** *hra traT chuA terC*
AN6	ESBL/AmpC	A	167 (CC 10)	**IncI1-I(Alpha)** Col156 IncFIB IncFIA (AP001918) IncFII IncX1 IncX4 p0111	H9:**O101**	***irp2 cib celb fyuA gad iucC iutA senB sitA traT*** *terC capU iss*
AN8	ESBL	A	10 (CC 10)	**IncFII** Col156	H9:**O9a**	***irp2 terC*** *cea fyuA iss*
AN3	ESBL	D	69 (CC 69) ExPEC	**IncFII** IncFIB (AP001918)	**H18**O15	***air chuA cia cvaC eilA etsC fyuA gad hlyF iroN irp2 iss iutA kpsE lpfA*** *terC* ***kpsMII_K5 mchF*** o***mpT sitA traT*** *iha iucC*
AN4	ESBL/AmpC	A	10 (CC 10)	**Col(KPHS6)** Col156 IncB/O/K/Z IncFII IncFII(pHN7A8) IncI2 (Delta)	H10:**O29**	***celb fyuA irp2 iucC iutA mcbA mchC terC traT*** *mchF sigA*
AN13	ESBL/AmpC	D	1299	IncFIB(AP001918) IncFII(pCoo) IncFII(pSE11) IncX1	H14:O175	***astA*** *chuA gad hra terC*
AN7	ESBL	F	457 ExPEC	IncFIB(AP001918)	O11	***cea chuA cma iss iucC iutA kpsMII ompT sitA traT yfcV*** *eilA gad hra terC kpsE lpfA papA papC*
AN10	ESBL	D	38 (CC 38)	Col156 IncFIB(AP001918) IncFII	**H18**:O86	***chuA fyuA irp2 iss kpsE senB sitA*** *eilA hra terC*
AN11	ESBL/AmpC	B2	131 (CC 131) ExPEC	IncFIB(AP001918) IncFII IncFIA	**H4**:O25	***chuA cnf1 fyuA hrA ihA irp2 iss iucC iutA kpsE kpsMII_K5 ompT papC sat sitA traT yfcV*** *terC gad*
AN12	ESBL	A	46 (CC 46)	IncFIB (AP001918) IncFIB(H89-PhagePlasmid)	H4:O8	***traT*** *iss terC*

Plasmid replicons were considered present if length coverage and identity to the reference sequence were 100% and ≥ 95%, respectively. Virulence genes were reported if length coverage and identity to the reference sequence were 100% and ≥ 90%, respectively. Genes indicated in black were identified with 100% coverage length and identity ≥ 95% for serotype. Genes indicated in black and bold were identified with 100% length coverage and 100% sequence identity. ExPEC, Extra-intestinal pathogenic *Escherichia coli*; ETEC, Enterotoxigenic *E. coli*.

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TABLE 5 Phylogroups of 11 isolates of *E. coli* producers of ESBL or ESBL/AmpC from clam samples.

Phylogroup	ESBL (%)	AmpC (%)	ESBL/AmpC (%)	No. of isolates (%)
A	3 (43%)	0	2 (50%)	5 (45%)
B2	0 (0%)	0	1 (25%)	1 (9%)
D	3 (43%)	0	1 (25%)	4 (36%)
F	1 (14%)	0	0	1 (9%)
Total No. of isolates	7	0	4	11

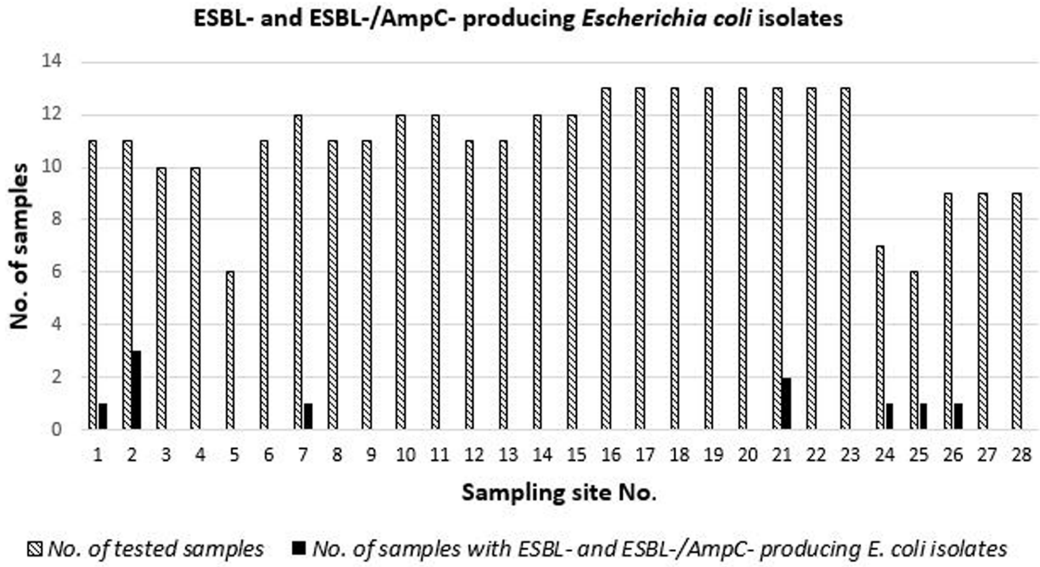

FIGURE 1
Occurence of ESBL-producing *E. coli* isolotes at 28 sampling sites of the Central Adriatic in 308 samples of clams collected between July 2018 and November 2019.

The occurrence of ESBL-/ESBL- and AmpC-producing *E. coli* STs at the sampling sites is reported in Table 6. Variability of STs and resistance to antimicrobials were found in strains isolated from clams sampled over time. One site (sampling point 2) had the greatest variability, with isolates of different STs harboring different ESBL-producing genes (Table 6).

Escherichia coli Levels and ESBL-producing *Escherichia* isolates in clams

The fecal indicator *E. coli* was quantified (MPN/100 g) in the 308 clam samples. *E. coli* numbers of clam samples were grouped into two *E. coli* contamination levels (<230 MPN/100 g and ≥ 230 MPN/100 g) and were further divided depending on the detection or not of ESBL-producing *E. coli* isolates (Table 7). ESBL-producing *E. coli* isolates were significantly more likely to be present ($p = 0.008$) among clam samples with *E. coli* > 230 MPN/100 g (4 out of 28 samples, 14% C.I.95: 5–33%) than in those with *E. coli* ≤ 230 MPN/100 g (6 out of 280 samples, 2% C.I.95: 1–5%).

The ETEC and two of the three ExPEC strains were isolated from samples with *E. coli* contamination levels >230 MPN/100 g. The majority (7 out of 11, 63%) of ESBL- and ESBL/AmpC-producing *E. coli* isolates were recovered from samples of areas classified as not suitable for direct human consumption.

Discussion

Gut bacteria from humans or terrestrial animals, including those resistant to antimicrobials, can enter aquatic environments through various routes. Thus, bivalves, which are filter-feeder animals, may act as possible carriers of bacteria derived from fecal pollution. Antimicrobial resistance monitoring programs in the EU focus on terrestrial animals, while studies on the presence of ESBL-producing bacteria in seafood products are limited.

We report here on the presence of ESBL-producing *E. coli* strains in bivalves collected in Italy between 2018 and 2019 from sampling points of production areas of the Central Adriatic Sea. To our knowledge, this is one of the few studies performed worldwide to investigate the occurrence of ESBL-producing *E. coli* strains in bivalves from production areas (Rees et al., 2015; Bueris et al., 2022). Moreover, this study investigated the relationship between levels of *E. coli*, the bacterial indicator of fecal contamination, and the presence of *E. coli* strains with an ESBL phenotype in bivalves. Overall, *E. coli* producers of ESBL were recovered from 3% of clam samples collected at several

TABLE 6 ESBL- (ESBL-/ESBL- and AmpC-) producing *E. coli* recovered from 308 samples of clams collected from harvesting areas of the Central Adriatic between July 2018 and November 2019.

Sampling point	Sample No.	Season	Phylogroup	ST	Phenotypic Resistance	Resistance genes
SP 1	AN2	Autumn	D	7401	AMP, AZI (NS), FOT, NAL, TET, SMX, TMP (MDR)	$bla_{CTX-M-27}$, *tet(A), aph (3″)-Ib, aph(6)-Id, sul1, sul2, dfrA17, aadA5, mph(A)*
SP 2	AN6	Winter	A	167	AMP, FOT, TAZ, NAL, TET, FOX, CIP (MDR)	$bla_{TEM-126}$/$bla_{TEM-106}$*, bla_{CMY-2}, bla_{OXA-1}, *aac(6′)-Ib-cr, tet(A),* bla_{TEM-1B}
	AN8	Winter	A	10	AMP, AZI (NS), FOT, FEP (SSD)	$bla_{CTX-M-14}$, *aac(6′)-Ib-cr, aac(6′)-Ib3, cmlA1, mph(A)*
	AN1	Autumn	A	398	AMP, FOT, TAZ, CHL, NAL, TET, SMX (MDR)	bla_{SHV12}, *tetB, tetA, aadA1, aadA2b, sul3, cmlA1*
SP 7	AN10	Spring	D	38	AMP, FOT, TAZ (I), FEP, SMX	$bla_{CTX-M-15}$, bla_{TEM-35}
SP 21	AN13	Spring	D	1299	AMP, FOT, SMX, FOX, FEP (SSD) (MDR)	$bla_{CTX-M-1}$, *sul2*
	AN3	Autumn	D	69	AMP, FOT, TMP, TET, SMX, FEP (SSD) (MDR)	$bla_{CTX-M-1}$, bla_{TEM-1B}, *qnrS1, tet(A), aph(3″)-Ib, aph(6)-Id, aadA2b, sul2, dfrA5, mph(A)*
	AN4	Autumn	A	10	AMP, FOT, TAZ (I), NAL, FOX, FEP (SSD)	$bla_{CTX-M-14}$, bla_{CMY-2}, bla_{TEM-1B}
SP 24	AN12	Spring	A	46	AMP, FOT, CHL, TMP, TET, GEN SMX, FEP (SSD) (MDR)	$bla_{CTX-M-15}$, bla_{TEM-1B}, *qnrS1, tet(M), tet(A), aph(3′)-Ia, aph(3″)-Ib, aph(6)-Id, aadA1, aac(3)-IId, sul2, sul3, cmlA1, dfrA14, dfrA12, lnu(F)*
SP 25	AN11	Spring	B2	131	AMP, FOT, CIP, TAZ, NAL, GEN, SMX, FOX (I), FEP (MDR)	$bla_{CTX-M-15}$, bla_{OXA-1}, bla_{TEM-1B}, *aac(6′)-Ib-cr, aac(3)-IIa*
SP 26	AN7	Winter	F	457	AMP, FOT, CIP, TAZ (I), CHL, NAL, TMP, TET, SMX, FEP(SSD) (MDR)	$bla_{CTX-M-55}$, bla_{TEM-1B}, *tet(A), aph(3′)-Ia, aph(3″)-Ib, aph(6)-Id, sul2, catA2, dfrA14*

*bla_{TEM} gene with a sequence identity of 99.8% to $bla_{TEM-106}$ (859/861 bp) and $bla_{TEM-126}$ (859/861 bp).

TABLE 7 Presence/absence of ESBL- (ESBL- and ESBL-/AmpC-) producing *E. coli* isolates in 308 samples of clams according to the levels of *E. coli* (<230 MPN/100 g and ≥ 230 MPN/100 g).

E. coli MPN/100 g	No. of samples analyzed	No. of positive samples for ESBL-producing *E. coli* *(%)
>230	28	4 (14%)
≤230	280	6 (2%)
Total	308	10 (3%)

sampling sites in the studied period; of these, 2% and 1% harbored ESBL- and ESBL-/AmpC-producing *E. coli* isolates, respectively. Previous studies in bivalve production areas were conducted in other countries and were limited in the sample collection period (Rees et al., 2015; Bueris et al., 2022). In a study conducted in Canada (Rees et al., 2015), with a study period of 2 months, ESBL-producing *E. coli* isolates were not recovered from oysters harvested from sampling points of an open oyster fishery and a restricted zone for bivalves (Rees et al., 2015). In another study from Brazil, ceftriaxone-resistant *E. coli* isolates with an ESBL phenotype were cultured from edible bivalves (oysters and brown mussels) collected from three locations of a polluted area on the South American Atlantic coast (Bueris et al., 2022). Other studies reporting on the prevalence of ESBL-producing *E. coli* in bivalves were performed at retail in European countries (Boss et al., 2016; Vu et al., 2018). In the latter studies, *E. coli* producers of ESBL or AmpC were not recovered from retail sampled oysters ($n = 10$) in Switzerland (Boss et al., 2016), whereas in Germany, ESBL-producing Enterobacteriaceae were isolated in 20% of bivalve samples collected at retail level in Berlin, with the bivalves originating from several European countries, including Italy (Vu et al., 2018). Another study from North Africa reported a prevalence of 1.6% of ESBL-producing Enterobacterales (mostly *E. coli*) among 215 analyzed pools of 5 clams (*Ruditapes decussatus*), which were sampled in unrelated markets in four different regions of Tunisia (Sola et al., 2022).

All ESBL- or ESBL-/AmpC-producing *E. coli* isolated in this study from bivalves were resistant to cefotaxime, a third-generation cephalosporin, and, to a lesser extent, to ceftazidime (27%) and fourth-generation cephalosporin cefepime (18%). Moreover, resistance to fluoroquinolones, another antibiotic class recognized as the highest priority critically important antimicrobials (HPCIAs) in human medicine (World Health Organization, 2019), was recorded in 27% of the ESBL- or ESBL-/AmpC-producing *E. coli* isolates.

Other studies have reported the proportion of third- or fourth-generation cephalosporin-resistant isolates of *E. coli* relative to the total number of isolates recovered from bivalve samples from production areas (Vignaroli et al., 2016; Grevskott et al., 2017; Miotto et al., 2019; Jeong et al., 2021). However, a screening method of a selective medium with an antibiotic to isolate third-generation cephalosporin-resistant *E. coli* strains was not applied in these studies; therefore, the prevalence of ESBL in samples collected at harvesting areas was not determined. In a systematic review and meta-analysis on antimicrobial resistance in marine bivalves from our group, resistance to third/fourth/fifth-generation cephalosporins and fluoroquinolones was recorded in approximately 10% of *E. coli* isolates, while resistance to carbapenems was not reported in *E. coli* strains from bivalves (Albini et al., 2022). Accordingly, resistance to carbapenems (ertapenem, imipenem, and meropenem) was not found in the ESBL- and ESBL-/AmpC-producing *E. coli* strains we isolated from bivalves.

CTX-M-type enzymes are the most common global ESBL in *E. coli*; among these, CTX-M-15 is the most frequent, followed by CTX-M-14 (Peirano and Pitout, 2019). Noteworthy, a recent study performed in Italy has reported CTX-M types as prevalent in both ExPEC human and animal isolates, and among these, the CTX-M-15 enzyme is largely predominant in human isolates and in a consistent percentage of the isolates from different animal species (Giufrè et al., 2021). According to the same study, the second most common CTX-M enzyme in Italian isolates was CTX-M-27 in humans and CTX-M-1 in animals. Previous studies performed on bivalve mollusks

reported that the ESBL phenotype in *E. coli* was predominantly due to the presence of bla_{CTX-M} genes (Vu et al., 2018; Bueris et al., 2022; Sola et al., 2022), with the most frequent one detected in isolates from clams sampled at retail in Tunisia being $bla_{CTX-M-15}$, followed by $bla_{CTX-M-1}$ and $bla_{CTX-M-14}$ (Sola et al., 2022). In agreement with these studies, our analysis of ESBL genes revealed that the most prevalent gene found in *E. coli* isolates from bivalves of the Central Adriatic Sea in the studied period was the $bla_{CTX-M-15}$ gene, followed by $bla_{CTX-M-14}$ and $bla_{CTX-M-1}$, whereas $bla_{CTX-M27}$ and $bla_{CTX-M55}$ were less represented. Interestingly, other ESBL-producing genes were found in a minor percentage of the *E. coli* isolates from clams (bla_{SHV12} and bla_{TEM} genes). Additionally, 18% of ESBL-producing *E. coli* isolates from clams possessed the bla_{CMY-2} gene for the plasmidic class C β-lactamases. Of the latter, only the CMY-2 enzyme was identified in clams. Analysis of the chromosomal or plasmid location evidenced that the majority of ESBL- /AmpC-encoding genes were harbored in sequences classified as plasmid-derived by the MOB suite. The genomic location of ESBL- / AmpC-encoding genes on plasmids in *E. coli* from the marine environment is worrying from a public health perspective as plasmids play an important role in the horizontal transfer of resistance genes. Moreover, mobile genetic elements such as insertion sequences (IS*Ecp1*, synonym of ISEc9, and IS6100) and transposons (Tn2) were also found in the same contigs of the bla_{CTX-M} (in five isolates) and the bla_{CMY-2} genes (in two isolates).

Phylogenetic group analysis showed that phylogroup A was the most prevalent (45%) in ESBL- and ESBL/AmpC-producing *E. coli* isolates, followed by other phylogroups that include isolates associated with human extra-intestinal infections (D, F, and B2). Among the sequenced ESBL- and ESBL-/AmpC-producing *E. coli* isolates, a high genomic diversity (10 different STs in 11 isolates) was observed, yet some clinically important STs were identified (ST131, ST38, ST10, ST69, ST457, and ST398). Of these, *E. coli* ST131, ST69, and ST457 showed ExPEC status and carried bla_{CTX-M} variants. The *E. coli* ST131 isolate from clams was MDR and had genomic features of clade C of the pandemic *E. coli* ST131 lineage (Denamur et al., 2021), which is the most prevalent ExPEC clonal group isolated in extra-intestinal infections in humans (Nicolas-Chanoine et al., 2014). The ST131 clone has previously been reported in water environments (Colomer-Lluch et al., 2013; Nicolas-Chanoine et al., 2014; Jørgensen et al., 2017), influent (Nicolas-Chanoine et al., 2014; Jørgensen et al., 2017), effluent treated wastewaters of water treatment plants (Zhi et al., 2020; Sekizuka et al., 2022), and bivalves (Vignaroli et al., 2016; Bueris et al., 2022; Sola et al., 2022). The ST457 is a broad host range, globally disseminated diverse *E. coli* lineage that can cause human extra-intestinal disease (Nesporova et al., 2021). A study focusing on ST457 evidenced that Australian human clinical and silver gull strains were closely related, suggesting that ST457 was an emerging ESBL lineage with reservoirs in wildlife and food-producing animals (Nesporova et al., 2021). ST38, ST10, and ST69 found in clams in a study conducted in Italy were reported to be more frequently detected in both human and animal isolates (Giufrè et al., 2021). Moreover, ST10 was one of the most represented STs among isolates from cattle and pigs, while ST 69 was also largely represented in isolates from pigs (Giufrè et al., 2021).

In this study, other ESBL-producing *Escherichia* spp., *E. marmotae* (Liu et al., 2015) and *E. ruysiae* (Van der Putten et al., 2021), were identified. Phylogroup analysis had previously assigned these species to *Escherichia* cryptic clades V and III, respectively. Previous studies on *Escherichia* cryptic clades have speculated that these may represent environmentally adapted *Escherichia* lineages that may be more abundant outside the gastrointestinal tract of the host (Walk et al., 2009; Ingle et al., 2011). Cryptic lineages of *Escherichia* were unlikely to be detected in human fecal samples and were more abundant in animal feces, ranging from 3% to 8% in non-human mammals to 8–28% in birds (Clermont et al., 2011). *E. marmotae* isolated from the feces of wild rodents (*Marmota himalayana*) has been reported as a potential invasive pathogen (Liu et al., 2019). In addition, human-invasive infections caused by *E. marmotae* have recently been described, and this *Escherichia* species has only recently been identified as a new common pathogen because it can be easily misidentified as *E. coli* in routine diagnostic laboratories (Sivertsen et al., 2022). Like animal isolates from wild rodents (Liu et al., 2019) and clinical isolates from human-invasive infections (Sivertsen et al., 2022), the ESBL-producing isolate of *E. marmotae* from clams harbored the enterotoxin-encoding gene *astA*. Moreover, the *E. coli in silico* serotyper evidenced that it contained the *fliC*-H56 flagellar antigen gene, as did the human clinical isolates (Sivertsen et al., 2022). Available data on *E. marmotae* isolates from different sources showed infrequent occurrences of antimicrobial resistance (Sivertsen et al., 2022); isolates from reported human-invasive infections were phenotypically susceptible to tested antimicrobials, and resistance genes were not identified in their genomes (Sivertsen et al., 2022). Differently from these findings, the *E. marmotae* strain from clams was an ESBL-producing isolate, phenotypically resistant to cefotaxime, which possessed the $bla_{CTX-M-1}$ gene.

Escherichia ruysiae sp. nov. was proposed by van der Putten et al. (2021) as a novel species, encompassing *Escherichia* cryptic clades III and IV. The strain of *E. ruysiae* was isolated from the fecal material of an international traveler, harbored the $bla_{CTX-M-14}$ gene, and was of cryptic clade IV (van der Putten et al., 2021). Differently from the human isolate, the isolate of ESBL-producing *E. ruysiae* from clams in this study harbored the $bla_{CTX-M-15}$ gene, the enterotoxin-encoding gene *astA*, and belonged to cryptic clade III.

In the EU, the microbiological safety of bivalve mollusks is based on the classification and monitoring of production areas. Assessing the sources and types of fecal contamination in the vicinity of the areas and how these affect mollusk production areas, combined with the quantitative monitoring of the fecal indicator organism *E. coli*, is critical to providing an estimate of the risk of contamination of an area by microbial pathogens.

To the best of our knowledge, this study was the first to investigate the correlation between the presence of ESBL-producing *E. coli* and the bacterial indicator of fecal contamination of *E. coli* in bivalve mollusks. Over the studied period, ESBL (including ESBL-/AmpC-)-producing *E. coli* were not isolated in clams from most (75%) of the studied sampling sites ($n = 28$), while the highest frequency of isolation (27%) was observed in an area requiring post-harvest treatment to reduce microbiological contamination before human consumption. A significant correlation was found between the indicator of fecal contamination by *E. coli* above 230 MPN/100 g and the presence of ESBL-producing *E. coli*. Thus, this study provided evidence of *E. coli* in molluscan shellfish as an index of the potential presence of ESBL-producing *E. coli* isolates, which are bacteria resistant to a critically important class of highest-priority antimicrobials.

Considering seasonality, ESBL-producing *E. coli* were not isolated in clam samples collected in summer but in other seasons (8% in spring, 5% in winter, and 4% in autumn), although these differences

in prevalence were not found to be significant. One area had the greatest variability in STs and ESBL-producing genes, whereas the $bla_{CTX\text{-}M\text{-}1}$ variant was present in one specific area. These findings can be explained by the variability in the presence and type of pollution sources affecting the microbiological contamination of bivalve mollusk areas and by environmental effects (e.g., rainfalls, winds, and tidal currents) on pollution sources. A large number of uncertainties exist with respect to the sources and transmission routes of antimicrobial-resistant bacteria and antimicrobial-resistance genes in food-producing environments.

In future studies, we will investigate the presence in bivalves of ESBL-producing *E. coli*, other antimicrobial-resistant bacteria, and antimicrobial-resistance genes of importance in human health in relation to the sources and types of pollution, seasonal variations, and climatic factors that influence production areas.

In conclusion, this study presents novel observations on the prevalence, seasonality, genomic, and phenotypic characteristics of ESBL-producing *E. coli* isolates in bivalves from production areas. ESBL-producing *E. coli* isolates were significantly more likely to be present among clam samples with higher levels of *E. coli* contamination (> 230 MPN/100 g) than among those with lower levels (≤ 230 MPN/100 g). Furthermore, potentially pathogenic ESBL-producing *E. coli* strains (ETEC and ExPEC) were isolated mainly in samples with *E. coli* contamination levels above 230 MPN/100 g. These findings provided evidence in support of *E. coli* as an index organism for the presence of ESBL-producing *E. coli*.

Data availability statement

The dataset is available in Appendix 1 of Supplementary Material. Sequencing data are published in online repositories and accession numbers are specified in the material and methods section.

Author contributions

FL: study conception. FL, CM, FB, FM, EA, LC, and CC: methodology. FL, CM, and FB: validation and study design. LS, GA, SP, EA, and FM: laboratory analysis. FL, AR, and FM: bioinformatics analysis. LC: isolate preparation for NGS analysis. CC and SP: NGS sequencing. FB: statistical analysis. FL, FB, EA, and FM: data curation. AD and FL: resources, project administration, and funding acquisition. FL: writing–original draft preparation. CM, FB, EA, and FM: writing–reviewing and editing supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1219008/full#supplementary-material

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Antimicrobial resistance and genetic diversity of *Klebsiella pneumoniae* strains from different clinical sources in horses

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Introduction: *Klebsiella pneumoniae* is a major cause of infections and reproductive disorders among horses, ranked in recent French studies as the sixth most frequently isolated bacterial pathogen in equine clinical samples. The proportion of multidrug-resistant (MDR) *K. pneumoniae* is therefore significant in a context where MDR *K. pneumoniae* strains are considered a major global concern by the World Health Organization.

Methods: In this study, we used a genomic approach to characterize a population of 119 equine *K. pneumoniae* strains collected by two laboratories specialized in animal health in Normandy (France). We describe the main antibiotic resistance profiles and acquired resistance genes, and specify the proportion of virulence-encoding genes carried by these strains. The originality of our panel of strains lies in the broad collection period covered, ranging from 1996 to 2020, and the variety of sample sources: necropsies, suspected bacterial infections (e.g., genital, wound, allantochorion, and umbilical artery samples), and contagious equine metritis analyses.

Results: Our results reveal a remarkable level of genomic diversity among the strains studied and we report the presence of 39% MDR and 9% hypervirulent strains (including 5% that are both MDR and hypervirulent).

Discussion: These findings clearly emphasize the importance of improving the surveillance of *K. pneumoniae* in routine equine diagnostic tests to detect high-risk MDR-hypervirulent *Klebsiella pneumoniae* strains. The circulation of these worrisome strains reveals that they are not being detected by the simple K1, K2, and K5 serotype approach currently implemented in the French horse-breeding sector.

KEYWORDS

***Klebsiella pneumoniae*, horse, K-antigen and O-antigen, MLST, multidrug resistance, whole genome sequencing**

Introduction

Klebsiella pneumoniae is ubiquitously isolated from humans, animals, and environmental samples and is the common cause of various infections.

In humans, *K. pneumoniae* is the third most common cause of healthcare-associated infections such as bacteremia, ventilator-associated pneumonia, and urinary tract infections. It has also emerged as an agent of severe community-acquired infections presenting as pyogenic liver abscesses, meningitis, and fasciitis (Holt et al., 2015). These two clinical presentations have been associated with two distinct evolutionary *K. pneumoniae* populations: one of them is multidrug-resistant (MDR), including to carbapenem, and causes nosocomial infections in immunocompromised patients; the other is a mainly wild hypervirulent type (i.e., producing only its chromosomal penicillinase) that causes severe infections in individuals from the community, who are often healthy. MDR *K. pneumoniae* isolates, which produce extended-spectrum β-lactamases (ESBLs) and/or carbapenemases belong to particular clones (Wyres et al., 2020). Thus, the majority of carbapenemase-producing *K. pneumoniae* worldwide belong to clonal group (CG) CG258 (including ST258, ST11, ST340, ST437, and ST512). Several other CGs are also globally distributed and associated with MDR *K. pneumoniae* (Dong et al., 2022). Invasive community-acquired isolates, usually named hypervirulent *K. pneumoniae* were initially described in Asian studies in the 1980s but are increasingly reported worldwide. These isolates differ in clonal background from MDR isolates and appear to predominantly belong to capsular serotypes K1 in CG23 and K2 in other unrelated CGs (Russo and Marr, 2019; Wyres et al., 2020). The hypervirulent *K. pneumoniae* virulomes are also well documented, in particular the importance of the pLVPK-like virulence plasmid and/or pathogenicity island in mucoid phenotype expression. Worryingly, the evolutionary convergence of MDR and hypervirulent *K. pneumoniae* high-risk clones raises serious therapeutic challenges. Its recent global spread has been associated with a diverse genetic background (Arcari and Carattoli, 2023).

In animals, most clinical manifestations of *K. pneumoniae* infections concern the urinary and respiratory tracts, which could lead to sepsis. In addition, among horses, *K. pneumoniae* is a major cause of abortion (Laugier et al., 2011; Akter et al., 2021) or metritis (Platt and Atherton, 1976), and may occasionally cause infertility issues in inseminated mares (Malaluang et al., 2021). Three recent French retrospective studies showed that *K. pneumoniae* is the sixth most isolated bacterial pathogen of equine clinical samples (Duchesne et al., 2019; Bourély et al., 2020; Léon et al., 2020), with an annual frequency of MDR strains ranging from 11.7 to 51.5% over the 2006–2019 period (Duchesne et al., 2019; Léon et al., 2020). A recent Israeli case control study on 3GC-resistant *Enterobacterales* infections in hospitalized horses and donkeys showed that the *Klebsiella* spp. were the most common 3GC-resistant *Enterobacterales* detected (Shnaiderman-Torban et al., 2021). However, it remains difficult to detect high-risk clonal lineages of *K. pneumoniae* through epidemiological and genomic analyses in horses (e.g., Trigo da Roza et al., 2019; Loncaric et al., 2020; Shnaiderman-Torban et al., 2021). A recent molecular study compared genomes from hypervirulent CG23 *K. pneumoniae* strains of both human and horse origins (Lam et al., 2018), but very little information is currently available regarding the hypervirulence of *K. pneumoniae* in horses.

The objectives of the present study were to (i) characterize by a genomic approach a population of 119 equine *K. pneumoniae* strains collected by two laboratories specialized in animal health in Normandy (France), and responsible for various manifestations of infection, (ii) describe the main antibiotic resistance profiles and acquired resistance genes, and (iii) describe the proportion of virulence-encoding genes carried by these strains. In addition to the numerous strains analyzed, the originality of this equine *K. pneumoniae* population lies in the broad collection period covered, ranging from 1996 to 2020, and the variety of sample sources: necropsies, suspected bacterial infections (e.g., genital, wound, allantochorion, and umbilical artery samples), and contagious equine metritis (CEM) analyses.

Materials and methods

Bacterial isolates

In all, 119 French *K. pneumoniae* strains were investigated in the present study, all from horses (Supplementary material). Between 1996 and 2020, the ANSES Normandy Laboratory for Animal Health isolated 118 *K. pneumoniae* strains from 117 necropsies and one analysis of the allantochorion from an abortion (2.6% of total necropsies performed with an aerobic bacteriological search between 1996 and 2020). Among the strains investigated, 23 were excluded from the study: ten were no longer cultivable and 13 were in fact not members of the *K. pneumoniae* species according to the *in-silico* analyses. The ANSES laboratory thus supplied 94 strains considered here as "necropsy-associated strains." The microbiology diagnostic unit of LABÉO laboratory provided a total of 25 strains isolated between 2011 and 2020, considered here as "non-necropsy-associated strains." These strains were isolated from genital samples ($n = 20$), which included 12 CEM cultures, wounds ($n = 4$), and the umbilical artery ($n = 1$). They were selected based on their antibiotic susceptibility profile and genital origin, which was the major source of sample origin for *K. pneumoniae* at LABÉO (Duchesne et al., 2019; Léon et al., 2020). ANSES's strains were stored using CryoBeads™ (bioMérieux) at a temperature $\leq -65°C$ and LABÉO's strains were conserved at-80°C on brain heart infusion broth with 10% glycerol. Before phenotypic analyses and genome sequencing, all the strains were cultivated on ready-to-use 5% sheep blood agar (bioMérieux) incubated at $35 \pm 2°C$ for 24 h.

Phenotypic antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion method on Mueller-Hinton agar plates according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline.[1] The panel of 35 antibiotics (BioRad) tested included amikacin, amoxicillin-clavulanate, aztreonam, cefalexin, cefamandole, cefepime, cefoxitin, ceftazidime, ceftiofur,

1 http://www.eucast.org

ceftolozane-tazobactam, ceftriaxone, chloramphenicol, ciprofloxacin, ertapenem, fosfomycin, gentamicin, imipenem, kanamycin, levofloxacin, marbofloxacin, mecillinam, meropenem, nalidixic acid, netilmicin, norfloxacin, piperacillin-tazobactam, spectinomycin, streptomycin, temocillin, tetracycline, ticarcillin-clavulanate, tigecycline, tobramycin, trimethoprim and trimethoprim-sulfamethoxazole. They were then interpreted as susceptible, intermediate, or resistant using the breakpoints for *Enterobacterales* available in the 2021 veterinary recommandations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CASFM, 2021) or, failing that, those available for *Enterobacterales* strains of human origin (CASFM, 2013; CASFM/EUCAST, 2021) (Supplementary material). Of note, strains that were categorized as intermediate were considered as resistant for further descriptive and comparative statistical analyses.

MDR strains have been defined as antimicrobial resistance shown by a species of microorganism to at least one antimicrobial drug in three or more antimicrobial categories as previously described (Magiorakos et al., 2012). The MDR classification complied with the 35 antibiotics.

K125 PCR

A multiplex PCR was performed using targets within the serotype-specific region of the capsular polysaccharide synthesis gene cluster of serotypes K1, K2, and K5 using primers described previously (Turton et al., 2008). Before the amplification, each strain was suspended in 500 µL of PBS solution compliant with McFarland 0.5 and heated to 95°C for 10 min then centrifuged at 1800 g for 1 min. PCR assays were performed using the Multiplex PCR kit (Qiagen) according to the manufacturer's recommendation. In brief, after an initial 15-min step at 95°C, the analyses were run on a Verity thermal cycler (Applied Biosystems, Thermo Fisher). There were 45 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 90 s and elongation at 72°C for 90 s, completed by a final 10-min step at 72°C. To ensure the validity of the assays, positive and negative controls were run in parallel. The length of the amplicons (1,283, 641 and 276 bp for the K1, K2, and K5 capsular polysaccharide synthesis gene, respectively) was verified using the Qiaxcel Advanced System (Qiagen).

Whole genome sequencing

Isolates were subcultured for 6 h in Luria-Bertani broth at 35 ± 2°C, then 1 mL was centrifuged for 10 min at 4,000 rpm. The pellet was then resuspended in 100 µL PBS 1X. Cells were lysed using MagNA Pure Lysis Buffer (Roche) as well as Proteinase K recombinant PCR Grade (Roche). DNA was extracted from each isolate using the MagNA Pure system (Roche).

Dual-indexed Illumina sequencing libraries were constructed from each sample using the Nextera XT DNA library preparation kit (Illumina), pooled, then sequenced on the Illumina NextSeq 500 platform (Plateforme de Microbiologie Mutualisée P2M, Institut Pasteur, Paris, France). Sequencing was performed according to the manufacturer's instructions using a 2 × 150-bp paired-end configuration with a minimum coverage of 30x. Quality control was carried out on the raw reads using FastQC (Andrews, 2010) and MultiQC (Ewels et al., 2016). Adapters and reads with a median Phred score lower than 25 and/or shorter than 70 pb were removed from the fastq files using fqCleanER.[2] Genomes were assembled using the SPAdes Genome Assembler v.3.12.0 with parameters recommended for 150 pb paired-end reads (k-mer lengths of 21, 33, 55, 77) (Bankevich et al., 2012). The quality of the assemblies was assessed in Quast (Gurevich et al., 2013). Genomes and associated metadata are available on the *Klebsiella* BIGSdb-Pasteur database[3] in the "Klebsequi-projet" public project. Genomes are also available on the NCBI database using the Bioproject number PRJNA1054041.

In silico WGS analysis

Several *in silico* analyses were performed for each genome: (i) species were checked using the rMLST software (Jolley et al., 2012); (ii) sequence types (STs) were attributed according to the multi locus sequence typing (MLST) scheme previously described using the BIGSdb-Kp database (Diancourt et al., 2005). Unknown or new STs were submitted to the BIGSdb-Kp database for curation (see footnote 3). A Venn diagram was plotted to illustrate overlaps of the source types/hosts (i.e., human, horse, animal other than horse, environment, and food) for STs identified in this study and already present in the BIGSdb-Kp database; (iii) the lipopolysaccharide O-antigen was characterized by the nucleotide sequence of the *wzm* gene using the Kaptive command line tool[4] (Follador et al., 2016; Wick et al., 2018) with "Good" as the confidence threshold (any result below the threshold was "not assigned"); (iv) the capsular polysaccharide K-antigen was determined by the nucleotide sequence of the *wzi* gene (Brisse et al., 2013) using the BIGSdb-Kp database; (v) a search was conducted for all the virulence-encoding genes into the BIGSdb-Kp database with a 90% identity threshold using the KMA software (Bialek-Davenet et al., 2014; Clausen et al., 2018); (vi) acquired antibiotic resistance genes and chromosomal mutations were determined using both ResFinder and rgi databases with a 90% identity and coverage threshold, respectively (Zankari et al., 2012; Alcock et al., 2020); (vii) the PlasmidFinder tool (Carattoli et al., 2014) was used to look for families of plasmids carried by the strains, including the pK2044 and pLVPK-like virulence plasmids. The tool plasmidfinder look for replicon sequences from plasmid among fastq reads or assembled sequences. Using small replicon sequences allow the identification of plasmids sequences using short-read sequencing data. Hypervirulent strains were defined based on the presence of several biomarkers according to Russo et al. (2018) (e.g., *peg-344*, *iroB*, *iucA*, *rmpA*, and *rpmA2*).

To further distinguish the isolates, cgMLST analysis using the chewBBACA (Silva et al., 2018) "*K. pneumoniae sensu lato* cgMLST" version 1.0 scheme with a total of 2,358 genes[5] was performed using all the genomes. Only genes for which alleles were found in all isolates were kept for analysis. A minimum spanning tree (MST) was constructed using GrapeTree (Zhou et al., 2018). Sublineage and clonal groups (CGs) were attributed for each strain using the core

2 https://gitlab.pasteur.fr/GIPhy/fqCleanER
3 https://bigsdb.pasteur.fr/klebsiella/
4 https://github.com/katholt/Kleborate
5 https://www.cgmlst.org/ncs/schema/2187931/

genome MLST scheme based on 629 genes previously described (Hennart et al., 2022).

Statistical analysis

Statistical analysis was performed using RStudio version 2022.12.0. Association between category variables and the MDR status of strains was tested with Pearson's Chi-squared test and Fisher's exact test where appropriate ($n < 5$). We did not impute unknown values and defined statistical significance as a p-value <0.05.

Results

Description of the equine *Klebsiella pneumoniae* population

The 94 (79.0%) necropsy-associated strains and 25 (21.0%) non-necropsy-associated strains studied were from 119 horses located in nine French regions, though predominantly from Normandy (79.8%) in accordance with the location of the two laboratories that isolated the strains. At least eight horse breeds were represented, with an overdistribution of Trotter or French Trotter (39.5%) and Thoroughbred (33.6%) mostly skewed by the necropsy recruitment. The sex (male: female) ratio of 0.63 is skewed mostly due to the dominance of genital origins for the non-necropsy-associated strains, and stands at 0.82 when only necropsy-associated strains are taken into account. Age ranged from fetus to 22 years old, and year of strain isolation ranged from 1996 to 2020: (i) necropsy-associated strains were from 18 fetuses (median, 8.5 months of gestation; range, 3.75–9.5 months of gestation), 63 foals (median, 1 month; range, 0–17 months), and 13 adult horses (median, 8 years; range, 3–22 years), and were isolated from 1996 to 2020 (median, 2007); (ii) non-necropsy-associated strains were from seven unknown and 18 adult horses (median, 11 years; range, 4–20 years), and were isolated from 2011 to 2020 (median, 2019). The clinical source of strain isolations was varied (Supplementary material) but it could be grouped into sepsis (21.8%), digestive (20.2%), genital (17.6%), abortion (15.1%), respiratory (13.5%, including *Rhodococcus equi* infections), cutaneous (3.4%), and other (8.4%, including fractures, anoxia/asphyxia, viral/parasitic infections, umbilical artery, cardiac tamponade, hepatic tear, cervical trauma, myopathy, and unknown). Furthermore, co-infections with other bacteria were found in 89.9% of cases (Supplementary material); the most frequent co-infections were with *Escherichia coli* (37%), *Staphylococcus xylosus* (15%) and *Streptococcus equi* subsp. *zooepidemicus* (14%), followed by, e.g., *Streptococcus* other than *S. zooepidemicus* (8%), *Enterococcus* spp. (8%), *Rhodococcus equi* (7%), *Clostridium difficile* (4%), *Pseudomonas aeruginosa* (4%) (Supplementary material).

For the 94 necropsy-associated strains, lesions related to the cause of death were observed in 69.1% of cases, while lesions unrelated to the cause of death (12.8%) and absence of lesions (17.0%) were also observed.

Antimicrobial susceptibilities

All 119 strains were resistant to at least one class of antibiotics tested, with a median of 1/35 antibiotics categorized as resistant per strain, and 46 (38.7%) presented an MDR phenotype according to the definition mentioned above, with a median of 17/35 molecules categorized resistant (Supplementary material). The most frequent resistant classes were ß-lactams, aminoglycosides and tetracyclines, followed by pyrimidines, phenicols, quinolones, and phosphoric acid (Table 1). The *in silico* WGS analysis showed the presence of three to 20 resistance genes per strain, with a median of four when all strains were considered and a median of 14 when only MDR strains were considered (Supplementary material).

Among ß-lactams, 83 strains (69.7%) presented a single resistance to temocillin, and they only produced the chromosomal narrow spectrum ß-lactamase SHV-1 or its variants. Thirty-one strains (26.1%) were resistant to at least one 3/4GC tested. Ceftazidime was the most frequent hydrolyzed 3/4GC, followed by ceftriaxone, ceftiofur, and cefepime (respectively 25.2, 24.4, 19.3, and 17.6%) (Table 1). Mechanisms involved other than the 3/4GC resistance phenotype were mainly ESBL production ($n = 22$, 71.0%), followed by plasmid-mediated AmpC cephalosporinase ($n = 9$, 29.0%). Among the plasmid-mediated AmpC cephalosporinase, the $bla_{\text{CMY-2}}$ gene was the most frequently found ($n = 7$), followed by $bla_{\text{DHA-1}}$ ($n = 2$). Regarding the ESBL-encoding genes, $bla_{\text{CTX-M-15}}$ ($n = 14$) was the most frequently encountered gene, followed by $bla_{\text{CTX-M-2}}$ (n = 3), $bla_{\text{CTX-M-3}}$ ($n = 2$), and $bla_{\text{SHV-12}}$ ($n = 2$), respectively (Supplementary material). Only one strain (strain 009) was resistant to ertapenem and none to imipenem or meropenem; this strain did not produce any carbapenemase, but the production of DHA-1 could lead to low-level resistance to ertapenem (Jacoby, 2009), especially if the cephalosporinase is associated with efflux pump overexpression and/or porin loss (Table 1; Supplementary material). Finally, only two strains were resistant to ceftolozan-tazobactam: one produced DHA-1 (strain 124) and the other ESBL, CTX-M-15, and SHV-12 (strain 131) (Table 1; Supplementary material).

Regarding the aminoglycoside class, streptomycin, gentamicin, kanamycin, and netilmicin were the most frequent ineffective antibiotics with the following resistance rates: 38.7, 22.7, 21.0, and 21.0%, respectively (Table 1). In contrast, only one strain (strain 126) was considered resistant to amikacin. Twenty aminoglycoside resistance genes were recovered from 48/119 strains, the most prevalent being *aph(6)-Id* ($n = 39$), *aph(3″)-Ib* ($n = 32$), and *aph(3′)-Ia* ($n = 17$) (Supplementary material).

One third of the strains were resistant to tetracyclines due to the presence of genes coding for a major facilitator superfamily (MFS) antibiotic efflux pump, respectively, *tet(A)* ($n = 31$), *tet(B)* ($n = 5$), *tet(D)* ($n = 3$), and *tet(C)* ($n = 1$) (Supplementary material). One resistant strain (strain 048) did not harbor a *tet* family gene but had a mutation in the *kpnF* gene which codes a subunit of a two-component system, KnpEF, involved in broad-spectrum antimicrobial resistance (Srinivasan and Rajamohan, 2013).

There was substantial resistance to pyrimidines: 27.8% to trimethoprim and 26.9% to trimethoprim-sulfamethoxazole (Table 1). Thirteen strains had a *sul* gene alone (*sul1*, $n = 6$; *sul2*, $n = 7$) with no phenotypic consequences for cotrimoxazole. In contrast, 32 pyrimidine-resistant strains had *sul* genes associated with dihydrofolate reductase encoding *dfrA*. The most frequent *dfrA* genes were *dfrA14* ($n = 12$), *dfrA12* ($n = 10$), *dfrA1* ($n = 6$), and *dfrA27* ($n = 4$), respectively (Supplementary material).

Concerning quinolone and fluoroquinolone, the most frequent resistant molecules were ciprofloxacin, nalidixic acid, and levofloxacin,

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TABLE 1 Antimicrobial susceptibility profiles of the 119 *K. pneumoniae* strains studied.

Antibiotic class	Family	Antibiotic	Susceptible [*n* (%)]	Resistant [*n* (%)]
β-lactams	Penicillin	MEC	114 (95.8)	5 (4.2)
		TEM	0 (0)	119 (100)
	Penicillin/β-lactamase inhibitor	AMC	91 (76.5)	28 (23.5)
		TIL	90 (75.6)	29 (24.4)
		PIT	112 (94.1)	7 (5.9)
	1GC	CLE	88 (73.9)	31 (26.1)
	2GC	CMA	88 (73.9)	31 (26.1)
		CXI	109 (91.6)	10 (8.4)
	3GC	CTZ	89 (74.8)	30 (25.2)
		CTR	90 (75.6)	29 (24.4)
		CTF	96 (80.7)	23 (19.3)
	4GC	CEP	98 (82.4)	21 (17.6)
	Cephalosporin/β-lactamase inhibitor	CTT	117 (98.3)	2 (1.7)
	Carbapenem	ERT	118 (99.2)	1 (0.8)
		IMI	119 (100)	0 (0)
		MER	119 (100)	0 (0)
	Monobactam	AZT	97 (81.5)	22 (18.5)
Aminoglycosides		STR	73 (61.3)	46 (38.7)
		GEN	92 (77.3)	27 (22.7)
		NET	94 (79.0)	25 (21.0)
		KAN	94 (79.0)	25 (21.0)
		TOB	96 (80.7)	23 (19.3)
		SPE	100 (84.0)	19 (16.0)
		AMI	118 (99.2)	1 (0.8)
Quinolones	Quinolone	NAL	105 (88.2)	14 (11.8)
	Fluoroquinolone	CIP	97 (81.5)	22 (18.5)
		LEV	105 (88.2)	14 (11.8)
		NOR	108 (90.8)	11 (9.2)
		MAR	111 (93.3)	8 (6.7)
Pyrimidines		TRI	86 (72.3)	33 (27.7)
		TRS	87 (73.1)	32 (26.9)
Phosphoric acid		FOS	116 (97.5)	3 (2.5)
Phenicol		CHL	98 (82.4)	21 (17.6)
Tetracyclines		TET	81 (68.1)	38 (31.9)
		TIG	118 (99.2)	1 (0.8)

MEC, mecillinam; TEM, temocillin; AMC, amoxicillin-clavulanate; TIL, ticarcillin-clavulanate; PIT, piperacillin-tazobactam; CLE, cefalexin; CMA, cefamandole; CXI, cefoxitin; CTZ, ceftazidime; CTR, ceftriaxone; CTF, ceftiofur; CEP, cefepime; CTT, ceftolozane-tazobactam; ERT, ertapenem; IMI, imipenem; MER, meropenem; AZT, aztreonam; STR, streptomycin; GEN, gentamicin; NET, netilmicin; KAN, kanamycin; TOB, tobramycin; SPE, spectinomycin; AMI, amikacin; NAL, nalidixic acid; CIP, ciprofloxacin; LEV, levofloxacin; NOR, norfloxacin; MAR, marbofloxacin; TRI, trimethoprim; TRS, trimethoprim-sulfamethoxazole; FOS, fosfomycin; CHL, chloramphenicol; TET, tetracycline; TIG, tigecycline.

respectively (18.5, 11.8, and 11.8%) (Table 1). Several mechanisms were involved; some strains had the gyrA-83I and parC-80I mutations inside the quinolone resistance-determining region leading to a complete resistance class ($n = 4$), while others had quinolone resistance genes (*qnr*), mainly *qnrS1* ($n = 11$), *qnrB1* ($n = 8$), and *qnrB4* ($n = 2$) (Supplementary material). Moreover, several efflux pump systems were found to be involved, particularly among nalidixic acid-resistant strains: A repressor of the MdtEF pump called CRP was mutated, as was subunit KpnG of the KpnHH efflux pump (Supplementary material). Marbofloxacin was the most active fluoroquinolone tested, 93.3% of the strains being susceptible to it (Table 1).

Twenty-one strains (17.6%) were resistant to chloramphenicol. The FloR exporter was the most frequent chloramphenicol resistance

mechanism ($n = 12$), followed by the presence of chloramphenicol acetyltransferase *cat* genes ($n = 7$) and dysregulations among RND pumps ($n = 4$): Mutations in *marA* and *acrB* (Supplementary material).

Aside from two strains (strains 35 and 63), *fosA* genes were found in the genome of all the strains (98.3%). The most frequent was *fosA6* ($n = 85$), followed by *fosA5* ($n = 26$), *fosA7* ($n = 5$), and *fosA3* ($n = 1$) (Supplementary material). Despite the quasi omnipresence of the *fosA* gene in the genomes, only three strains were resistant to fosfomycin (Table 1). Among tetracyclines, 38 strains (31.9%) were resistant to tetracycline but only one was found to be resistant to tigecycline, which was a very active molecule (Table 1). Even if the correlation between genotype and phenotype was not performed due to natural resistance for macrolides and rifampicin, there were many resistance-encoding genes. Regarding the macrolide, lincosamide and streptogramin molecules, gene-coding macrolide phosphotransferases were predominant: *mph(A)* ($n = 21$), *mph(E)* ($n = 2$), and *mph(B)* ($n = 1$), followed by *ere(A)* and *msr(E)* ($n = 2$ for both). On the rifampicin side, two different types of rifampin ADP-ribosyltransferase were found among the genomes, *arr-3* ($n = 12$) and *arr-8* ($n = 1$). Finally, one *mcr-9* gene conferring resistance to colistin (molecule not tested in the present study) was found in strain 132 (Supplementary material).

Antimicrobial susceptibilities and MDR prevalence in necropsy-associated strains

The focus on the 94 necropsy-associated strains showed lower resistance to those observed when all 119 strains are considered. However, this difference cannot be taken into consideration as almost all non-necropsy-associated strains were selected based on their antimicrobial drug profile. It is the same way for the lower MDR proportion observed among the necropsy-associated strains ($n = 27$, 28.7%) compared to the MDR proportion in all 119 strains studied ($n = 46$, 38.7%). Interestingly, a large difference was observed between the 1996–2007 and 2008–2020 periods of necropsy-associated strain isolations (Figure 1). The 2008–2020 period showed that resistances had increased by 2.2 to 30.5% for 29/35 antibiotics compared with the 1996–2007 period; only amikacin, ceftolozane-tazobactam, imipenem, meropenem, and temocillin showed equivalent resistances, and tigecycline resistance had decreased by 2.1%. The increased resistances during the 2008–2020 period were statistically significant for 12 antibiotics, with *p*-values from 0.0001 to 0.0392 (represented in red in Figure 1). This difference was also observed considering the MDR proportion between the 1996–2007 period (nine MDR/48, 18.8%) and 2008–2020 period (18 MDR/46, 39.1%) of necropsy-associated strain isolations. The bigger MDR proportion for the 2008–2020 period than the 1996–2007 period was statistically significant (Table 2). The MDR distribution in the necropsy-associated strains was also analyzed considering the information on horses (sex, age and breed) and clinical presentations (Table 2). Thus, statistically significant differences were observed, such as a higher MDR proportion for categories "foal," "Thoroughbred," "digestive," and "presence of lesions not related to the cause of death," and a lower MDR proportion for categories "fetus," "abortion" and "co-infection with *Streptococcus zooepidemicus*." The lower MDR proportion in category "presence of lesions related to the cause of death" was not statistically significant (Chi^2, $p = 0.0539$).

High diversity of MLST genotypes confirmed by cgMLST

The population studied reveals a remarkable level of genomic diversity since 83 different STs were assigned among the 119 genomes, including 63 singletons. ST127 ($n = 7$), ST2813 ($n = 6$), ST25, ST60, ST145 ($n = 4$ each), and ST2454 ($n = 3$) were the most prevalent STs and concerned nearly a quarter of the strains (Figure 2A, Supplementary material). ST25 (Fisher's test, $p = 0.021$), ST127 (Fisher's test, $p = 0.013$) and ST2813 (Fisher's test, $p = 0.003$) were statistically more frequent in MDR strains. The analysis of the source types/hosts of the STs already present in the BIGSdb-Kp database showed that 49 STs had been previously reported in humans, 33 in animals other than horses, 23 in the environment, 16 in horses (33 STs of equine origin in the BIGSdb-Kp database were not taken into account since they were new STs identified in this study) and nine in food, with multiple overlaps (Supplementary material, Figure 2B).

The genome comparison by cgMLST was carried out on 1809 genes after excluding 549 genes with missing values. In total, 66,402 single alleles were identified with a median of 38 alleles per gene (range 1–87). The median distance between the strains was 1,520 alleles with a maximum distance of 1,580 alleles, reflecting a very diverse population. Only a few strains were much closer to each other, e.g., both strains of ST5415 shared the same 1809 alleles, strains 101 and 102 of ST2454 were distant by only two alleles, all six strains of ST2813 had a maximum distance of seven alleles, strains 82, 83, and 107 had a maximum distance of nine alleles, and all four strains of ST25 had a maximum distance of 11 alleles (Supplementary material). Phylogenetic relations between strains assessed using cgMLST are visualized as MST according to ST distribution (Figure 3), clinical sources (Figure 4), and MDR phenotype (Figure 5). Among the 72 CGs assigned (Supplementary material), the five most frequent CGs were CG10451 (seven ST127 strains from diverse clinical sources, including six that were MDR), CG12510 (six ST2813 and MDR strains from genital and abortion sources), CG25 (four ST25 and MDR strains, three of which were from a genital source), CG60 (four ST60 strains from diverse clinical sources, half of which were MDR), and CG10451 (four ST145 strains from genital and digestive sources, half of which were MDR).

Diversity of O-and K-antigens and comparison with K125 PCR detection

Ten different O-antigens were assigned for 88/119 genomes; O1v1 ($n = 23$, 19.3%) was the most prevalent followed by O3b ($n = 15$, 12.6%), O2v1 ($n = 14$, 11.8%), O2v2, O3/O3a ($n = 10$, 8.4% each), O5 ($n = 6$, 5.0%), OL101, OL103 ($n = 3$, 2.5% each), O1v2, and O1/O2v1 ($n = 2$, 1.7% each) (Figure 6A). Sixty-one *wzi* alleles were detected and 29 led to the assigning of 24 different K-antigens for 66/119 genomes, including the most prevalent K22.37 ($n = 13$, 10.9%), K30 ($n = 7$, 5.9%), K2, K3 ($n = 6$, 5.0% each), K1 ($n = 4$, 3.4%), and K5, K9 ($n = 3$, 2.5% each) (Figure 6B). Among the 32 *wzi* alleles that did not lead to the assigning of K-antigens for 53/119 genomes, *wzi*-274 ($n = 12$, 10.1%), *wzi*-173 ($n = 3$, 2.5%), and *wzi*-419 ($n = 3$, 2.5%) were the most prevalent (Figure 6B). Only 48 strains (40.3%) had a complete O:K serotype for a total of 24 different profiles, including the most prevalent O1v1:K22.37 ($n = 8$, 17.0%), O2v2:K30 ($n = 5$, 10.7%), and

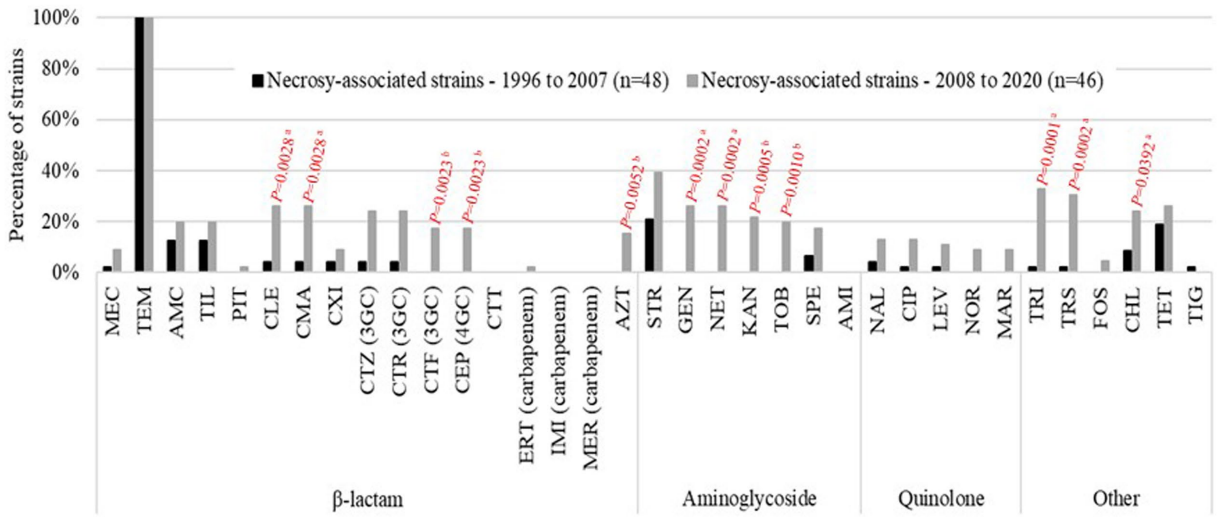

FIGURE 1
Percentage of resistant results for the equine necropsy-associated *K. pneumoniae* strains studied. Comparison between the 1996–2007 period ($n = 48$; black bars) and the 2008–2020 period ($n = 46$; gray bars). Statistically significant differences are indicated in red ([a]Pearson's Chi-squared test; [b]Fisher's exact test).

O1v1:K1 ($n = 3$, 6.4%) (Supplementary material). The O1v1:K22.37 profile was more frequent in MDR strains but this observation was not statistically significant (Fisher's test, $p = 0.0538$).

The K1, K2, and K5 antigen assignments by WGS were compared with K125 PCR detection (Table 3). The results were consistent for 11/13 strains where a K1, K2 or K5 antigen had been assigned; the remaining two strains with a K2 antigen assigned by WGS resulted in a negative K125 PCR. Conversely, the K125 PCRs were positive for three strains carrying a *wzi*-181 variant (K1 PCR positive), an assigned K3 antigen (K2 PCR positive) and a *wzi*-482 variant (K5 PCR positive), respectively; however, the *wzi*-181, *wzi*-482 and K3 antigen assignment did not consistently show a positive K125 PCR result (Table 3).

Distribution and identification of plasmids and virulence factors

Among the 119 strains studied, 29 plasmids were detected one to 34 times each, and 543 virulence genes and variants were detected one to 55 times each. Each strain showed the presence of zero to six plasmids with a median of one, and of eight to 60 virulence genes with a median of 14 (Supplementary material).

Plasmids are detailed in Table 4. The results revealed a high diversity of the incompatibility (Inc) groups. IncFIB(K) was detected in 28.6% ($n = 34$) of strains, including IncFIA(HI1) ($n = 2$), IncFIB(pNDM-Mar) ($n = 2$), and IncFIB(pKPHS1) ($n = 1$) co-detections. Twenty-two strains without IncFIB(K) harbored one or two other IncFI members, including IncFIA(HI1) ($n = 7$), IncFIB(K) (pCAV1099-114) ($n = 12$), IncFIB(pKPHS1) ($n = 8$), and IncFIB(pNDM-Mar) ($n = 4$). IncFII(K) was detected in 14.3% ($n = 17$) and three other IncFII members in 5.9% ($n = 7$). Other plasmids of an Inc. group were more infrequently detected, including five IncHI members, IncC, IncI1-I(Gamma), IncM1, IncN, IncQ1, IncR, and IncX3. Six Col plasmids, FIA(pBK30683), and RepB were also detected, the most frequent being Col(pHAD28) ($n = 19$, 16.0%) and ColRNAI ($n = 9$, 7.6%). Four Inc. plasmids and ColRNAI were statistically more frequent in MDR strains; IncHI1A and IncHI1B(R27) were more frequent in MDR strains but without statistical significance (Table 4).

Virulence factors are detailed in Table 5. As expected, the *mrk* type 3 fimbriae cluster was detected in 98.3% ($n = 117$) of strains, the remaining 1.7% ($n = 2$) carrying an incomplete cluster, *mrkACDFHIJ*. Aerobactin receptor *iutA* was detected in 95.0% ($n = 115$) but only one strain (strain 132) harbored both *iutA* and aerobactin cluster *iucABCD*. Yersiniabactin cluster *ybt-fyuA-irp* was detected in 31.1% ($n = 37$) and partially detected in 3.4% ($n = 4$), ferric uptake system *kfuABC* was detected in 28.6% ($n = 34$) and partially in 2.5% ($n = 3$), two-composent system *kvgAS* was detected in 22.7% ($n = 27$) and partially in 5.0% ($n = 6$), the *mce* microcin E492 cluster was detected in 10.1% ($n = 12$) and partially in one strain, salmochelin cluster *iro* was detected in 7.6% ($n = 9$), and mucoid phenotype regulators *rmpA* and *rmpA2* were detected in 7.6% ($n = 9$). Genes involved in the allantoin metabolism (*allABCDRS*, *arcC*, *fdrA*, *gcl*, *glxKR*, *hyi*, *ybbWY*, *ylbEF*) were co-detected with KP1_1364 and KP1_1371 in 10.9% ($n = 13$) of strains, while one strain harbored only *allB*. Colibactin cluster *clb* was detected in only two strains but was partially detected in 7.6% (eight strains did not harbor *clbK* and one strain harbored only *clbE*). Interestingly, yersiniabactin-and colibactin-encoding genes were statistically more frequent in MDR strains (Table 5).

The presence of a large virulence plasmid was sought by sequence homology with pLVPK (219,385 bp) and pK2044 (224,152 bp). The result suggests the presence of a plasmid of approximately 165 kb in strain 132 (75.2% coverage with pLVPK and 73.1% with pK2044), while all other strains showed coverage ranging from zero to 33.5% (Supplementary material).

Considering these findings, we highlighted 11 hypervirulent strains (9.2%) (indicated in blue in Figure 7); five of these were also MDR; including 3GC-resistant strain 132 (Figure 7). Nine strains

TABLE 2 Distribution of MDR and non-MDR necropsy-associated *K. pneumoniae* strains according to clinical presentation and co-infection.

Parameter	MDR (n = 27) [n (%)]	no MDR (n = 67) [n (%)]	p-value
Isolation period			
1996–2007	9 (33.3)	39 (58.2)	**0.0290**[b]
2008–2020	18 (66.7)	28 (41.8)	**0.0290**[b]
Sex[a]			
Female	14 (51.9)	37 (55.2)	0.7112[b]
Male	13 (48.1)	29 (43.3)	0.7112[b]
Age range			
Fetus	1 (3.7)	17 (25.4)	**0.0157**[b]
Foal	23 (85.2)	40 (59.7)	**0.0174**[b]
Adult	3 (11.1)	10 (14.9)	0.7509[c]
Breed[a]			
Thoroughbred	13 (48.1)	16 (23.9)	**0.0239**[b]
Trotter	12 (44.5)	34 (50.7)	0.5359[b]
Saddlebred	2 (7.4)	11 (16.4)	0.3333[c]
Other	0 (0.0)	5 (7.5)	0.3165[c]
Clinical source			
Abortion	1 (3.7)	17 (25.4)	**0.0157**[b]
Digestive	13 (48.2)	11 (16.4)	**0.0014**[b]
Sepsis	5 (18.5)	21 (31.3)	0.2085[b]
Respiratory	5 (18.5)	11 (16.4)	0.0933[c]
Other	3 (11.1)	7 (10.5)	1.0000[c]
Lesions observed at the necropsy			
Absence	5 (18.5)	11 (16.4)	1.0000[c]
Presence not related to the cause of death	7 (25.9)	5 (7.5)	**0.0353**[c]
Presence related to the cause of death	15 (55.6)	50 (74.6)	0.0539[b]
Co-bacterial identification[a]			
None	2 (7.4)	1 (1.5)	0.2011[c]
Actinobacillus equuli	0 (0.0)	3 (4.5)	0.5537[c]
Aeromonas spp.	0 (0.0)	2 (3.0)	1.0000[c]
Clostridium difficile	2 (7.4)	3 (4.5)	0.6256 [c]
Clostridium perfringens	0 (0.0)	2 (3.0)	1.0000[c]
Enterococcus spp.	3 (11.1)	4 (6.0)	0.3516[c]
Escherichia coli	10 (37.0)	28 (41.8)	0.7291[b]
Proteus vulgaris	1 (3.7)	0 (0.0)	0.2903[c]
Pseudomonas aeruginosa	1 (3.7)	2 (3.0)	1.0000[c]
Pseudomonas cepacia	0 (0.0)	2 (3.0)	1.0000[c]
Rhodococcus equi	2 (7.4)	6 (9.0)	1.0000[c]
Salmonella spp.	1 (3.7)	3 (4.5)	1.0000[c]
Serratia rubidaea	0 (0.0)	1 (1.5)	1.0000[c]
Staphylococcus aureus	3 (11.1)	1 (1.5)	0.0721[c]
Staphylococcus xylosus	4 (14.8)	14 (20.9)	0.4784[b]
Steptococcus equi	0 (0.0)	1 (1.5)	1.0000[c]
Streptococcus equisimilis	2 (7.4)	4 (6.0)	1.0000[c]
Streptococcus zooepidemicus	0 (0.0)	16 (23.9)	**0.0046**[c]
Candida famata	0 (0.0)	1 (1.5)	1.0000[c]

[a]One unknown data excluded (no MDR strain 114).
[b]Pearson's Chi-squared test.
[c]Fisher's exact test.

harbored at least two to three biomarkers reported by Russo et al. (2018); in more detail, *mrk*, *iutA*, *ybt-fyuA-irp*, *iro*, and *rmpA* (eight strains) or *rmpA2* (strain 132) loci were all detected, in addition to *iucABCD* and incomplete *clbABCDEFGHIJLMNOPQR* loci in K20-ST268 strain 132, *kfuABC* and *kvgAS* loci in *wzi 274*-ST145 strains 023, 115, and 137, and K55-ST145 strain 129, together with the *kfuABC* locus in K5-ST60 strains 066 and 087, and *wzi 482*-ST60 strains 073 and 135 (Figure 7); of note, a K125 PCR revealed K5 antigens in strain 135 (Table 3, Supplementary material). Five of these strains were necropsy-associated, and lesions related to the cause of death were reported at the necropsy for three of them (strains 23, 66, and 87) (Figure 7). Two additional necropsy-associated K2-ST131 strains—035 and 063—were also considered hypervirulent despite the absence of *peg-344*, *iroB*, *iucA*, *rmpA*, and *rpmA2* biomarkers reported by Russo et al. (2018) because they harbored *mrk*, *iutA*, *ybt-fyuA-irp*, *kfuABC*, *kvgAS*, *clb*, and *mce* loci; in both cases, lesions related to the cause of death were reported at the necropsy (Figure 7).

Other STs (e.g., ST25, ST2813) or K-antigens (e.g., K30, K22.37) may also be of interest due to the MDR status and the presence of several virulence genes (Figure 7).

Discussion

The originality of our study is based on the high number of *K. pneumoniae* strains from both necropsy-associated and clinical isolates in horses. This panel of strains was isolated over a long collection period (1996 to 2020) covering the whole of France (i.e., national scale) and from various sample sources: necropsies, suspected bacterial infections (genital, wound, allantochorion, and umbilical artery samples) and contagious equine metritis analyses. This panel highlights the proportion of hypervirulent and/or MDR *K. pneumoniae* strains that have been circulating in French horses over the past few decades and ideally complements the limited number of publications dedicated to the detection of high-risk clonal lineages of *K. pneumoniae* in horses (e.g., Trigo da Roza et al., 2019; Loncaric et al., 2020; Shnaiderman-Torban et al., 2021).

The main finding of this study is the remarkable level of genomic diversity and the atypical panel among the 119 strains studied,

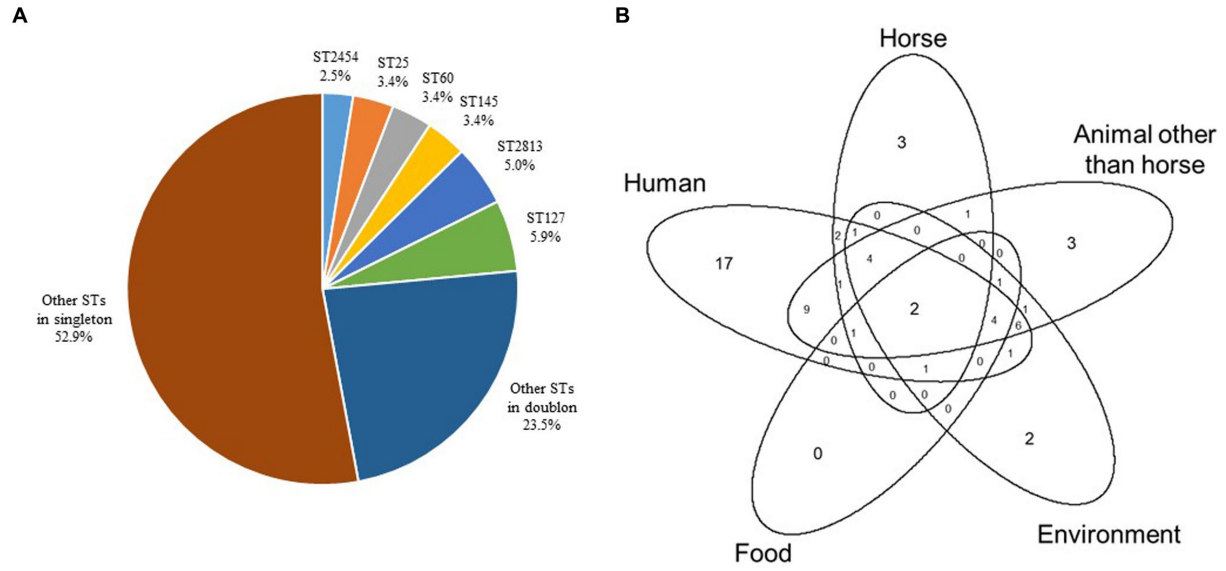

FIGURE 2
Distribution of ST genotypes in the 119 equine *K. pneumoniae* strains studied. **(A)** Distribution (%) of ST genotypes. **(B)** Venn diagram to illustrate overlaps of the source types/hosts (i.e., human, horse, animal other than horse, environment, and food) of STs identified in this study and already present in the BIGSdb-Kp database (https://bigsdb.pasteur.fr/klebsiella/).

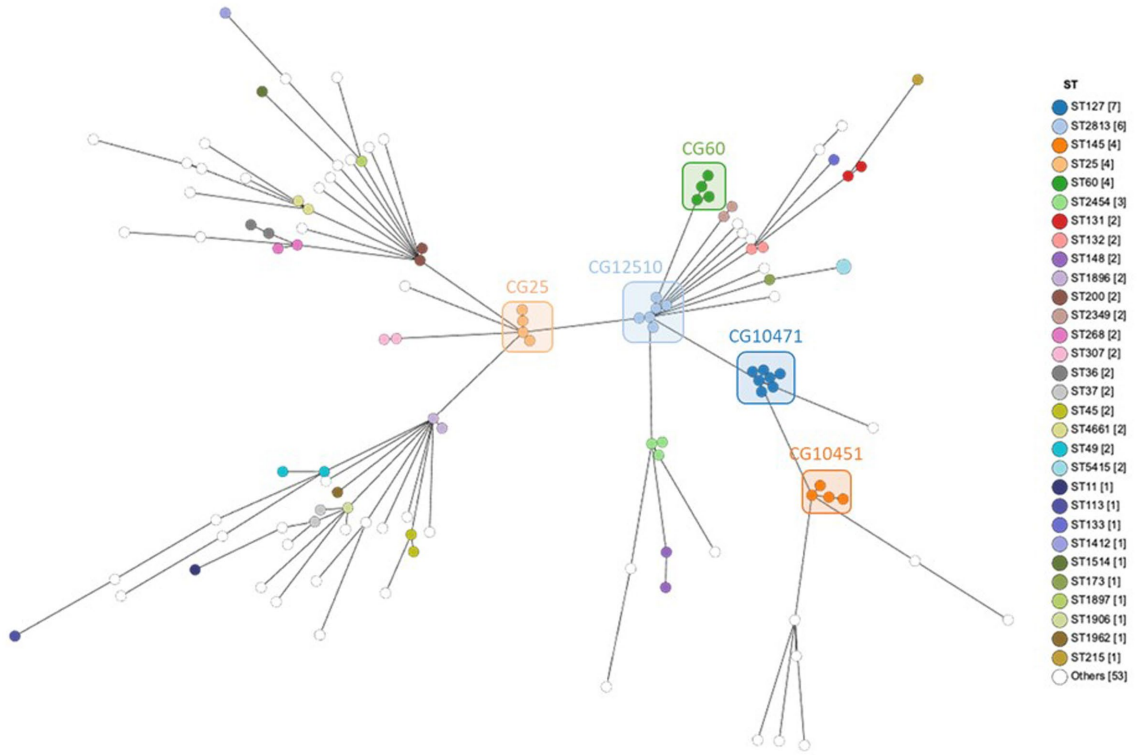

FIGURE 3
MST showing cgMLST analysis of 119 equine *K. pneumoniae* studied (1809 genes without missing data) according to the ST assignment. Node color indicates the ST assignment. The five most frequent CGs are boxed.

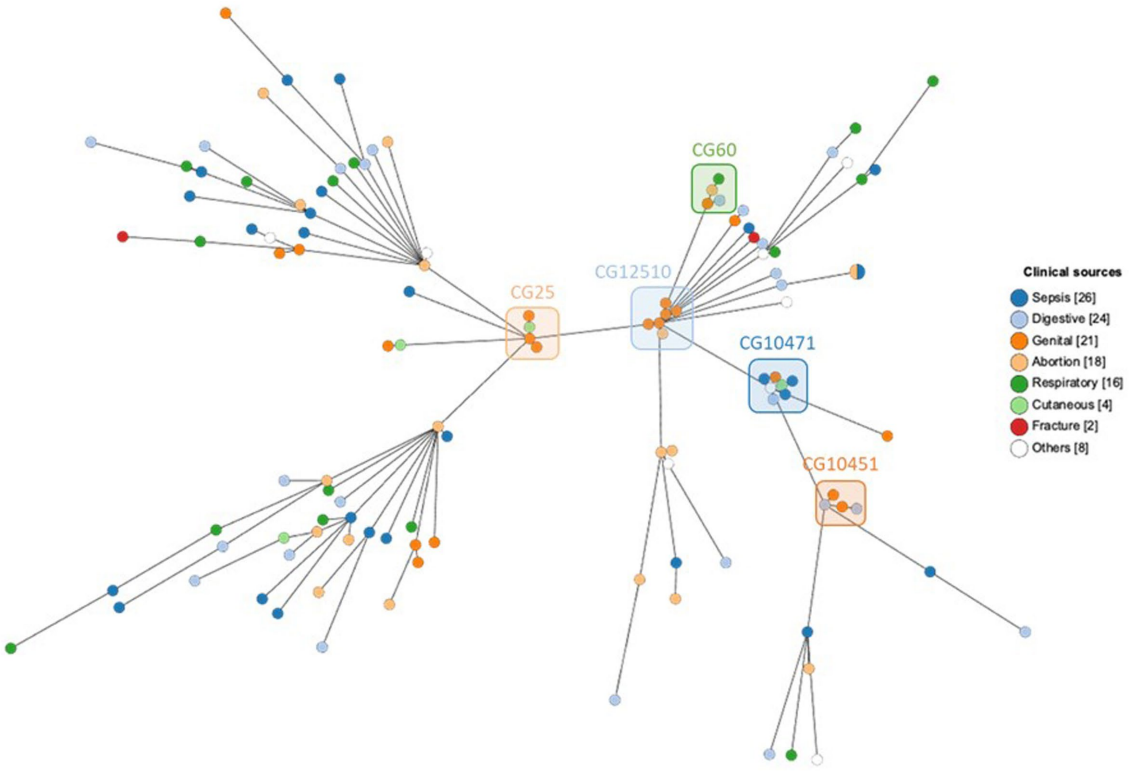

FIGURE 4
MST showing cgMLST analysis of 119 equine *K. pneumoniae* studied (1809 genes without missing data) according to the clinical sources. Node color indicates the clinical sources. The five most frequent CGs are boxed.

TABLE 3 Comparison of assigned antigens K1, K2 and K5 by WGS and K125 PCR detection.

WGS		K125 PCR			
wzi variant	Assigned K-antigen	K1	K2	K5	Not detected
wzi-12	K1	4	0	0	0
wzi-181	Not assigned	1	0	0	1
wzi-2	K2	0	0	0	2
wzi-72	K2	0	4	0	0
wzi-59	K3	0	1	0	2
wzi-5	K5	0	0	3	0
wzi-482	Not assigned	0	0	1	1

suggesting a community acquisition of this pathogen that is frequently associated with equine clinical samples (Duchesne et al., 2019; Bourély et al., 2020; Léon et al., 2020). Thus, the most frequent populations were rarely described elsewhere with CG10451 (seven K30-ST127), CG12510 (six K22.37-ST2813), CG25 (four K2-ST25) composed of non-hypervirulent but MDR strains, and CG60 (four *wzi-482* or K5-ST60), CG10451 (four *wzi-274* or K55-ST145) composed of hypervirulent strains of which half were MDR. This finding does not confirm the conventional consideration of two individual populations associated with MDR strains (mainly belonging to CG258) and hypervirulent strains (mainly belonging to K1-CG23 and K2-CG25; note that four K2-CG25 strains were identified in our study, all were MDR but not hypervirulent) of *K. pneumoniae* found in humans (Holt et al., 2015; Russo and Marr, 2019; Wyres et al., 2020; Dong et al., 2022). In particular, we did not find any K1-ST23 strains while Lam et al.'s, 2018 study performed a comparative study of 97 genomes from both human and equine hypervirulent CG23 strains, including 15 equine K1-ST23 strains isolated from 1980 to 2004 in France. This epidemiological difference could be explained by the fact that their *K. pneumoniae* population was collected from genital samples, e.g., cervix, fetus, genital tract, mare metritis, stallion sperm (Lam et al., 2018), which was an origin that represented only 18% of the samples in our study. Furthermore, the only four K1 strains we identified were not of a genital origin. However, it is important to note that we found 11/119 (9.2%) hypervirulent strains based on virulence genes including the biomarkers described by Russo et al. (2018). Interestingly, seven of these strains were necropsy-associated. They could play a role in virulence and severe infections but did not cover all 65 horses (out of 94 necropsy-associated, 69.1%) of our panel where the lesions observed at necropsy were related to the cause of death (Supplementary material). Numerous genetic factors contribute to the ability of *K. pneumoniae* strains to cause severe diseases and probably most of them are still not known and/or not well enough known to affirm that they enhance severity, in particular in an unexplored host, like a horse. In our work, we revealed substantial allelic and gene content heterogeneity and sometimes partial coverage of well-described virulent plasmids such as pLVPK and pK2044 (e.g., MDR-hypervirulent strain 132). The extent and clinical impact of allelic and/or truncated virulence genes remain uncertain. In addition

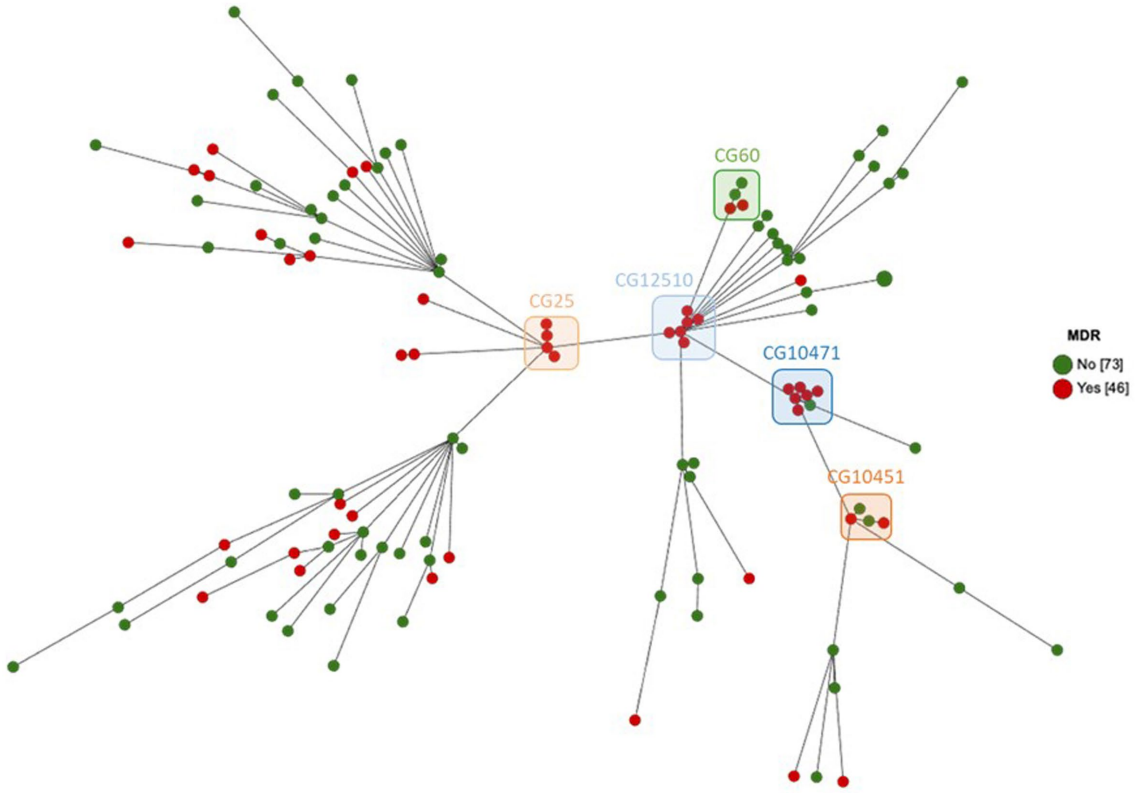

FIGURE 5
MST showing cgMLST analysis of 119 equine *K. pneumoniae* studied (1809 genes without missing data) according to the MDR phenotype. Node color indicates the MDR phenotype. The five most frequent CGs are boxed.

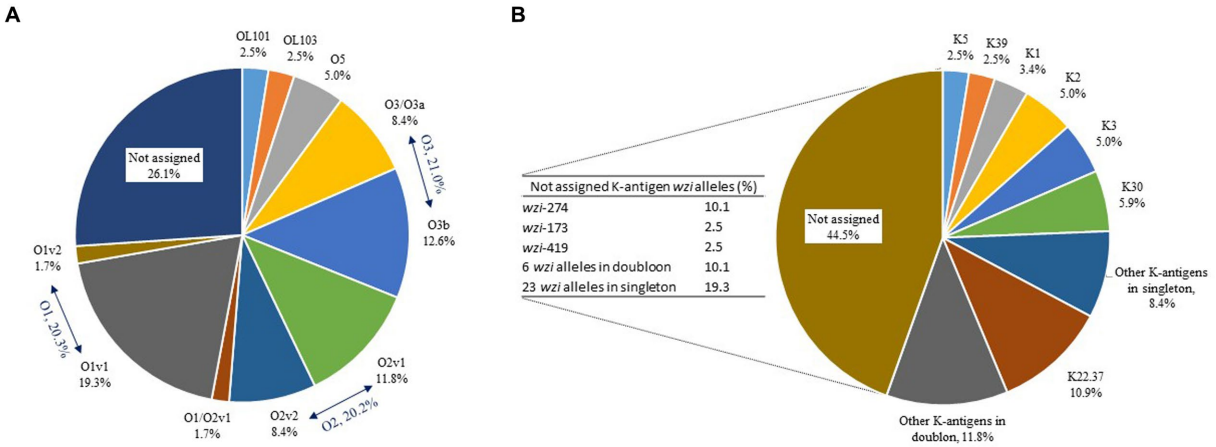

FIGURE 6
Distribution (%) of O-antigens **(A)** and K-antigens **(B)** in the 119 equine *K. pneumoniae* strains studied. Distribution (%) of *wzi* variants corresponding to the 44.5% of K-antigens not assigned is presented in the table to the left of the pie chart.

to this, we also need to determine by further studies the potential role of the other co-infecting bacteria found with *K. pneumoniae* (Supplementary material).

A highlight of our study is the description of five *K. pneumoniae* strains that are both MDR and hypervirulent, thus exacerbating the threat posed by very limited treatment options. The highly mosaic nature of *K. pneumoniae* plasmids creates the risk of MDR and virulence determinants converging within a single plasmid (Lam et al., 2019). However, this convergence was concentrated within a small number of STs comprising well-known hypervirulent (e.g., ST23, ST86, ST65) or MDR lineages (e.g., ST11, ST15, ST231, and ST147) (Lam et al., 2021). Geographically, the focal point for

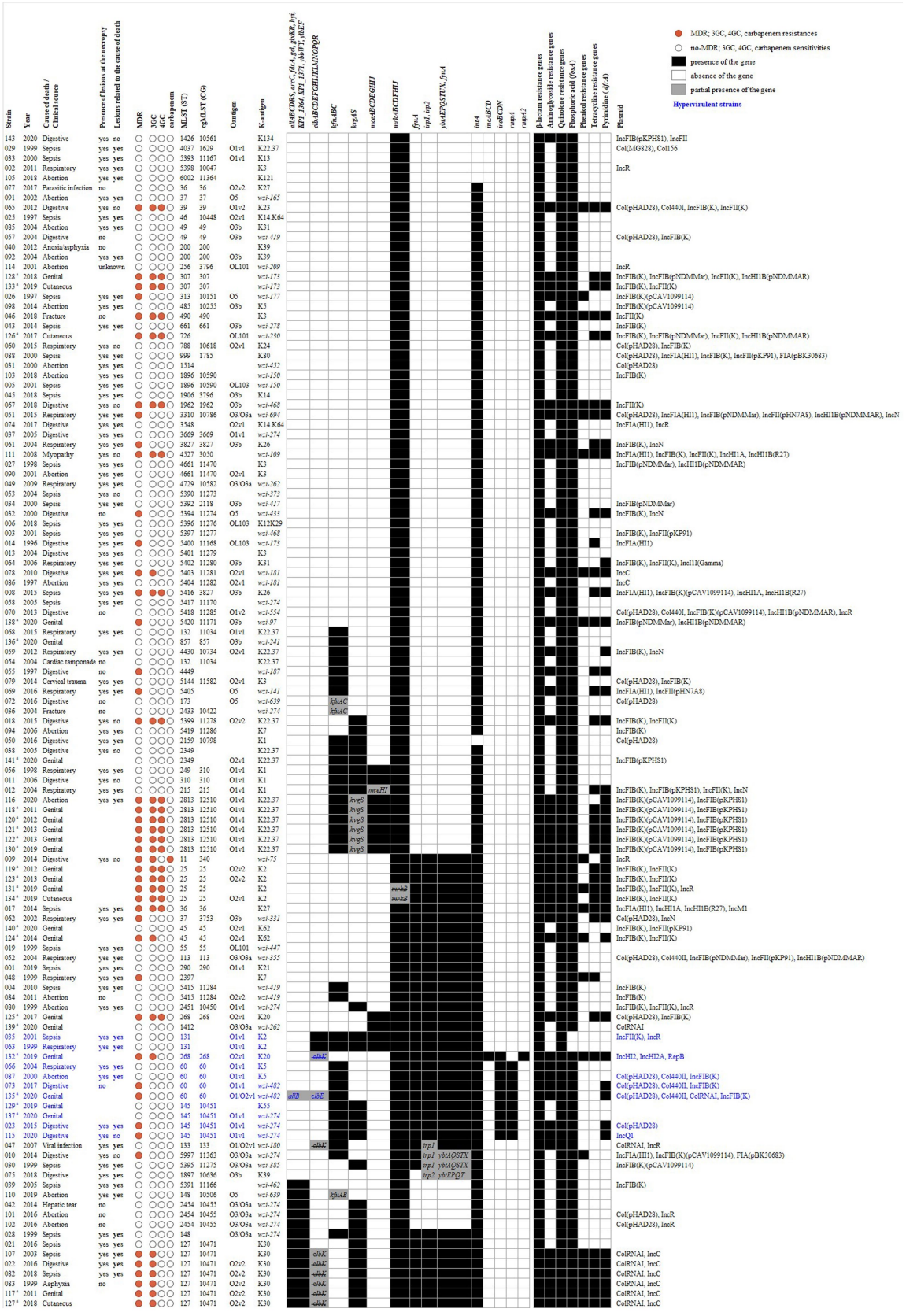

FIGURE 7
Heatmap of MDR, 3GC, 4GC, and carbapenem status, ST, O-antigen, K-antigen, virulence gene/cluster and antimicrobial resistance gene content of *K. pneumoniae* isolates ($n = 119$). The circular symbols represent MDR, 3GC, 4GC and carbapenem status as either MDR/resistance (red) or not MDR/sensitivity (white). The presence (black) or absence (white) of virulence genes/clusters and antimicrobial resistance genes is indicated. The partial

(Continued)

FIGURE 7 (Continued)
presence of virulence clusters is highlighted in gray with a mention of the gene(s) present (gene not crossed out) or absent (gene crossed out). The antimicrobial resistance genes were grouped by antibiotic family and a positive result can concern more than one gene. Hypervirulent strains based on genotypic virulence profile are indicated in blue. Non-necropsy-associated strains ($n = 25$) are marked with an[a].

TABLE 4 Plasmid identification.

Plasmid	All strains ($n = 119$)	MDR ($n = 46$)	not MDR ($n = 73$)	p-value[a]
Col (MG828)	1	0	1	1.0000[c]
Col (pHAD28)	19	7	12	0.8595[b]
Col156	1	0	1	1.0000[c]
Col440I	2	1	1	1.0000[c]
Col440II	4	2	2	0.6397[c]
ColRNAI	9	7	2	**0.0267**[c]
IncC	8	7	1	**0.0053**[c]
IncFIA (HI1)	9	7	2	**0.0267**[c]
IncFIB (K)	34	16	18	0.2338[b]
IncFIB (K) (pCAV1099-114)	12	9	3	**0.0104**[c]
IncFIB (pKPHS1)	9	6	3	0.0869[c]
IncFIB (pNDM-Mar)	6	4	2	0.2042[c]
IncFII	1	0	1	1.0000[c]
IncFII (K)	17	14	3	**0.0001**[b]
IncFII (pHN7A8)	2	2	0	0.1474[c]
IncFII(pKP91)	4	0	4	0.1576[c]
IncHI1A	3	3	0	0.0554[c]
IncHI1B (pNDM-MAR)	7	4	3	0.4275[c]
IncHI1B (R27)	3	3	0	0.0554[c]
IncHI2	1	1	0	0.3866[c]
IncHI2A	1	1	0	0.3866[c]
IncI1-I (Gamma)	1	0	1	1.0000[c]
IncM1	1	1	0	0.3866[c]
IncN	6	4	2	0.2042[c]
IncQ1	1	1	0	0.3866[c]
IncR	9	1	8	0.1508[c]
IncX3	2	1	1	1.0000[c]
FIA (pBK30683)	2	1	1	1.0000[c]
RepB	1	1	0	0.3866[c]

[a]MDR strains were compared with strains that were not MDR.
[b]Pearson's Chi-squared test.
[c]Fisher's exact test.

convergence appears to be Asia, where both MDR and hypervirulence are common (Wyres et al., 2020). Interestingly, four MDR-hypervirulent *K. pneumoniae* found in our horse panel belong to other STs (*wzi 482*-ST60 and *wzi 274*-ST145) that originate from France but had never been previously described. Only one strain in our panel—K20-ST268 strain 132—with 3GC resistance had already been described in a human healthcare surveillance system in China (Tang et al., 2020). The circulation of MDR-hypervirulent *K. pneumoniae* in horses in France is of concern from a One Health perspective, and requires greater awareness on a national scale.

MDR *K. pneumoniae* clones display much greater diversity and are known to be associated with a common cause of opportunistic infections in hospitalized patients. Highly-resistant lineages (including those resistant to 3GC and/or carbapenems) spread around the world rapidly. These MDR *K. pneumoniae* populations belong to CG258, CG15, CG29, CG37, CG147, and CG101 (Wyres et al., 2020). Here again, our work indicates sporadic or localized spread (confirmed by

TABLE 5 Virulence gene identification.

Gene	Virulence factor	All strains (*n* = 119)	MDR (*n* = 46)	Not MDR (*n* = 73)	*p*-value[a]
mrkABCDFHIJ	Type-3 fimbriae synthesis	117, 2 *mrkACDFHIJ*	44, 2 *mrkACDFHIJ*	73	1.0000[b]
iutA	Aerobactin synthesis	113	46	67	**0.0460**[b]
irp1, irp2	Iron regulatory protein (yersiniabactin-encoding gene)	38, 3 *irp1*, 1 *irp2*	21, 1 *irp1*	17, 2 *irp1*, 1 *irp2*	**0.0232**[b]
ybtAEPQSTUX	Yersiniabactin biosynthesis	38, 2 *ybtAQSTX*, 1 *ybtEPQT*	21, 1 *ybtAQSTX*	17, 1 *ybtAQSTX*, 1 *ybtEPQT*	**0.0148**[b]
fyuA	Yersiniabactin uptake receptor / Iron uptake	40	22	18	**0.0092**[b]
kfuABC	Ferric ionic-uptake system / Iron uptake	34, 1 *kfuB*, 2 *kfuC*	13	21, 1 *kfuB*, 2 *kfuC*	0.5963[b]
kvgAS	Two-component system KvgAS	27, 6 *kvgS*	9, 6 *kvgS*	18	0.3454[b]
allABCDRS	Allantoin metabolism	13, 1 *allB*	6, 1 allB	7	0.3534[b]
arc	Carbamoyl phosphate degradation	13	6	7	0.5564[b]
fdrA	Succinate-CoA ligase	13	6	7	0.5564[b]
gcl, glxKR	Glycolate degradation	13	6	7	0.5564[b]
Hyi	Glyoxylate metabolic process	13	6	7	0.5564[b]
KP1_1364, KP1_1371	Unknown function	13	6	7	0.5564[b]
ybbWY	Allantoin permease / Purine permease	13	6	7	0.5564[b]
ylbEF	Catabolic oxamate carbamoyltransferase	13	6	7	0.5564[b]
mceABCDEGHIJ	Microcin E492 (pore-forming bacteriocin) synthesis	12, 1 *mceHI*	7	5, 1 mceHI	0.2334[b]
clbABCDEFGHIJKLMNOPQR	Colibactin synthesis	2, 8 *clbABCDEFGHIJLMNOPQR*, 1 *clbE*	7 *clbABCDEFGHIJLMNOPQR*, *1 clbE*	2, 1 *clbABCDEFGHIJLMNOPQR*	**0.0218**[c]
iroBCDN	Salmochelin synthesis	9	5	4	0.3051[c]
rmpA, rmpA2	Regulator of mucoid phenotype A/A2	8 *rmpA*, 1 *rmpA2*	5	4	0.3051[c]
iucABCD	Aerobactin biosynthesis	1	1	0	0.3866[c]

[a]MDR strains were compared with strains that were not MDR.
[b]Pearson's Chi-squared test.
[c]Fisher's exact test.

the cgMLST approach implemented) of rare and distinct STs that have rarely been described in the literature. Furthermore, we found a fairly high proportion of MDR strains out of the 119 studied (*n* = 46, 38.7%; including 31 strains (26.1%) resistant to at least one 3/4GC tested, mainly due to plasmid acquisition containing ESBL or AmpC genes). However, we also report the presence of several genotypes that are globally distributed and associated with MDR in humans (Dong et al., 2022), like the ST11 (CG258) carbapenemase producer, or ST307 (CG307) and K2-ST25, which concern, respectively, one, two and four strains among our panel of 3GC-MDR strains. A recent Israeli case control study on 3GC-resistant *Enterobacterales* infections in hospitalized horses and donkeys showed that the *Klebsiella* spp. were the most common 3GC-resistant *Enterobacterales* detected (Shnaiderman-Torban et al., 2021). Interestingly, we note a large

increase in MDR between the 1996–2007 (nine MDR/48, 18.8%) and 2008–2020 (18 MDR/46, 39.1%) periods among the 94 necropsy-associated strains isolated. Seven hypervirulent strains out of 11 were isolated in the 2008–2020 period, which is also the case for all five MDR-hypervirulent strains studied.

A limitation of our work is that it is a retrospective study designed to include two distinct but complementary sources of *K. pneumoniae* strains of equine origin. The first one is based on nationwide diagnostic necropsy activity from 1996 to 2020, and the second one provides an antibiotic susceptibility profile and a selection of strains of genital origin isolated during the routine diagnostic activity of a regional veterinary laboratory, since *K. pneumoniae* is one of the pathogens sought before and during the breeding season among thoroughbreds, due to its propensity to cause metritis, infertility and abortion in mares (Léon et al., 2020).

Nevertheless, our analyses provide valuable insights and essential data to motivate enhanced public health surveillance among horses.

Conclusion

In conclusion, our results clearly emphasize the importance of improving the surveillance of *K. pneumoniae* strains in routine equine diagnostic tests to detect high-risk MDR and/or hypervirulent strains. A better understanding of the epidemiological reservoirs of high-risk *K. pneumoniae* is needed to control their dissemination and provide essential data to public health surveillance bodies, both for humans and animals considering the One Health perspective. Furthermore, the circulation of these worrisome MDR-hypervirulent *K. pneumoniae* strains highlights the fact that they remain undetected by a simple diagnostic approach using K1, K2, and K5 serotypes as is implemented in the French horse-breeding sector. Further studies by genomic analyses are needed to propose better tools for improved epidemiological surveillance to estimate the burden of pathogenic *K. pneumoniae* strains in horses.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://bigsdb.pasteur.fr/klebsiella/, (Klebsequi public projet id 15662-15676, 15689-15696, 21799-21801, 57541-57634). Genomes are also available on the NCBI database using the Bioproject number PRJNA1054041.

Author contributions

FG: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing, Resources, Visualization. CS: Investigation, Validation, Writing – review & editing, Resources. SC: Investigation, Writing – review & editing. NF: Investigation, Writing – review & editing, Resources. KM: Writing – review & editing, Resources. JT: Writing – review & editing, Resources. AL: Validation, Writing – review & editing, Resources. BL: Data curation, Writing – review & editing, Investigation, Software. SL: Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing, Conceptualization, Methodology, Project administration, Visualization. SP: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing, Project administration, Visualization.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1334555/full#supplementary-material

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Clonal and plasmidic dissemination of critical antimicrobial resistance genes through clinically relevant ExPEC and APEC-like lineages (ST) in the dairy cattle population of Québec, Canada

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Antimicrobial resistance can be effectively limited by improving the judicious use of antimicrobials in food production. However, its effect on the spread of AMR genes in animal populations is not well described. In the province of Québec, Canada, a new legislation implemented in 2019 has led to an unprecedented reduction in the use of critical antimicrobials in dairy production. We aimed to investigate the potential link between ESBL/AmpC *E. coli* isolated before and after legislation and to determine the presence of plasmids carrying genes responsible for critical AMR. We collected fecal samples from calves, cows, and manure pit from 87 Québec dairy farms approximately 2 years before and 2 years after the legislation came into effect. The whole genomes of 183 presumptive ESBL/AmpC *E. coli* isolated after cefotaxime enrichment were sequenced. Their phylogenetic characteristics (MLST, serogroup, cgMLST) and the presence of virulence and resistance genes and replicons were examined. A maximum likelihood phylogenetic tree was constructed based on single nucleotide polymorphism (SNPs). We identified 10 clonal lineages (same cgMLST) and 7 clones (SNPs $\leq$ 52). Isolates belonging to these clones could be found on different farms before and after the legislation, strongly suggesting a clonal spread of AMR genes in the population during this 4-year period. All isolates were multidrug resistant (MDR), with clone 2 being notable for the

presence of macrolide, fluoroquinolone, and third-generation cephalosporin resistance genes. We also identified clinically relevant ExPEC (ST10) and APEC-like lineages (ST117, ST58, ST88) associated with the presence of ExPEC and APEC virulence genes, respectively. Our data also suggests the presence of one epidemic plasmid belonging to the IncY incompatibility group and carrying *qnrs1* and $bla_{CTX-M-15}$. We demonstrated that AMR genes spread through farms and can persist over a 4-year period in the dairy cattle population through both plasmids and *E. coli* clones, despite the restriction of critical antimicrobial use. MDR ExPEC and APEC-like STs are present in the normal microbiota of cattle (more frequently in calves). These data increase our knowledge on gene dissemination dynamics and highlight the fact that biosecurity measures should be enhanced in this industry to limit such dissemination.

KEYWORDS

***Escherichia coli*, gene spread, manure pit, legislation, calf**

1 Introduction

The global burden attributable to bacterial antimicrobial resistance (AMR) has been estimated at 1.27 million human deaths in 2019 (Antimicrobial Resistance Collaborators, 2022). Therefore, tackling AMR has become a public health priority worldwide. In recent years, several international organizations [World Health Organization (WHO), Food and Agriculture Organization (FAO), World Organization for Animal Health (WOAH)] and several countries have developed strategies to fight AMR (United Nation General Assembly, 2016). Improving the judicious usage of antimicrobials (AM) in food production is one of them (Tang et al., 2017). The province of Québec (Canada) adopted a new legislation in February 2019, to limit usage of category I AMs (e.g., third generation cephalosporins, fluoroquinolones or polymyxin B) of the Health Canada classification (Government of Canada, 2009) in production animals (Roy et al., 2020). This new regulation has been very effective in reducing the use of these AMs (Millar et al., 2022). However, the effect of modification of antimicrobial use in food-producing animals on AMR gene dissemination in animal populations is not well described. It should be noted, however, that WHO and Health Canada categorizations are similar but exhibit some differences (Lardé et al., 2021). For example, Category I AM, as defined by Health Canada, aligns with the highest-priority critically important antimicrobials (HPCIA) outlined by the WHO, except for macrolides, which the WHO categorizes as HPCIA whereas they are classified as Category II AM in Canada.

Escherichia coli is a ubiquitous Gram-negative rod. It is mostly commensal and can be found in the gut of all mammals (Gyles et al., 2010). However, depending on the presence of specific virulence and/or resistance genes, it can also cause multiple diseases (from mild diarrhea to fatal sepsis) in both humans and animals. High risk clones were described within the last decades (Mathers et al., 2015; De Lagarde et al., 2021). They are defined as emergent, multi-drug resistant (MDR), highly pathogenic and capable of potent dissemination (De Lagarde et al., 2021), and they are recognized as a cause of major disease outbreaks worldwide (Kocsis et al., 2022). Successful dissemination of these drug-resistant and pathogenic *E. coli* depends on the acquisition and carriage of niche-specific characteristics that allow for steady colonization and persistence. A critical evolutionary step in the emergence of these MDR clones, is the acquisition of multi-drug resistance and/or fitness genes through plasmids. The study of the emergence of these clones and the diverse plasmids carrying resistance and virulence genes present in the animal population is essential to develop fighting strategies such as vaccines and limit the spread through improvement of biosecurity measures.

Extraintestinal pathogenic *E. coli* (ExPEC) are responsible for a significant number of human infections worldwide (Poolman and Wacker, 2015). These strains typically reside in the intestinal microbiota, and from there, they emerge to cause infections outside the intestines. A few specific lineages of ExPEC classified with their sequence type (ST) are responsible for many of these infections. The top five clinically relevant STs are ST131, ST69, ST10, ST405, and ST38 (Manges et al., 2019). Certain sets of ExPEC are causing specific colibacilloses in poultry and have been therefore designed as avian pathogenic *E. coli* (APEC). The APEC predominant lineages are ST131, ST117, ST23, ST428, ST355 (Johnson et al., 2022). Interestingly, the ST131 lineage can be of importance in both poultry and humans. These ExPEC-APEC strains are also known for their association with the acquisition of new and concerning AMR genes. The clinical and economic impact of ExPEC infections, as well as their optimal management in the face of increasing AMR, pose significant challenges that are not fully recognized. Understanding the genetic factors that contribute to the persistence, predominance, and competitiveness of ExPEC strains within the gut microbiota remains unclear but may provide insights into the success of these lineages. Cattle are not recognized as reservoir for ExPEC, however, there are recent evidence that this information might need to be revised (Salaheen et al., 2023).

Our research team already established the portrait of both antimicrobial usage (AMU) and AMR in dairy farms in Québec prior to the regulation implementation (Lardé et al., 2021; Massé et al., 2021), and the impact that the regulation had on AMU and AMR in *E. coli* isolates (De Lagarde et al., 2022; Millar et al., 2022). We also characterized antimicrobial resistance genes in

ESBL/AmpC isolates before the regulation implementation and determined the correlation between phenotypes and genotypes in these isolates (Massé et al., 2023). However, we have not yet characterized the ESBL/AmpC *E. coli* isolated after the restriction of category I AMU, and the persistence of resistance genes, clones, and plasmids. Therefore, the first objective of this study was to characterize and assess the putative phylogenic link between ESBL/AmpC *E. coli* gathered over a 4-year period (pre- and post-regulation implementation). Secondly, we aimed to determine the presence of plasmids carrying genes responsible for resistance to critical AM in these isolates, and whether these plasmids were able to persist over a 4-year period.

2 Materials and methods

2.1 Selection of herds and sample collection

The herd selection and sample collection were described previously (Massé et al., 2021; De Lagarde et al., 2022). Briefly, we used an observational prospective cohort study on 87 commercial dairy farms. Prior to initiating the research, the protocol was approved by the Animal Use Ethics and the Research Ethics Committees of the Université de Montréal (20-Rech-2085). The 87 farms were in Montérégie, Centre-du-Québec and Estrie, Québec, Canada, three of the main dairy areas of Québec. These regions were selected based on the proximity to the veterinary faculty of the Université de Montréal. The farms were randomly selected in the three regions from a list of dairy farms provided by the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ; Québec's department of agriculture, fisheries, and food). In total, four samplings were carried out. The two first samplings were performed approximately 2 years before the regulation implementation (April to June 2017, October to November 2017). Two additional samplings were performed approximately 2 years after the regulation implementation (August to September 2020 and February to March 2021). The timeline of the sampling was previously illustrated (De Lagarde et al., 2022).

The sampling protocol was previously described (De Lagarde et al., 2022). Briefly, on each visit, fecal samples were collected from five pre-weaned calves and mixed to obtain a composite sample. Fecal samples of five lactating cows were also collected and mixed to obtain another composite sample. On each farm, a convenience sample was assembled based on accessibility of the calves and cows. Fecal samples were obtained directly from the rectum for calves and freshly voided cow feces were obtained from the floor. A composite manure sample was also collected from two convenient locations in the manure pit. For each composite sample, approximately 25 g of feces or manure were placed in a 50 mL sterile tube and stored immediately on ice at the farm. Samples were processed in the laboratory within < 24 h. A preservative medium (peptone water with 30% glycerol) was added to the sample at a 1:1 volume to weight ratio; samples were then homogenized and frozen at −80°C.

As part of a wider project on AMR and AMU, we also gathered information on farm location and veterinarian care.

2.2 Bacterial isolation and presumptive ESBL/AmpC *E. coli* identification

The protocol for bacterial isolation was previously described (Massé et al., 2021). Briefly, composite fecal samples were processed according to the laboratory protocol of the European Union Reference Laboratory on Antimicrobial Resistance for the recovery of ESBL-, AmpC- and carbapenemase-producing *E. coli* from composite fecal samples. The protocol is available online at https://www.eurl-ar.eu/protocols.aspx. One gram of each composite fecal or manure sample was added to 9 mL of Buffered Peptone Water, then incubated at 37°C for 20 h. One loop (10 µl) was streaked onto a MacConkey agar plate containing 1 mg mL-1 of cefotaxime, then incubated at 44°C for 20 h. Lactose positive colonies were subcultured onto Columbia agar with 5% sheep blood, and then incubated overnight at 37°C. Identification of *E. coli* was confirmed by MALDI-TOF MS. Composite samples with at least one *E. coli* colony isolated with this technique were labeled as presumptive ESBL/AmpC *E. coli*. All *E. coli* selected were incubated for 24 h at 37°C in Luria-Bertani (LB) broth then mixed 50:50 with 30% glycerol and stored at −80°C.

2.3 DNA extraction, library preparation and whole genome sequencing

Due to financial and logistic restrictions, we sequenced 183 isolates in total. Most isolates were randomly selected within the collection pre- and post- regulation (178/183), ensuring that we had a similar number of isolates for both periods. Additionally, a subset of 5 isolates in the pre-regulation collection were selected because they presented an atypical phenotype (Massé et al., 2023). Genomic DNA was extracted using QIAamp DNA Mini Kit for DNA following manufacturer's guidelines (Qiagen, Hilden, Germany). The isolates gathered before the regulation were sequenced with MiSeq platform with 2 × 300 paired end runs after library preparation with the Illumina Nextera XT DNA Library preparation kit, according to the manufacturer's instructions. The isolates gathered after the regulation were sequenced on the Illumina (San Diego, CA) iSeq100 platform with 2 × 150 paired end runs after library preparation with the Illumina DNA prep kit (former Nextera Flex kit), according to the manufacturer's instructions.

Illumina platform was used to assemble genomes using SPADES 3.9.0 (Bankevich et al., 2012). An assembly was rejected if the number of contigs (> 500 bp) was > 400 or if the N50 was $< 50{,}000$. Details of data assembly quality are available in **Supplementary Table 1**.

2.4 Multi locus sequence typing (MLST), serotype, phylogroup and adhesin *fimH*

Multi locus sequence typing (MLST) (Larsen et al., 2012) (minimal depth for the detection of an MLST allele was 5x), O and H serotypes (Joensen et al., 2015) (85% identity and 60% coverage to count as a hit) and the *fimH* subtype (Roer et al., 2017) (95%

identity to count as a hit) were determined by analysis of generated FASTA files using the Center of Genomic Epidemiology (CGE) platform.[1] The *fimH* gene is part of the *fim* operon, which encodes for type 1 fimbriae found in most *E. coli* strains.

Phylogroups were determined with *in silico* PCR using the Clermont Typing platform[2] (Beghain et al., 2018). These parameters (MLST, serotype, phylogroup and *fimH* subtype) will be referred to as phylogenetic characteristics.

2.5 Virulence and resistance genes and replicons

To determine the presence of virulence genes, AMR genes and point mutations, Virulence finder, Res Finder 4.0 and Point Finder were used on the CGE platform (Bortolaia et al., 2020). The default parameters were used for each application (90% identity and 60% coverage).

PlasmidFinder (Carattoli et al., 2014) was used to determine the presence of replicons (95% identity and 60% coverage). Mobile genetic element (MGE) (Johansson et al., 2020) was used to identify mobile genetic elements and their relation to antimicrobial resistance genes and virulence factors. The CGE platform was used for both tools.

2.6 Phylogenetic analysis

Phylogenetic analysis was performed with an "in-house" pipeline. We used the Digital Research Alliance of Canada computing servers (alliancecan.ca). Raw data preprocessing and phylogenetic analysis were performed as follow: first, we trimmed low quality reads using Trimmomatic (Bolger et al., 2014) with default parameters. Second, trimmed reads were mapped on the reference genome (isolate *E. coli* K12, NC 000913) using BWA (Li and Durbin, 2009). Mapping files were further converted from bam to vcf format and were filtered out using vcftools (Danecek et al., 2011). Finally, Fasttree2 (Price et al., 2010) was used to generate the selection of best models and phylogenetical analyses. Conversion of multiple formats between these different steps has been performed using a combination of freebayes (Garrison and Marth, 2012), samtools (Danecek et al., 2021), gatk software (Van Der Auwera and O'Connor, 2020) and Fasta2Phylip perl program.[3] The complete python pipeline is available at https://github.com/yterrat/AMR_FMV/blob/main/pipeline_mapping.py.

The SNP phylogenies were annotated with the relevant metadata using iTOL[4] (Letunic and Bork, 2016).

A clonal lineage was defined as a group of isolates that belong to the same core genome MLST (cgMLST). We used the cgMLSTfinder 1.2 application (Clausen et al., 2018; Zhou et al., 2020) available on CGE platform.

Clones were defined as previously described (De Lagarde et al., 2021). Briefly, only branches from nodes with a bootstrap value of 1 and groups of three or more isolates were considered. Furthermore, the maximum number of SNPs between pairs of isolates within a group, defined as the SNP_{max} was $< M \times T \times P$ where M is the mutation rate of *E. coli*, which has been described as 3×10^{-6} per year per site (Grad et al., 2012), T is the number of years between two isolates and P is the total number of nucleotide sites analyzed in all genomes for each isolate.

Singletons were defined as unique isolates in terms of phylogenetic characteristics (MLST, serotype, *fimH* gene and phylogroup).

Each farm where an ESBL/AmpC isolate was retrieved, was geolocated at the centroid of the 3-digit postal code area with GeoPinpointTM Suite (DMTI Spatial Inc.). Then geographical distribution of farms for clones was performed in ArcGIS (version 10.8.1).

3 Results

3.1 *E. coli* collection description

During the sampling prior to the regulation implementation, 599 fecal samples were collected from 101 dairy farms. A total of 214 ESBL/AmpC-producing *E. coli* were obtained using the selective protocol.

All farmers were contacted in July 2020 and asked to participate in the post-regulation sampling. A total of fourteen farmers either refused or were not able to join the second part of the study. Among the 516 fecal composite samples collected from 87 dairy farms, we recovered 162 presumptive ESBL/AmpC *E. coli*.

The genomes of 182 isolates belonging to the pre- and post-regulation collections (91 in each collection) were completely sequenced. As already published, 85 and 82% of herds were positive for presumptive ESBL/AmpC-producing *E. coli* in at least one sample in 2017 (Massé et al., 2021) and 2020–2021 (De Lagarde et al., 2022), respectively, with no significant differences detected between the two periods (De Lagarde et al., 2022).

3.2 Identification of 6 clones over the 4-year period

Overall phylogeny is presented in **Figure 1**. Among the 182 isolates, the predominant phylogroups were A (n = 61) and B1 (n = 57). The phylogroups C, D, E, F and G were also represented. Various serogroups were identified, the most predominant serogroup being O101:H9 (n = 15). Numerous MLST were also determined, with the predominant one being the ST10 (n = 28). More than 30 types of *fimH* were identified (data not shown on **Figure 1**). The most predominant was *fimH*54 (n = 28), however, it was not possible to associate any of them specifically with any other phylogenetic characteristics. Phylogenetic characteristics of all isolates are also available in **Supplementary Table 1**.

Ten clonal lineages (sets of isolates belonging to the same cgMLST) were identified. The maximal number of SNPs used to

1 http://www.genomicepidemiology.org/

2 http://clermontyping.iame-research.center/

3 https://github.com/josephhughes/Sequence-manipulation/blob/master/Fasta2Phylip.pl

4 http://itol.embl.de

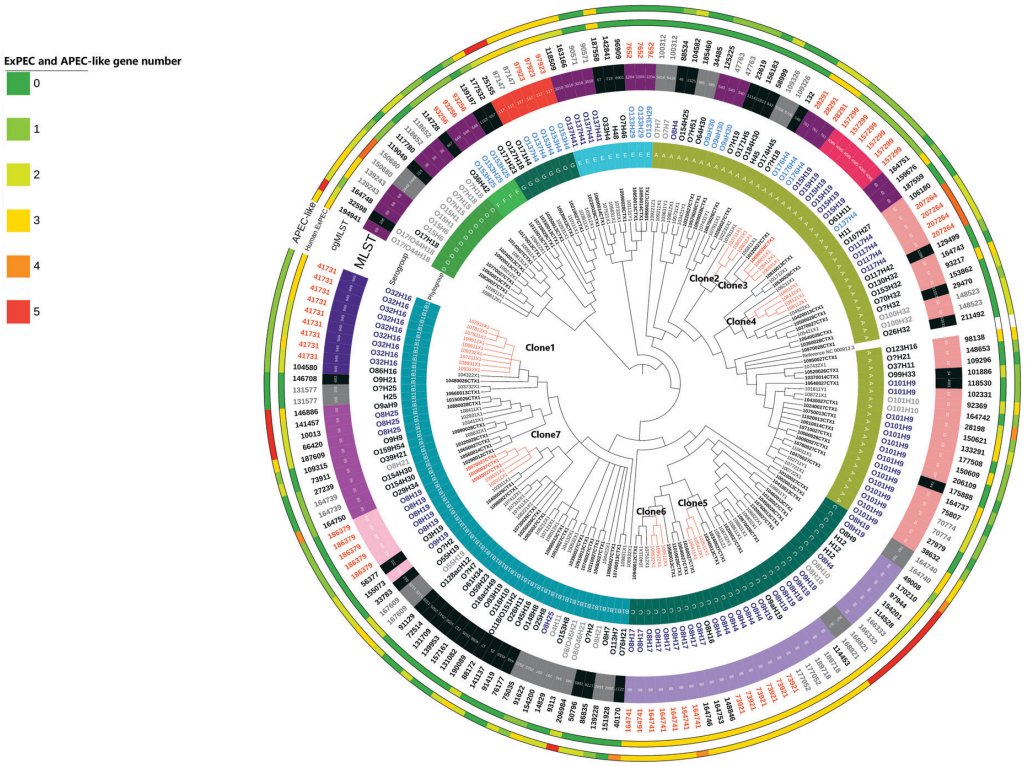

FIGURE 1
Overall phylogeny based on SNP distance of 182 ESBL/AmpC *E. coli* isolates. The length of the branches is not proportional to the phylogenetic distance. Each branch with a bootstrap value under 1 was collapsed. Samples with ID labels in bold indicate those collected prior to the implementation of the regulation. Isolates are considered as clones if there are not different one from another with more than 52 SNPs. The presence of the 5 APEC-like and 5 human ExPEC predictor genes (Johnson et al., 2003, 2008) is codified by color (from 0 to 5 corresponding to green to red).

define a clone using our definition was 52. With this definition, we identified 7 clones (illustrated with the maximum number of SNPs between two isolates in **Figure 1**). Distance matrix is provided in **Supplementary Table 2**. On the 31 isolates belonging to the 7 different clones, 20 (65%) were identified in calves' samples, 8 (25%) were identified in manure pits and 3 (10%) were identified in cows' samples. Isolates belonging to clone 6 (n = 3) were identified only on farm #95. Isolates belonging to other clones (1, 2, 3, 4, 5, 7) were identified across multiple farms (from 2 to 7), suggesting clonal dissemination within farms. As illustrated in **Figure 2**, clones 3 and 7 (in orange) were composed of isolates identified before and after the regulation implementation, suggesting the persistence of this clone over the 4-year period (from April 2017 to March 2021).

3.3 AMR profiles

All isolates were resistant to third generation cephalosporins, as they were selected following enrichment with cefotaxime. All but 4 isolates carried at least one gene or mutation among the following: bla_{SHV-12}, $bla_{CTX-M-1}$, $bla_{CTX-M-15}$, $bla_{CTX-M-27}$, $bla_{CTX-M-55}$, $bla_{CTX-M-65}$, $bla_{CTX-M-124}$, bla_{CMY-2}, and a mutation in the ampC promoter (42C). Using CARD (McArthur et al., 2013), we identified a determinant of efflux mechanism that might explain the cephalosporin resistance in the 4 other isolates. The AmpC promoter mutation was exclusively identified in ST88 isolates (data not shown).

Most identified resistance genes were similar between seasons (fall vs. spring) and between periods (pre- and post-regulation implementation). However, the *ereA* gene (n = 11) (responsible for macrolide resistance), the bla_{SHV-12} gene (n = 2) (responsible for ESBL resistance), the *qnrB4* (n = 1) and *qnrB19* genes (n = 2) (responsible for fluoroquinolone resistance), the *aac(6′)-II-c* (n = 2), the *aac(6′)-Ib-cr* (n = 2), the *aac(6′)-Ib-3* (n = 2) and the *bleO* (n = 1) genes (responsible for aminoglycoside resistance), the bla_{CARB2} (n = 10) and bla_{TEM-1C} (n = 2) genes (responsible for penicillin resistance), the *tetD* gene (n = 2) (responsible for tetracycline resistance) and the *dfr15* (n = 1) and *dfr16* (n = 11) genes (responsible for trimethoprim resistance) were only identified post-regulation. On the other hand, the *aadA24* gene (n = 1), $bla_{CTX-M-65}$ gene (n = 1), the *drf7* (n = 4) and *drf8* genes (n = 1) were only detected pre-regulation. The details of genes identified in each isolate can be found in **Figure 3**.

3.4 Virulence profiles of interest

As illustrated in **Figure 1**, 16 isolates carried the 5 APEC-like predictors (*iss*, *iutA*, *ompT*, *hlyF*, *iroN*) (Johnson et al., 2008), commonly present in APEC isolates and, therefore, presenting a putative risk for poultry infection. They were mainly associated

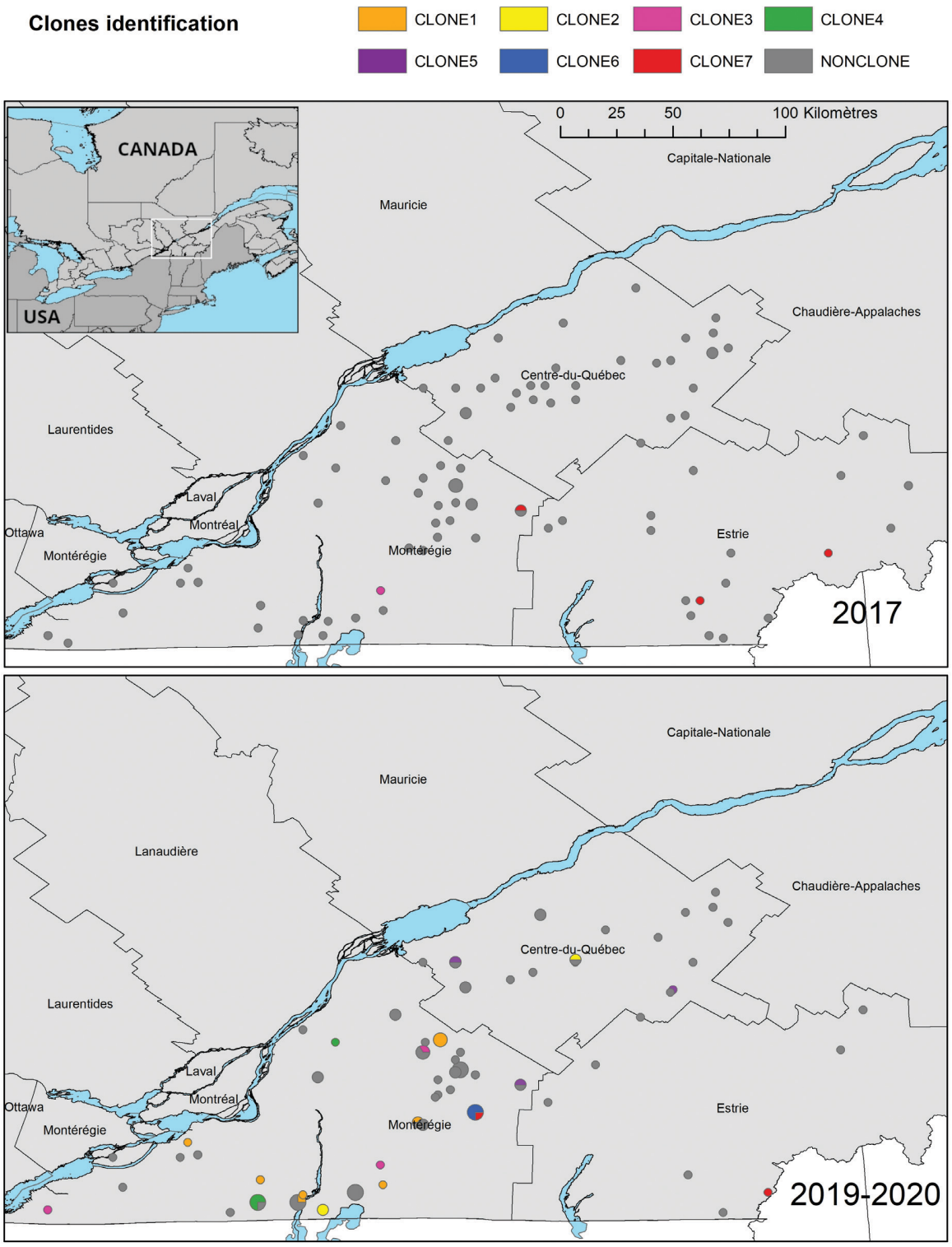

FIGURE 2
Geographical distribution of *E. coli* ESBL-AmpC isolates in the three study regions of the province of Québec, according to their sampling year. Clones were identified as a group of at least three isolates that differ by a maximum of 52 SNPs. A Lambert conformal conic projection (NAD 1983) was used for mapping.

with the ST117 which belongs to predominant pathogenic APEC STs, and to ST58, ST88 which have also been reported as pathogenic STs. The 5 genes commonly present in ExPEC pathogen for human are *afa*, *sfa*, *iutA*, *pap*, *kpsMII* (Johnson et al., 2003). In our isolates, the gene *sfa* was not detected, however, the four other genes were detected. Isolates presenting the maximum number of these genes were associated with the ST10 and the ST2449.

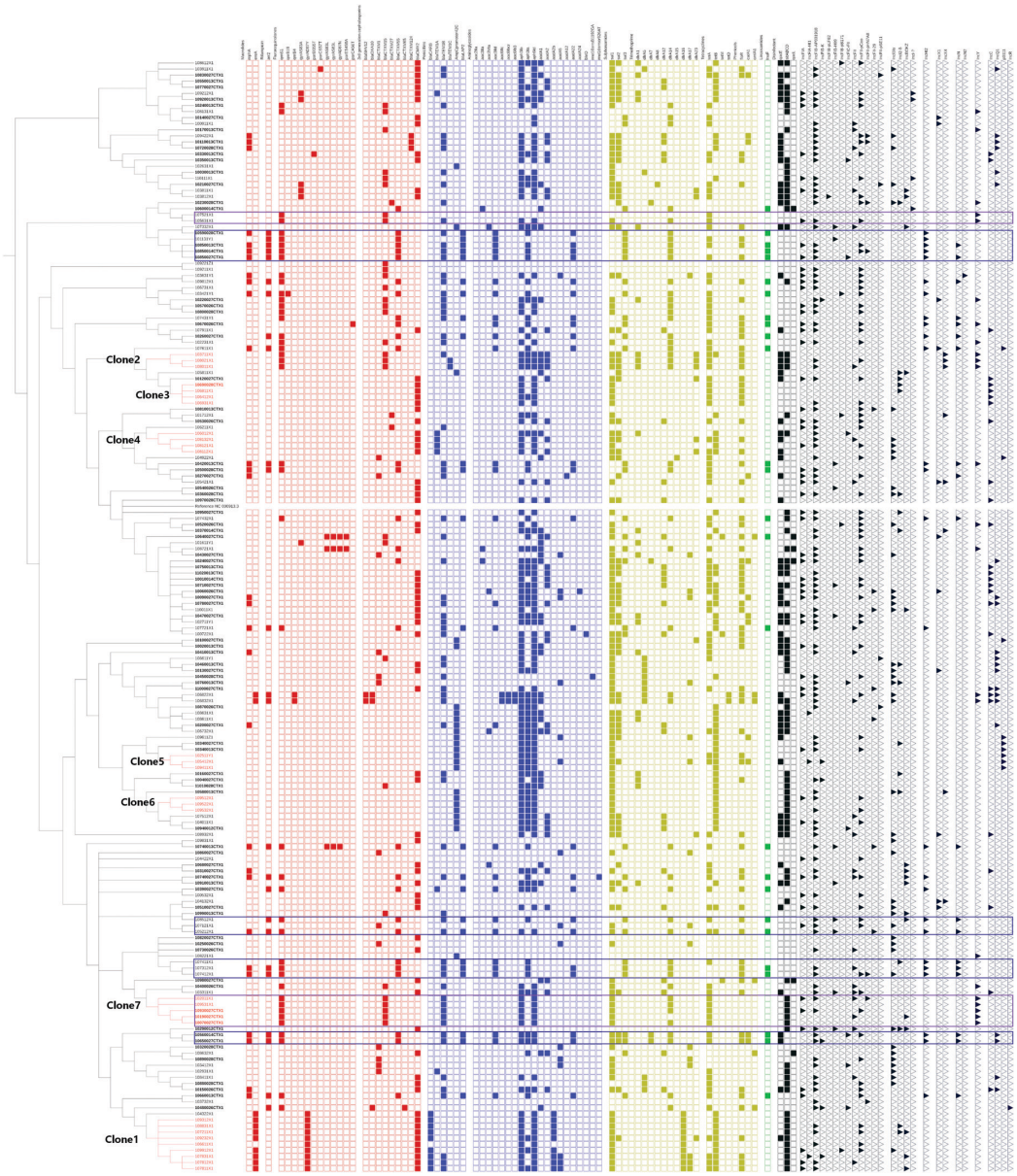

FIGURE 3
Overall phylogeny based on SNP distance of 182 ESBL *E. coli* isolates. The colored square indicates the presence of resistance genes. In red, critical high priority antimicrobials for human medicine. In blue, critical priority antimicrobials for human medicine. In yellow, high priority antimicrobials for human medicine. In green: antimicrobials important for human medicine. The importance of antimicrobials for human medicine was defined according to the World Health Organization. Black triangle indicates the presence of replicons. Violet frames aid the visualization of genes present when the replicon IncY is present. Blue frames aid the visualization of genes present when the replicon IncN and incHI2 are present. Samples with ID labels in bold indicate those collected prior to the implementation of the regulation.

One isolate (10990013CTX), belonging to the B1 phylogroup, O26:H11, MLST21 carried the *eae*, *espA*, *espB*, *espF*, and *tir* genes, therefore, was classified as an Enteropathogenic *E. coli* (EPEC) and was identified in a calf.

All isolates belonging to C (32/32), F (4/4) and G (8/8) phylogroups, all isolates but one belonging to the B1(56/57) phylogroup and 5/11 isolates belonging to phylogroup D (45%) carried the gene *lpfA*, encoding for the major fimbrial subunit of the log polar fimbriae (facilitating attachment) (Toma et al., 2006). Only two isolates carried *stx* toxins genes. These isolates were, respectively, O118:H2, ST17 belonging to B1 phylogroup and H32 (O was not identified) ST155 belonging to A phylogroup.

3.5 Plasmids carrying genes responsible for resistance to fluoroquinolones and 3rd generation cephalosporins

Our data strongly suggests that clone 7 carries a plasmid identified with the replicon IncY. It seems this plasmid carries the following resistance genes: *qnrS1*, $bla_{CTX-M-15}$, bla_{TEM-1},

aph31b, *aph61d*, *sul2*, *drf14*, *tetA*, and *sitABCD* (**Figure 3**, framed in violet), therefore carrying resistance to 6 families of antimicrobials, including 2 critical antimicrobials of category I (C3G and fluoroquinolones). Moreover, *qnrS1* and $bla_{CTX-M-15}$ are found on the same contig on these isolates indicating with certainty that they are linked and flanked with the insertion sequence ISKpn19. We also noticed that no virulence genes seemed linked to the plasmid IncY.

Isolates carrying replicons IncHI2 and IncN seem to also carry resistance genes to 10 antimicrobial families, although we could not directly associate one or the other replicon with the different genes, because they were not found on the same contig. This replicon was identified pre- and post-regulation. Similarly, when identified together in one isolate, $bla_{CTX-M-55}$ and *qnrs1* were not found on the same contig.

3.6 Clones of interest

Isolates belonging to clone 7 were identified pre- and post-regulation, in five different farms. Three out of five farms have bought cows within the last year at the time of the last sampling, and two were clients of the same veterinary clinic. However, no other link could be established between these farms. All isolates carried genes conferring resistance to 6 antimicrobial families and resistance to disinfectant and replicon IncY.

Isolates belonging to clone 1 were identified only post regulation, in 8 different farms, none of which were clients of the same veterinary clinic and two farms had bought cows within the past year at the time of the last sampling. Isolates all possessed a specific *gyrA* (D87Y) mutation found in no other isolate in the whole collection. They also carried genes conferring resistance to 6 other antimicrobial families (specifically bla_{CMY-2}), however, no replicon could be identified clearly in these isolates (see **Figure 2**). Isolates from this clone carried the *mcmA* and the *papC* and *papA* genes and several genes involved in iron modulation.

Isolates belonging to clone 5 were identified only post-regulation, in 3 different farms, none of which were clients of the same veterinary clinic and one farm had bought cows within the last year at the time of the last sampling. Isolates carried the *ampC* promoter mutation and genes conferring resistance to 3 other antimicrobial families. The virulence profile of this clone was particular because its isolates carried several genes usually identified in the ExPEC pathotype (Johnson et al., 2008; Kathayat et al., 2021), being the *iss*, *iutA*, *ompT*, *fyuA*, *hra*, *ireA*, *irp2*, and *iucC* genes. These results suggest that isolates belonging to clone 3 might have the potential to cause infection in poultry.

Isolates belonging to clone 2 were identified only post-regulation in 2 different farms which were not clients of the same veterinary clinic and had not bought cows within the last year at the time of the last sampling. These isolates carried genes conferring resistance to 7 antimicrobial families and the replicon IncY and IncX. As they harbor the same resistance profile and the same replicons, these data suggest that the plasmid present in clone 1 is also present in clone 2.

Isolates belonging to clone 4 were identified only post-regulation and in only one farm. They carried only the bla_{CMY-2} gene and an IncI replicon. The virulence profile of this clone was also of particular interest because these isolates carried several genes usually identified in the ExPEC (APEC-like) pathotype (Johnson et al., 2008; Kathayat et al., 2021), being the *afa*, *iutA*, *ompT*, *papC*, *papA*, *sitA*, *traT*, *cia*, *kpsE*, and *kpsM* genes. These results suggest that isolates belonging to clone 6 might have the potential to cause infection in both poultry and humans, as it carries 4 genes of the human ExPEC predictors (Johnson et al., 2003).

The geographical distribution of clones is illustrated in **Figure 2**. On this map, it is important to notice that clones 3 and 7 have been identified in 2017 and 2019-20, in different locations, suggesting they persisted through the 4-year period and disseminated in different farms. Clones 1, 2, 4, 5 and 6 were identified only in 2019-20. Moreover, clones 1 and 7 and have been identified all over the studied territory on farms over 100 km away.

4 Discussion

The main objective of this study was to characterize and assess the putative phylogenic link between ESBL/AmpC *E. coli* gathered on 87 dairy farms in Québec, over a 4-year period (pre- and post- regulation implementation). Our secondary objective was to determine the presence of plasmids carrying genes responsible for resistance to critical AM in these isolates, and to ascertain whether these plasmids were able to persist over a 4-year period. Indeed, deepening our knowledge on clonal and plasmid dissemination of resistance genes in the Québec cattle population would improve our ability to predict cross-resistance at the local level and provide data to improve biosecurity measures to limit dissemination.

Our analytical approach allowed us to examine the phylogenetic variation of ESBL/AmpC isolates from healthy cattle (calves, cows) and from their direct environment (manure pit). Firstly, the ESBL/AmpC isolates were very diverse. Indeed, all phylogroups were represented with a predominance of A and B1 which are the phylogroups commonly described in commensal *E. coli* in herbivores (Tenaillon et al., 2010). However, we also identified a variety of phylogroups (C to G) that are more commonly associated with pathogenic isolates (Denamur et al., 2021). We identified one EPEC (with the presence of *eae*) and several combinations of ExPEC genes, associated with known pathogenic STs. In terms of virulence, there were several sets of isolates of particular interest. Firstly, the 28 isolates belonging to ST88, phylogroup C, and presenting O8-O9 serogroups carried between 3 and 5 of the ExPEC predictors (Johnson et al., 2008) and were identified as putative pathogens in humans, domestic mammals and birds (Denamur et al., 2021). It is noteworthy that isolates of clone 3 belong to this group and carry resistance genes to 4 AM families. Secondly, 6 isolates belong to ST117 O137-O153/H4 that has been identified as a pathogen in birds (Denamur et al., 2021). Two isolates belong to phylogroup G, ST117, cgMLST 87147 and have been identified in calves. Isolates belonging to the same clonal lineage (same cgMLST) were identified causing omphalitis in Québec in poultry [unpublished data coming from the Animal Pathogenic and Zoonotic *E. coli* database (De Lagarde et al., 2020)]. Thirdly, the clone 6 belonged to the ST10 which has been associated with pathogenicity in Humans (Manges et al., 2019). Moreover, these latter isolates carry 4/5 genes predictors for human pathogenicity (Johnson et al., 2003) and thus, represent a putative

risk for human health. Fourthly, we identified isolates belonging to the ST58 which seems to be an emergent uropathogenic *E. coli* (UPEC) in humans. However, our isolates did not carry the colV replicon associated with virulence (as it is commonly found in isolates of bovine origin) (Reid et al., 2022). Presence of ExPEC genes in these isolates is not surprising because they enhance survival by providing protection against predation by protozoa [such as amoebae (Alsam et al., 2006) and *Tetrahymena* spp. (Steinberg and Levin, 2007)]. However, our results highlight the importance of the surveillance of these strains in the bovine population due to their putative capacity to cross species barriers and cause disease in humans.

Although we observed a variety of strains, it is essential also to notice that we identified several clones. Our definition of a clone is based on several criteria, with a maximal number of SNPs (De Lagarde et al., 2021). This definition might be open to discussion because the mutation rate is subject to environmental pressure and is difficult to establish (Sniegowski et al., 1997; Reeves et al., 2011). Nevertheless, it is still a very stringent definition, and we found clonal isolates on different farms and persisting over a 4-year period. This result suggests that current biosecurity measures are not sufficient to limit the propagation of AMR genes from one farm to another, even though we were not able to determine the vector of dissemination in these farms. Several hypotheses can be considered. Firstly, wild birds have been proposed as a putative vector to spread clonal bacteria over a territory (Skarżyńska et al., 2021). However, it might not be the only route of dissemination. Another possibility may be dissemination through insects and especially through flies (Zurek and Ghosh, 2014). Although a fly usually covers a 2 miles diameter sector, some have been able to travel between 5 and 20 miles (Townsend, 1997). Moreover, it is also possible that they become trapped in vehicles and travel further. Other human vectors (such as animal transporters, inseminators, or other stakeholders) as well as other wildlife species have not been investigated in our study and represent a possible dissemination mode between farms. All these hypotheses are avenues for action to limit the spread of resistance genes and improve biosecurity in dairy farms in Québec.

It will be important to continue surveillance for clone 1, the only clone in our study that possessed a mutation (D87Y) in the *gyrA* gene. This mutation is known to confer resistance to nalidixic acid in *E. coli* isolates (Weigel et al., 1998). Indeed, clone 1 isolates were resistant to nalidixic acid but susceptible to fluoroquinolones (data not shown). This type of mutation might increase the fitness of the clone (Marcusson et al., 2009) and its capacity to spread efficiently. In addition, this clone was the most frequently observed in this study and it only appeared post-regulation, suggesting that it may harbor elements associated with evolution advantage. Moreover, isolates of this clone already possess virulence genes such as *papA* and *papC*, which are a part of the *pap* genes cluster encoding for the proteins required for P-fimbrial synthesis. The P fimbria is recognized as an essential adhesin in UPEC (Lane and Mobley, 2007). The acquisition of a plasmid carrying additional virulence or resistance genes might allow it to become more worrisome for bovine or human health.

The presence of $bla_{CTX-M-15}$ and *qnrs1* on the same contig is quite alarming also because resistance genes to 3rd generation cephalosporins and fluoroquinolones are thus linked together. They confer, to isolates that carry them, resistance to 2 families of critical antimicrobials. Although short read data are limited to circularize plasmids, it is very likely that these two genes were carried by plasmids that carry also other resistance genes from other categories (possibly IncY). This type of plasmid has been detected over the world, in Nigeria, Africa (Alonso et al., 2017) and United Kingdom, Europe [Muna Anjum. abstract of the 9th symposium on antimicrobial resistance in animals and the environment (ARAE)]. This highlights their capacity of dissemination. Moreover, the co-resistance phenomenon enhances the importance of a judicious usage of all antimicrobials, and not only critical antimicrobial for human health. Indeed, through these putative plasmids, ESBL and fluoroquinolones genes might persist in the *E. coli* population even though their usage has stopped. The long-read sequencing of a few isolates of our collection would have been very instructive to document accurately these plasmids. It will be the subject of a subsequent study.

The *ampC* gene is located on *E. coli* chromosome and produces a class 1 cephalosporinase. In its normal state, the expression of *ampC* in wild-type cells is low and does not provide significant resistance to beta-lactam antimicrobials (Jacoby, 2009). However, various genetic changes can lead to increased expression of *ampC*, a condition known as *ampC* hyperexpression. In the 80s, *ampC* hyperproduction in *E. coli* was the main mechanism of resistance to third generation cephalosporins at that time. In recent years, with the emergence of plasmid-mediated *ampC*s (such as bla_{CMY}), which are genes encoding for enzymes that confer resistance to a broader range of beta-lactam antimicrobials, *ampC* hyperproduction is no longer the dominant mechanism of resistance in *E. coli* (Findlay et al., 2020). However, recently it has regained interest in the scientific community as it has been identified in livestock and humans in the UK (Alzayn et al., 2020), in Netherlands (Ceccarelli et al., 2019), and in Belgium (Guerin et al., 2021). In several of these studies, the *ampC* hyperproduction mechanisms is due to the mutation −42 (C > T) and seems associated with the ST88. We identified similar phenomenon in our isolates in Québec Canada, which suggests that they might disseminate through clonal lineage around the world. This would need further investigation in a large international study.

It is interesting to note that most of the clonal isolates were sampled in calves. This information corroborates what had already been demonstrated previously (Horton et al., 2016; De Lagarde et al., 2022). To explain this, we can hypothesize that the immaturity of the calves' microbiome is more prone to the persistence of bacteria and a fortiori *E. coli* clones with increased fitness. It seems also that feeding waste milk containing antimicrobial residues increase the number of resistant bacteria shed in feces in calves (Brunton et al., 2014). Another possibility is that calves receive more systemic oral treatment than cows, which tend to be treated locally, intravenously, or intramuscularly. Oral treatments are susceptible to modify the intestinal microbiome. Regardless of the reason, it means that calves should be manipulated more cautiously to limit the dissemination and the putative transmission of MDR clones to humans.

Our study presents limitations. Indeed, even if we gathered a lot of information's in our questionnaires (Lardé et al., 2021), we still were not able to identify physical vectors for clones. In the future, it would be of interest to obtain data on animals' movements. Flies and wild birds should also be considered in future samplings. These types of data should be gathered in the future to clarify the epidemiological link between farms which is essential to elaborate

effective biosecurity measures to limit clonal spread. The other major limitation of our study, as already mentioned, is the lack of long read sequencing, which greatly limited our study and therefore our analysis on plasmid dissemination.

As a conclusion, we demonstrated that MDR ExPEC are present in the normal microbiota of cattle (more frequently in calves) and that AMR genes spread through farms. These genes can persist over a 4-year period in the dairy cattle population through both plasmids and *E. coli* clones despite important changes in AMU following the implementation of a new restricting regulation. In a previous paper, we demonstrated that the number of MDR isolates decreased between the two periods (De Lagarde et al., 2022). Taken together, these data demonstrate that, although efficient, the decrease in AMU is not enough to fight against AMR because gene dissemination is a complex phenomenon. Resistance gene surveillance should include the study of clones, their virulence, and their fitness. These data advocate changes to current AMR monitoring methods and highlight that biosecurity measures should be enhanced in this industry to limit this dissemination.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA716674; https://www.ncbi.nlm.nih.gov/, PRJNA1022465.

Ethics statement

The animal studies were approved by the Animal Use Ethics and the Research Ethics Committees of the Université de Montréal. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

ML: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. JF: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Supervision, Visualization, Writing – review & editing. MA: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. SD: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – review & editing. DF: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing. JM: Investigation, Methodology, Writing – review & editing. HL: Data curation, Investigation, Methodology, Writing – review & editing. CA: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. M-EP: Funding acquisition, Project administration, Resources, Writing – review & editing. YT: Formal analysis, Writing – review & editing. J-PR: Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1304678/full#supplementary-material

SUPPLEMENTARY TABLE 1
Quality data and phylogenetic characteristics.

SUPPLEMENTARY TABLE 2
Distance matrix.

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Polyether ionophore resistance in a one health perspective

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Antimicrobial resistance is a major threat to human health and must be approached from a One Health perspective. Use of antimicrobials in animal husbandry can lead to dissemination and persistence of resistance in human pathogens. Polyether ionophores (PIs) have antimicrobial activities and are among the most extensively used feed additives for major production animals. Recent discoveries of genetically encoded PI resistance mechanisms and co-localization of resistance mechanisms against PIs and antimicrobials used in human medicine on transferrable plasmids, have raised concerns that use of PIs as feed additives bear potential risks for human health. This review summarizes the current knowledge on PI resistance and discusses the potential consequences of PI-usage as feed additives in a One Health perspective.

KEYWORDS

antimicrobial resistance, polyether ionophore, one health, vancomycin resistance, anticoccidials, coccidiostats, growth promoters

1 Introduction

Antimicrobial resistance (AMR) poses a serious threat to human health. It has been estimated that 1.27 million deaths were caused by bacterial AMR in 2019 (Murray et al., 2022) and the negative effect on human welfare is predicted to escalate in the next decades. Although the main focus on AMR has been on health care settings, it is recognized that veterinary medicine, plant- and animal production, and environmental sectors play an important role in the origin, persistence, and spread of AMR.

Polyether ionophores (PIs) have been used as feed additives for production animals since the early 1970 (Chapman et al., 2010). PIs possess both antibacterial and antiprotozoal activity and are currently used in poultry production worldwide to control severe diseases such as coccidiosis caused by *Eimeria* spp. and necrotic enteritis caused by *Clostridium perfringens* (Martins et al., 2022). The antibacterial activity of PIs has also proven useful to improve feed conversion in ruminants (Callaway et al., 2003; Kim et al., 2014; Scharen et al., 2017). PIs are not used in human medicine due to their cytotoxicity. However, PIs and PI-derivatives with low toxicity are considered for therapeutic treatment of cancer (Huczyński, 2012; Kaushik et al., 2018; Wang et al., 2021) and infectious diseases caused by bacteria (Wollesen et al., 2023), fungi, protozoa, and even virus (Huczyński, 2012; Lin et al., 2021).

Sales and use of PIs are not systematically reported in most countries, making it difficult to estimate the global consumption level (Hansen et al., 2009; Mulchandani et al., 2023). However, based on available data from countries in Europe, North America, and Australia, PIs are among the most extensively used antimicrobial feed additives for production animals

across the world (DANMAP 2015, 2016; PHAC, 2016; SWEDRES/SVARM 2016, 2017; FDA, 2023a).

Polyether ionophores are produced and secreted by bacteria of the class Actinomycetia. They are highly lipophilic compounds that form lipid soluble complexes with cations and facilitate their diffusion through biological membranes. This disrupts chemical gradients across membranes and interferes with essential biological processes. The PIs display different ion selectivity for cations abundant in biological systems depending on their structure. Most commonly used PIs in animal husbandry can bind both K^+ and Na^+ under artificial conditions, and with the exception of monensin, these PIs prefer K^+ over Na^+. Lasalocid, however, has been shown to form complexes with both mono- and divalent cations (Table 1).

The antimicrobial mechanism(s) of these PIs are not fully understood. Independent of the cation preference in artificial systems, it appears that the effect of most PIs used in animal husbandry is disruption of the Na^+/K^+ homeostasis and a concomitant change in cytosolic pH (Russell and Strobel, 1989). This could theoretically be a direct effect of the PIs ability to bind both Na^+ and K^+ and thereby facilitate transport of Na^+ into the cell and K^+ out of the cell, and that the change in pH results from induction of endogenous membrane transport systems to restore the chemical gradients of Na^+ and K^+. However, the general opinion is that PIs transport a cation (Na^+/K^+) in one direction and H^+ in the opposite direction. The primary effect would therefore be an intracellular change in Na^+ or K^+ gradient and a simultaneous change in pH. It has been hypothesized that the resulting disruption of membrane cation gradients induces compensatory mechanisms to restore cation-homeostasis, including Na^+/K^+ ATPase and F-ATPase. The activity of these compensatory mechanisms results in a secondary effect of disrupted Na^+, K^+, and H^+ homeostasis, increased demand for ATP and subsequent tertiary effects on essential cellular processes (Figures 1A,B; Smith and Galloway, 1983; Russell and Strobel, 1989).

It is likely that the PI-mediated disruption of cation gradients directly or indirectly inhibits both primary and secondary membrane transport proteins resulting in disrupted import and export of nutrients, metabolites and xenobiotics. In addition, the disturbances in the intracellular cation concentration may inhibit enzymes involved in essential cellular processes. Although it has been suggested that bacterial growth inhibition could be caused either by energy depletion due to increased demand for ATP or by cell acidification as a result of influx of H^+ (Russell, 1987), other mechanisms may also be involved.

Polyether ionophores affect bacterial metabolism of carbohydrates, amino acids, and fatty acids. The specific effect of disrupting the chemical gradients across the cytoplasmic membrane varies depending on the cation-dependent processes and the metabolic requirements of the organism. Chow et al. showed that monensin and lasalocid inhibited growth of the gram-negative bacterium *Fibrobacter succinogenes*. They showed that the ATP synthesis was decreased, most likely as an effect of an inability to take up glucose via Na^+/glucose symporters (Chow and Russell, 1992). PIs have also been shown to reduce amino acid transport in ruminal bacteria (Chen and Russell, 1989; Russell and Strobel, 1989; Van Kessel and Russell, 1992). In contrast, while monensin exposure caused cessation of growth of *Streptococcus bovis in vitro* (Russell, 1987), glucose transport was not inhibited and glucose fermentation continued resulting in continuous ATP production (Russell, 1987). The hypothesized explanation to this observation was that *S. bovis* can use the phosphotransferase system as well as facilitated diffusion for glucose uptake (Russell et al., 1990).

Similar biological effects have been observed in the protozoan parasite *Eimeria tenella* where monensin caused an increase in intracellular concentrations of both Na^+ and K^+ (Smith and Galloway, 1983). As specific inhibition of the Na^+/K^+-pump increased the K^+ level, it was proposed that monensin caused an initial uptake of Na^+ followed by an exchange of intracellular Na^+ for extracellular K^+ (Figure 1B). Several anticoccidial modes of PIs have been suggested, such as energy depletion, mitochondrial stress, and inhibition of invasion of enterocytes. However, parasite swelling, and eventual bursting, have been suggested as the most likely mode of action (Smith et al., 1981; Chapman et al., 2010). Whether the osmotic stress survival of bacteria is influenced by PIs has to our knowledge not been shown experimentally.

The specific activity of PIs is influenced by the extracellular conditions. Extracellular cation concentrations promoting the natural electrochemical gradients across the cytoplasm (high $[Na^+]$, $[H^+]$, $[Ca^{2+}]$) enhances the activity of ionophores, whereas extracellular cation concentrations equilibrating the intracellular levels (high $[K^+]$) decrease the ionophore-activities (Dawson and Boling, 1987; Russell

TABLE 1 Polyether ionophores commonly used in animal husbandry and their ion selectivity in artificial systems.

Ionophore	Concentration in feed	Producer organism	Mr	Selectivity sequence	References
Narasin	60–70 mg/kg†	*Kitasatospora aureofaciens*	765	$K^+ > Na^+$	Caughey et al. (1986)
Salinomycin	50–70 mg/kg†	*Streptomyces albus*	751	$K^+ > Na^+$	Rokitskaya et al. (2023)
Lasalocid (X-537A)	75–125 mg/kg†	*Streptomyces lasalocidi*	591	$K^+ > Na^+ > Ca^{2+} > Mg^{2+}$	Pressman (1968); Antonenko and Yaguzhinsky (1988)
Maduramicin (X-14868A)	5–6 mg/kg†	*Actinomadura yumanensis*	934	$K^+ > Na^+$	Liu et al. (1983)
Monensin A	100–125 mg/kg†	*Streptomyces cinnamonensis*	671	$Na^+ > K^+$	Pressman (1968); Antonenko and Yaguzhinsky (1988)
Laidlomycin	~110 mg/kg#	*Streptovertilicillium olivoreticuli*	698	$K^+ > Na^+ > Ca^{2+}$	Gräfe et al. (1989)

†Minimum and maximum concentrations of active substance/kg in complete feed for broiler production in the EU (EUR-lex, 2023).
#Recommended concentration of active substance/kg for improved feed efficiency in cattle in the United States (FDA, 2023b).

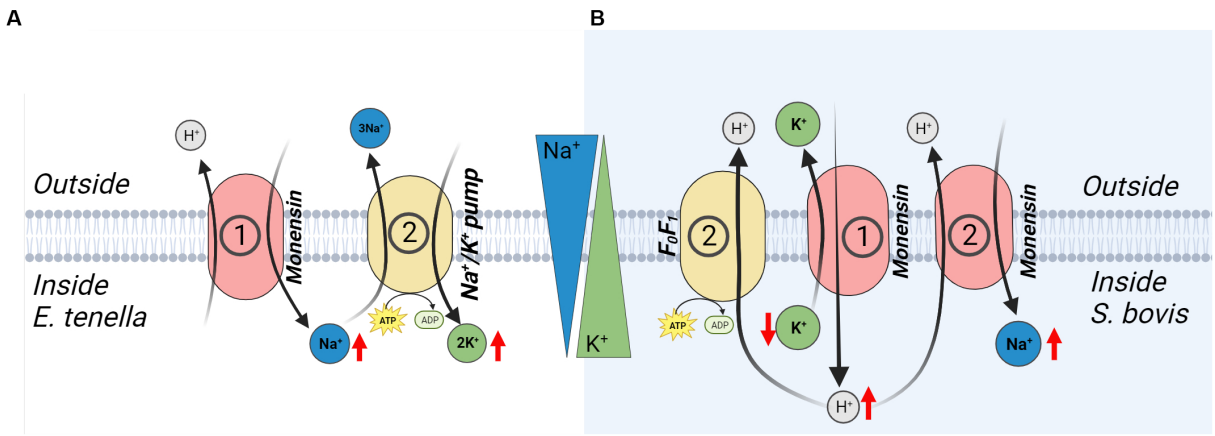

FIGURE 1
Proposed effects of monensin-mediated ion gradient disruption in *Eimeria tenella* (Smith and Galloway, 1983) parasites **(A)** and in gram-positive *Streptococcus bovis* (Russell, 1987) bacteria **(B)**. Encircled numbers represent suggested sequence of ion transport events. Red arrows indicate direction of change in cystosolic ion concentrations. Cation gradients across a bilayer is displayed by triangles.

et al., 1988; Chow and Russell, 1992; Van Kessel and Russell, 1992; Wollesen et al., 2023). Considering that the cation concentrations and pH varies along the length of the gastrointestinal tract of animals, it is likely that the antimicrobial effect of PIs differs in different parts of the gastrointestinal tract, and thereby exert different selection pressures on the local microbiota.

2 PI-resistance mechanisms in bacteria

Antimicrobial resistance is the ability of bacteria to survive and grow in the presence of antimicrobials (Balaban et al., 2019). Since polyether ionophores have not been used in human medicine, clinical cut-off values have not been established. When we discuss resistance to polyether ionophores in this review, we refer to survival and growth of: (1) a particular strain of bacteria at PI concentrations to which it was previously susceptible, (2) a species at concentrations above the epidemiological cut-off values for that species, or (3) a species at the highest concentration of PI tested in susceptibility assays *in vitro* (intrinsic resistance).

Resistance mechanisms in bacteria are attributed to either intrinsic resistance, where all individuals of a certain type of bacteria can survive and grow in the presence of a specific antimicrobial, or acquired resistance that can arise in a previously susceptible population due to mutations or horizontal transfer of resistance genes. In addition, bacteria can survive high concentrations of antimicrobials due to the formation of persister cells or biofilms. Persister cells are subpopulations of a species with a different physiology (metabolically quiescent) compared to the general bacterial population. Biofilms are bacterial communities embedded in an extracellular matrix consisting of physiologically diverging subpopulations of bacteria (Olivares et al., 2013).

Although bacterial resistance to PIs was described 30 years ago, the mechanism(s) of resistance are poorly understood. Table 2 summarizes the currently known putative and confirmed bacterial resistance mechanisms.

2.1 PI-resistance in gram-negative bacteria

Gram-negative bacteria generally display intrinsic resistance to PIs due to the nature of their cell envelope. The outer membrane of gram-negative bacteria is impenetrable to many macromolecules and allows passage of solutes through porins. Porins are hydrophilic channels embedded in the outer membrane with a size exclusion limit of approximately 600 daltons. Ionophores are highly lipophilic and in general larger than 600 daltons, making them unable to pass through the porins and the negatively charged LPS of the outer membrane (Nagaraja, 1995).

Intrinsic resistance to PIs is not universal to all gram-negative bacteria. Certain strains of *Bacteroides*, *Fibrobacter*, and *Prevotella* belonging to the ruminal microbiota were sensitive to monensin when grown *in vitro* (Chen and Wolin, 1979; Newbold et al., 1993; Callaway and Russell, 1999, 2000). Some sensitive strains developed resistance after exposure to sub-lethal monensin concentrations through unknown mechanisms, while others remained sensitive (Callaway and Russell, 1999, 2000). *Prevotella ruminicola* grown in the presence of increasing concentrations of tetronasin developed resistance to tetronasin, lasalocid, and monensin and to a lesser extent to the glycopeptide avoparcin. The resistant mutants did not lose the resistance phenotype after subculturing in the absence of ionophores and bound less radioactively labeled ionophore. Reduced metabolism of tetraphenylalanine (Mr = 607), but unaffected metabolism of triphenylalanine (Mr = 460), indicated reduced penetration through the outer membrane, and reduced porin exclusion limit was suggested as the mechanism of resistance (Newbold and Wallace, 1989).

2.2 PI-resistance in gram-positive bacteria and mycobacteria

In contrast to gram-negative bacteria, gram-positive bacteria do not possess a protective outer membrane. Although the outer peptidoglycan layer of gram-positive bacteria can be relatively thick, it is porous and permits diffusion of small molecules, and this allows

TABLE 2 Putative bacterial polyether ionophore-resistance mechanisms.

Resistance	Bacteria	Target PI	Putative mechanism	References
Intrinsic	Many gram (−)	All PIs	Reduced permeability due to an outer membrane with negatively charged LPS and porins	Nagaraja (1995)
Acquired	*Enterococcus faecium* (+)	Narasin, Salinomycin, and Maduramicin	Ionophore efflux by ABC type transporter encoded by *narAB*	Naemi et al. (2020)
	Enterococcus faecalis (+)			
	Streptomyces lividans (+)	Tetronasin[$]	Ionophore efflux by ABC type transporter encoded by *tnrB2/B3*	Linton et al. (1994)
	Staphylococcus aureus (+)	Nanchangmycin[$]	Regulation of potassium homeostasis by potassium importer encoded by *trkH*[†], Regulation of membrane integrity by proteins encoded by *sarV*[†], *mspA*[†]	Wollesen et al. (2023)
	S. aureus (+)	Nanchangmycin[$], Lasalocid, and Salinomycin	Altered metabolism due to mutations in genes *aroC*[#], *hemB*[#], *qoxABC*[#], *ndh2*[#], and *cyoE*[#]	Wollesen et al. (2023)
	S. aureus (+)	Monensin	Altered nucleotide metabolism due to mutations in *apt*[†], *purR*[†], and *R*egulation of Na^+/H^+ homeostasis by Na^+/H^+ antiporter encoded by *mnh*[†]	Dan I. Andersson (personal communication, March 9, 2023; published with permission)
	Mycolicibacterium aurum (AF)	Nigericin[$]	Altered gene regulation due to mutations in transcriptional regulator *tetR*[†], Regulation of Na^+/H^+ homeostasis by Na^+/H^+ antiporter encoded by *nhaA*[†]	Huang et al. (2017)
	M. aurum (AF)	Calcimycin[$]	Altered gene regulation due to mutations in transcriptional regulator *tetR*[†]	Huang et al. (2017)
Altered physiology	*Prevotella bryantii* (−)	Monensin	Cell wall thickening	Callaway and Russell (1999); Rychlik and Russell (2002); Simjee et al. (2012)
	E. faecium (+)			
	E. faecalis (+)			
	Clostridium aminophilum (+)			
	S. aureus (+)	Salinomycin, Narasin, Nanchangmycin[$], and Calcimycin§	Biofilm and persisters	Wollesen et al. (2023)

[†]Mutants isolated under laboratory conditions; [#]transposon insertion mutant, (−) gram-negative, (+) gram-positive, (AF) acid fast; [$]ionophores presented to provide insight into potential resistance mechanisms, but not applied in animal production.

for the lipophilic ionophores to readily dissolve into the cell membrane of gram-positive bacteria (Rutkowski and Brzezinski, 2013).

2.2.1 Plasmid mediated PI-resistance mechanisms

Although the biological role of PI production is not yet established (Bakker, 1979; Kevin et al., 2009), it is likely that the antibacterial activity of ionophores improves the competitiveness of PI secreting bacteria in their habitats. The secretion of ionophores into the environment suggests that PI producing bacteria concomitantly express protective mechanisms of self-resistance. A self-resistance mechanism against the PI tetronasin was identified in 1994 by screening of a genomic library from the tetronasin-resistant *Strepmomyces longisporoflavus* in the tetronasin-susceptible species *Streptomyces lividans* (Linton et al., 1994). Tetronasin-resistance was associated with a DNA region containing the *tnrB2/B3* operon encoding an ATP-binding cassette (ABC) transporter consisting of ATPase (TnrB2) and permease (TnrB3) subunits. Later, the presence of plasmid-encoded homologs of *tnrB2/B3* were identified in *Enterococcus faecium* isolates from Swedish and Norwegian poultry, and the presence of these genes on large mobile plasmids correlated with resistance to narasin (Nilsson et al., 2016). Naemi et al. (2020) cloned the putative narasin resistance genes, coined *narAB*, into a

cloning vector under control of its natural promoter and unequivocally showed that this operon was sufficient to confer resistance to the PIs narasin, salinomycin and maduramicin, but not to monensin. Interestingly, Naemi et al. also showed that the *narAB* operon was transcriptionally upregulated by exposure to narasin, which potentially reduces the fitness cost associated with carrying the operon in PI-free conditions.

In *E. faecium* isolated from broilers in Norway (Sletvold et al., 2007, 2008, 2010), Sweden (Nilsson et al., 2016), Denmark (Leinweber et al., 2018), and the Netherlands (Pikkemaat et al., 2022), the *narAB* operon was located on plasmids belonging to the broad-host-range inc18-group (Gilmore et al., 2014). Inc18 plasmids are widespread in isolates from the environment, the clinic, and domestic animals (Kohler et al., 2018). They are naturally occurring in streptococci and enterococci, often carry resistance genes, and have been shown to be transferable from enterococci to staphylococci (Kohler et al., 2018). The assembled NarAB-encoding plasmids published to date, vary in size and carry mobile elements such as transposons and insertion sequences. These plasmids share only limited gene synteny. However, the *narAB* operon is often associated with a full or truncated ω-ε-ζ toxin-antitoxin system and flanked by insertion sequences, such as IS1216 (Sletvold et al., 2007, 2008, 2010; Leinweber et al., 2018). Filter mating experiments demonstrated that NarAB encoding plasmids were transferable between *E. faecium* strains despite the lack of apparent plasmid encoded transfer systems (Dahl et al., 2007; Nilsson et al., 2012; Leinweber et al., 2018; Naemi et al., 2020). Leinweber et al. (2018) observed that the NarAB encoding plasmid was transferred by conjugation along with a larger co-residing conjugative plasmid suggesting that the larger plasmid acted as a helper plasmid. In Dutch *E. faecalis* isolates, *narAB* was most often localized on large plasmids of the RepA_N family (Pikkemaat et al., 2022). RepA_N plasmids display a relatively broad distribution but appear to be adapted to their host and display restricted transferability to other species (Weaver et al., 2009).

The resistance mechanism(s) of NarAB and TnrB2/B3 have not yet been characterized. The ABC transporter superfamily is an ancient family of membrane transporters that utilizes the energy released from hydrolysis of ATP to drive transport of substrates against a concentration gradient. ABC-transporters transport a wide range of substrates including ions, nutrients, xenobiotics, and secondary metabolites, including antibiotics. It is obvious to assume that NarAB and other TnrB2/B3 homologs function as drug efflux proteins, but this has not yet been proven experimentally.

The origin of *narAB* is not known. The occurrence of *narAB* in certain subpopulations of enterococci and the general localization of this operon on mobilizable plasmids suggests acquisition by horizontal gene transfer. The similar function of NarAB and TnrB2/B3 suggests that NarAB may have originated as a self-resistance mechanism in PI-producing bacteria. However, the permease subunit NarB (Accession: QHA94815.1) displays 33% identity to TnrB3 (Accession: CAA52013.1) of *Strepmomyces longisporoflavus* and 30% identity to a putative self-resistance gene (Accession: WP_030553101.1) of the narasin producing *Kitasatospora aureofaciens* (our unpublished data). This suggests that if the gene originates from horizontal gene transfer from PI-producing bacteria it would have been an early event predating the use of ionophores in animal husbandry. Alternatively, *narAB* originates from a so far unidentified bacterium, and it cannot be excluded that it has divergently evolved from an operon that is intrinsic to a species of enterococci.

2.2.2 Mutations giving rise to PI-resistance

In a screen for natural compounds displaying *in vitro* anti-mycobacterial activity, Huang et al. (2017) identified the PIs nigericin, calcimycin (A23187), and salinomycin as hits. Spontaneous mutants of *Mycobacterium* spp. resistant to nigericin and calcimycin were isolated on selective plates. Deleterious mutations in a *tetR* family regulator resulted in resistance to both PIs, while a non-synonymous mutation in *nhaA* resulted in resistance to nigericin. Mutation of the *tetR* regulator led to increased transcription of an RND family efflux pump hypothesized to export the PIs, while mutation in *nhaA* encoding a homolog of Na^+/H^+-antiporter likely compensates for a disrupted sodium gradient.

Wollesen et al. (2023) grew *Staphylococcus aureus* in the presence of the PI nanchangmycin with the intention to isolate mutants resistant to PIs and to identify potential resistance mechanisms. Nanchangmycin-resistant mutants carried mutations in genes encoding a potassium importer TrkH, a transcriptional activator involved in regulating autolysis SarV, and a membrane stabilizing protein MspA. Interestingly, the authors did not detect cross-resistance in these mutants to salinomycin, lasalocid, or calcimycin. To gain further insight into the resistance mechanisms, a methicillin-resistant *S. aureus* transposon mutant library was screened for increased sensitivity to lasalocid, salinomycin, calcimycin, and nanchangmycin. Mutations in the electron transport chain (ETC) genes *qoxABC*, *nhd2*, and *cyoE*, conferred a modest increase in sensitivity toward lasalocid, salinomycin, and nanchangmycin (Wollesen et al., 2023). These findings indicate a role of the ETC in PI-resistance in *S. aureus*, potentially to meet the high demand for energy to uphold cation homeostasis across the cell membrane.

Recently, spontaneous mutants with reduced susceptibility to monensin were isolated after growth of clinical *S. aureus* isolates under laboratory conditions. Mutations were found in different genes, such as *apt* encoding an adenine phosphoribosyltransferase, *purR*, encoding a repressor of nucleotide biosynthesis, and non-synonymous mutations in different genes of the *mnh* operon encoding a Na^+/H^+ antiporter (Dan I. Andersson, personal communication, March 9, 2023; published with permission). While *apt* and *purR* theoretically are involved in compensating for increased need for ATP to counteract a PI-induced cation imbalance, the *mnh* mutations are likely directly compensating for a disrupted transmembrane chemical gradient.

2.3 PI-resistance due to altered bacterial physiology

Persister cells and biofilm formation are mechanism considered to play key-roles in persistence of bacteria in different environments including the human host (chronic infections) and causing antimicrobial treatment failures (Olivares et al., 2013). Wollesen et al. (2023) analyzed the antimicrobial effect of PIs on a laboratory-induced persister phenotype and preformed biofilms of *S. aureus*. They observed that in general both persister cells and biofilms were more resistant to PIs compared to exponentially growing bacteria. Interestingly, persister cells were susceptible to lasalocid, and biofilms

were susceptible to lasalocid, calcimycin, and nanchangmycin (Wollesen et al., 2023).

In the gram-positive bacteria *Clostridium aminophilum F*, *Clostridium perfringens*, *E. faecalis*, and *E. faecium* and in the gram-negative species *Prevotella ruminants*, adaptation to growth in monensin was associated with thickening of the cell wall (Callaway and Russell, 1999; Rychlik and Russell, 2002; Simjee et al., 2012). The observed increase in cell wall thickness was reversed after passage in monensin-free medium and the authors concluded that monensin resistance was due to physiological changes rather than mutations (Simjee et al., 2012). However, it should be noted that this was not confirmed by whole genome sequencing.

3 Relationship between PI resistance and resistance to medically important antimicrobials

Since PIs are currently not utilized to combat bacterial infections in humans due to cytotoxicity, the prevalence of PI-resistance in bacterial isolates from animals has not been considered a threat to public health. However, use of PIs in animal husbandry and PI-resistance can contribute to resistance to medically important antimicrobials if PI-resistance confers cross-resistance or co-resistance to antimicrobials used to treat infections in humans. While cross-resistance occurs in a microbe carrying a resistance mechanism conferring resistance to structurally different antibiotics, co-resistance arises if genes encoding resistance mechanisms for different antimicrobials are genetically linked.

3.1 Sparse evidence of cross-resistance between PIs and medically important antimicrobials

The ruminal bacterium *C. aminophilum* that had been adapted to grow in monensin or lasalocid, was only resistant to the cell-wall acting bacitracin out of 16 tested medically relevant antibiotics (Houlihan and Russell, 2003). Interestingly, these results are in agreement with a strong association between narasin resistance and bacitracin resistance as discovered in *Enterococcus* spp. isolates from broilers in Norway (NORM/NORM-VET 2004, 2005). However, it was shown that NarAB does not confer cross-resistance to bacitracin, suggesting two separate AMR mechanisms for these antimicrobials in enterococci (Naemi et al., 2020). As described above, the gram-negative bacterium *P. ruminicola*, adapted to grow in the presence of tetronasin, displayed a moderately reduced (65%) susceptibility to the glycopeptide avoparcin (Newbold et al., 1992). The cross-resistance between tetronasin and avoparcin was likely due to changes in the porins of the outer membrane. Some of the mutants of *S. aureus* that are resistant to monensin due to mutations in *apt*, *purR*, or *mnh* displayed minor changes in resistance to antimicrobials used to treat infections in humans (Dan I. Andersson, personal communication, March 9, 2023; published with permission). These resistance mechanisms have not been characterized in detail and a mechanism of cross-resistance to medically relevant antimicrobials have not been confirmed.

Naemi et al. (2020) tested the resistance profile of *E. faecium* carrying the *narAB* operon on a plasmid under control of the natural promoter and compared it to the resistance profile of the isogenic strain carrying the vector control. They detected no difference between the two strains in minimum inhibitory concentration (MIC) values of any of the tested medically important antimicrobials, suggesting that NarAB does not confer resistance to antimicrobials used in human medicine (Naemi et al., 2020).

3.2 Co-resistance of PIs and medically important antimicrobials

Vancomycin-resistant *E. faecium* has regularly been isolated at low levels from broilers in Norway and Sweden using a selective isolation method (NORM/NORM-VET 2004, 2005; SWEDRES/SVARM 2013, 2014). All of these vancomycin-resistant enterococci (VRE), which carry *vanA*, were also resistant to narasin and carried the *narAB* genes (Nilsson et al., 2012; Simm et al., 2019). Filter mating experiments showed that vancomycin and narasin resistance were frequently co-transferred to a recipient (Nilsson et al., 2016; Naemi et al., 2020). Comparative genomics revealed that *narAB* and *vanA* can be physically linked on transferrable plasmids (Johnsen et al., 2005; Nilsson et al., 2016).

A study from the Netherlands on 35 *E. faecium* and 61 *Enterococcus faecalis* isolates from poultry products found statistically significant correlations between phenotypic resistance to salinomycin, tetracycline, and erythromycin in both species (Pikkemaat et al., 2022). Sequencing of a selection of 20 isolates revealed that *narAB* was present in all of the salinomycin-resistant isolates of both *E. faecium* and *E. faecalis*. In the *E. faecalis* isolates, *narAB* was physically linked with *ermB* (conferring macrolide resistance) and *tet(L)* and *tet(M)* or *tet(O)* (*tet*-genes confer resistance to tetracycline) on plasmids (Pikkemaat et al., 2022), confirming the co-occurrence of resistance genes. The correlation between salinomycin and tetracycline resistance aligns with a metagenomics study in which narasin-fed chickens were enriched for bacteria encoding tetracycline resistance genes, suggesting that narasin and tetracycline resistance are co-selected (Plata et al., 2022). Taken together these data provide strong evidence for transferrable co-resistance of PIs and medically important antimicrobials.

4 Prevalence of PI-resistant bacteria in animals and risks for human health

The worldwide prevalence of PI-resistance is challenging to assess because resistance to PIs is not reported systematically, and large-scale surveys of resistance has not been performed. However, a few countries in Europe have regularly reported on the prevalence of PI-resistance in indicator bacteria such as the opportunistic pathogens *E. faecium* and *E. faecalis* isolated from animals or animal products.

4.1 Prevalence of PI-resistant bacteria in animals

In a Dutch report from 2022, the authors analyzed the antimicrobial susceptibility of *E. faecium* and *E. faecalis* from an in-house collection of isolates gathered in the years 2013, 2016, 2018, and 2020. They reported that 31% of *E. faecium* and 23% of *E. faecalis*

isolates collected from broilers and broiler products of conventionally reared poultry displayed a MIC of salinomycin >2 mg/L, while 48.6% of *E. faecium* and 47.5% of *E. faecalis*, displayed a MIC value >1 mg/L. The authors performed whole genome sequencing of a subset of the isolates and discovered that all isolates with a MIC >1 mg/L carried plasmids encoding the PI-resistance genes *narAB* (Pikkemaat et al., 2022). Based on this, it was suggested that the cut-off value for salinomycin defining a non-wild-type should be adjusted to >1 mg/L. The Dutch AMR monitoring program (MARAN) historically used a MIC of 4 mg/L as cut-off for resistance and reported the number of resistant isolates for each year. The proportion of resistant *E. faecium* and *E. faecalis* isolates from Dutch broilers varied between 41.3–81.7% and 3.9–34.5%, for *E. faecium* and *E. faecalis*, respectively, in the years 2004–2014 (Figure 2; MARAN, 2023). Applying a cut-off of >1 mg/L in place of >4 mg/L to the MIC results from the last MARAN report to document salinomycin resistance, increased resistance rates from 5.6 to 61.9% and 38.5 to 84.6% for *E. faecalis* and *E. faecium*, respectively (MARAN, 2014). The Danish surveillance program (DANMAP) reported during the same period that their proportion of isolates from broilers with an MIC of salinomycin >4 mg/L varied between 50 and 74.8% for *E. faecium* and 0–10.5% for *E. faecalis* (Figure 2; DANMAP, 2023). Considering the results of Pikkemaat et al. that cut-off values as low as 1 mg/L correlated with occurrence of the *narAB* resistance operon, the occurrence of PI-resistant enterococci in both Denmark and the Netherlands must be regarded as significantly higher than previously reported.

In Norway and Sweden, narasin was used in the test-panels for surveillance of PI resistance in indicator enterococci. In Sweden, 77% of *E. faecium* and 27% of *E. faecalis* isolated from broilers in 2014 were considered narasin-resistant with MIC over the EUCAST epidemiological cut-off (ECOFF) value (>2 mg/L) (SWEDRES/SVARM 2013, 2014). The prevalence of resistant isolates varied in the years 2000–2014 with 77–93.3% for *E. faecium* and 22.7–44.9% for *E. faecalis* (Figure 2) (SWEDRES-SVARM, 2023). This is similar to the situation in Norway, where 61–91% of the *E. faecium* isolates from broilers were resistant to narasin in the years 2002–2014 (Figure 2) (NORM/NORM-VET, 2023). Since PIs are not currently used in human medicine, most countries have removed PIs from the AMR test panels in monitoring of indicator enterococci. However, due to a decision by the poultry industry in Norway to remove PIs as feed additives in conventional rearing of broilers in 2015, occurrence of narasin resistance in *E. faecium* and *E. faecalis* was monitored in 2018 and 2020 to follow the development after discontinuation. The occurrence of narasin-resistant *E. faecium* isolates was reduced from >90% in 2014 to 24.7% in 2018 and 15.6% in 2020 (NORM/NORM-VET 2018, 2019; Simm et al., 2019; NORM/NORM-VET 2020, 2021). These data strongly suggests that in-feed PIs select for PI-resistant enterococci in broilers, a conclusion that is supported by a controlled study comparing occurrence of PI-resistant enterococci in broilers fed diets with and without PIs (Simm et al., 2019).

DANMAP reported that 0 of 1,349 *E. faecalis* isolates and 1 of 1,217 *E. faecium* isolates collected from pigs between 2004 and 2013 had a MIC of salinomycin >4 mg/L (DANMAP, 2023). The Norwegian monitoring program for AMR in the veterinary sector (NORM-VET) reported that 0 and 3.2% of *E. faecalis* and *E. faecium* pig isolates were resistant to narasin (MIC >2 mg/L) in 2004, 2008, and 2009 (NORM/NORM-VET 2004, 2005; NORM/NORM-VET 2008, 2009; NORM/NORM-VET 2009, 2010). Similarly, narasin resistance was detected in only 2% of *E. faecium* isolates from layers in Norway in 2013 (NORM/NORM-VET 2013, 2014). *Enterococcus faecium* and *E. faecalis* collected from turkeys in Norway in 2007, 2013, and 2020 displayed 76.2 and 7.3% narasin-resistant isolates, respectively (NORM/NORM-VET 2007, 2008; NORM/NORM-VET 2013, 2014; NORM/NORM-VET 2020, 2021). PIs have not been used in rearing of pigs or layers in the sampling period, but turkeys were fed a diet containing monensin. In the Netherlands, the prevalence of

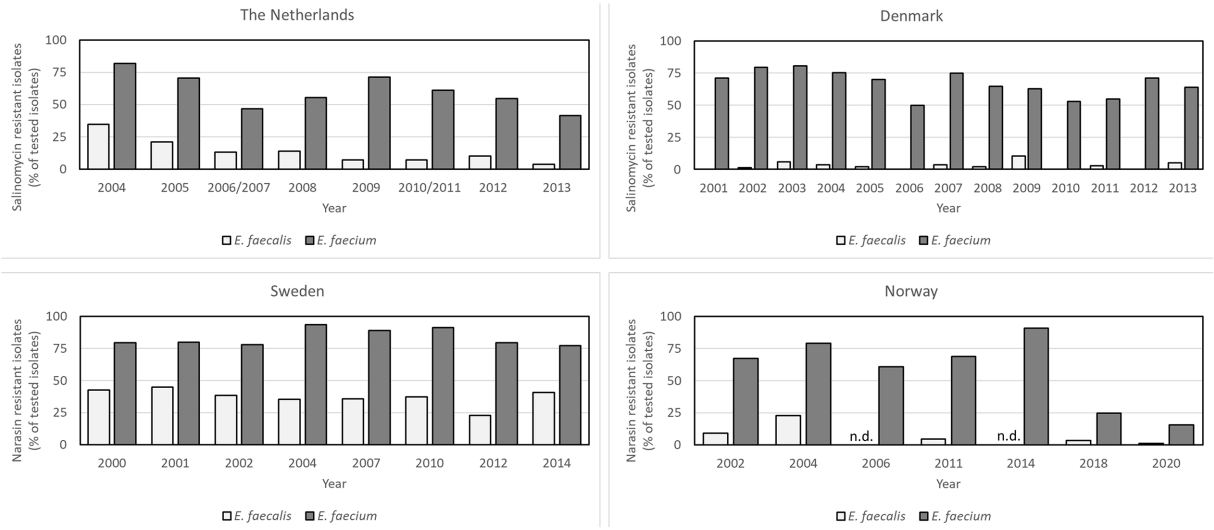

FIGURE 2
Comparison of the proportion of polyether ionophore resistant isolates from conventionally reared broilers of *Enterococcus faecium* and *Enterococcus faecalis* in four European countries sorted by year. The data were retrieved from the reports of the surveillance programs of each country (MARAN, The Netherlands; DANMAP, Denmark; SVARM, Sweden; NORM-VET, Norway). Cut-off values used were > 4 mg/L for salinomycin, and > 2 mg/L for narasin. Note that these cut-off values are higher than the values proposed to correlate with the *narAB* resistance genes by Pikkemaat et al. (2022) and Nilsson et al. (2016) and the results likely underestimate the true prevalence of PI resistant isolates in broiler populations.

salinomycin resistant enterococci has steadily declined since 2006, when use of salinomcyin was banned in rearing of pigs. These data further support that in-feed PIs select for PI-resistant bacteria.

In Norway, the glycopeptide avoparcin was used as a feed additive in broiler production between 1986 and 1995 (Borgen et al., 2000b). Vancomycin resistance provides cross resistance against avoparcin and use of avoparcin as a feed additive avidly selected for VRE in Norwegian broilers (Kruse et al., 1999). In 2000, it was still possible to isolate VRE from 99% of sampled broiler farms in Norway by plating samples directly onto agar supplemented with vancomycin (Borgen et al., 2000a). This selective method has also been used in the Swedish and Norwegian surveillance programs. Data from Norway showed decreasing detectable occurrence of VRE in broilers between 2002 and 2014 and non-detectable levels since 2018. Avoparcin has not been used in Sweden since the ban of antimicrobial growth promoters in 1986. The occurrence of VRE in Swedish broiler flocks first increased in the early 2000, reached a peak at >40% in 2005 (Nilsson et al., 2019) and has decreased since then. VRE were detected in 11% of samples from Swedish broilers in 2015 (Nilsson et al., 2019) and 6% of the samples in 2020 (SWEDRES/SVARM 2020, 2021). Corresponding data does not exist for other European countries since the national surveillance programs have not used the selective method of isolation.

Considering that all VRE in Norway and Sweden are co-resistant to narasin, the fact that VRE has not been detected in Norwegian broilers after the discontinuation of prophylactic use of in-feed narasin, and the continued detection of VRE in Swedish broilers fed narasin in the same time period, is strong evidence that narasin selects for VRE in these broiler production systems. It should be noted that there has been a continuous decline in occurrence of VRE in both Norway (between 2002 and 2014) and Sweden (between 2005 and 2020) despite use of narasin as a feed additive. This does not contradict the selection pressure of narasin for VRE co-resistant to PIs. VRE have only been detected occasionally and at very low levels by non-selective methods (0.14 and 0.75% in Sweden and Norway, respectively from 2000 to 2020). This means that VRE represent a very small proportion of the narasin resistant population of enterococci. Therefore, it is fair to assume that the apparent occurrence of VRE decreases over time even under narasin selection pressure as long as VRE are not re-introduced into the broiler production system.

4.2 Occurrence of PI-resistant bacteria in humans and risks for human health

One in 50 *E. faecium* isolates from healthy human volunteers sampled in Denmark in 2005 displayed an MIC of salinomycin >4 mg/L (DANMAP 2005, 2006). Interestingly, one isolate from the same material was also vancomycin resistant. Considering that all VRE isolated from broilers in Norway are also PI-resistant, it is tempting to speculate that the VRE isolate from healthy humans in Denmark was the same isolate that was salinomycin resistant. Furthermore, in depth analysis of WGS data from three large collections of enterococci reveals that *narAB* is also found in human isolates, including clinical isolates, though at low prevalence (Gouliouris et al., 2018; Arredondo-Alonso et al., 2020; Pöntinen et al., 2021). This clearly shows that even though PIs have never been used in human medicine, PI resistant isolates can colonize humans and cause invasive infections.

Several reports have demonstrated the temporary colonization of human intestine with antimicrobial-resistant *E. faecium* and *E. faecalis* transmitted by direct or indirect animal contact or by meat consumption (Bortolaia and Guardabassi, 2015) including narasin-resistant VRE (Johnsen et al., 2005; Sørum et al., 2006). Such temporary colonization could allow for transfer of antimicrobial resistance genes from isolates of animal origin to bacteria in the human host, such as in the case of *vanA*-encoding *E. faecium* (Lester et al., 2006). Plasmids encoding narasin and vancomycin resistance have been shown to transfer readily from poultry derived VRE to human isolates of *E. faecium* in a mouse model (Dahl et al., 2007). Transfer was observed within a day after inoculation of the donor strain. Plasmids from enterococci can also spread to other human pathogens. Experimental transfer of vancomycin resistance from *E. faecium* to *S. aureus* has for instance been demonstrated on mouse skin (Noble et al., 1992). Clinical isolates of vancomycin-resistant *S. aureus* (VRSA) from humans are often accompanied by VRE (Cong et al., 2020) and genomic comparisons have demonstrated that the *vanA* locus can transfer from plasmids of VRE and be stably integrated into the chromosome of *S. aureus* to create VRSA (Haas et al., 2023). This demonstrates that temporary colonization of humans by resistant bacteria of animal origin can pose a threat to human health even if the animal derived strain does not stably colonize the human host or cause infection in humans.

High prevalence of PI-resistance has mostly been documented for the indicator bacteria and prevalent nosocomial opportunistic pathogens, *E. faecium* and *E. faecalis*, collected from farm animals in Scandinavia and the Netherlands. However, PI resistance is likely globally widespread in enterococci from animals fed diets supplemented with PIs, and potentially also present in other opportunistic pathogens, such as *S. aureus*. So, although the available evidence does not indicate a major transmission of PI resistant bacteria from poultry to humans, transmission does occur and may be more pronounced in countries with practices for animal husbandry that differ from the regulations set by the European Union.

There are still major knowledge gaps on the risks associated with PI resistance for human health: (1) The global consumption level of PIs and hence the potential selection pressure for PI-resistance in different parts of the world are unknown since data on use of PIs in animal husbandry is not reported in most countries; (2) The frequency of PI resistance among isolates from human infections is unknown since these isolates are rarely tested for PI susceptibility; (3) The carriage rate of PI-resistant bacteria in humans is unknown. The proportion of PI-resistant bacteria in a microbiota not exposed to a selection pressure may be low and selective identification methods are required to assess the prevalence of PI resistance; (4) The distribution of PI resistance and the potential for dissemination of the resistance mechanism(s) among human pathogens are unknown. PI-resistance has mainly been tested in gram-positive indicator bacteria (enterococci) from production animals. Systematic studies from different environments and different parts of the world are needed to properly assess the risks; (5) The PI resistance mechanism(s) must be identified and characterized in detail and the full complement of resistance mechanisms to medically important antimicrobials that exist on PI-resistance plasmids should be determined.

5 Conclusion

Bacteria can become resistant to PIs through horizontal gene transfer of resistance genes and by mutations of intrinsic genes. So far, none of the putative resistance mechanisms have been characterized in detail. However, accumulating evidence suggests that several mechanisms can confer resistance to PIs, including efflux of PIs from the cell, upregulation of cation transporters counteracting the action of the PI and reduced permeability of PIs into the cell. The *narAB* operon is localized on transferrable plasmids in the human opportunistic pathogens *E. faecium* and *E. faecalis*. These plasmids have been shown to carry various resistance mechanisms to antimicrobials used in human medicine. Use of PIs in rearing of production animals provides a selection pressure that promotes expansion of a PI-resistant population of bacteria and persistence of co-localized resistance mechanisms. PI-resistant bacteria can colonize humans and cause invasive infections and the PI resistance plasmids can spread in bacterial populations, both *in vitro* and *in vivo*. Therefore, there is a potential risk associated with the use of in-feed PIs, though more research is needed to explore this further to be able to conduct a thorough risk assessment with a One Health perspective.

Author contributions

RF: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. JS: Writing – review & editing, Conceptualization. SG: Writing – review & editing, Conceptualization. KL: Supervision, Writing – review & editing, Conceptualization. MP: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing. AU: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. RS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Rapid typing of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* by Fourier-transform Infrared spectroscopy informs infection control in veterinary settings

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Introduction: The emergence of multi-drug resistant (MDR) pathogens linked to healthcare-associated infections (HCAIs) is an increasing concern in modern veterinary practice. Thus, rapid bacterial typing for real-time tracking of MDR hospital dissemination is still much needed to inform best infection control practices in a clinically relevant timeframe. To this end, the IR Biotyper using Fourier-Transform InfraRed (FTIR) spectroscopy has the potential to provide fast cluster analysis of potentially related organisms with substantial cost and turnaround time benefits.

Materials and methods: A collection of MDR bacterial isolates ($n = 199$, comprising 92 *Klebsiella pneumoniae* and 107 *Pseudomonas aeruginosa*) obtained from companion animal (i.e., dogs, cats and horses) clinical investigations, faecal and environmental screening from four veterinary facilities between 2012 and 2019 was analysed retrospectively by FTIR spectroscopy. Its performance was compared against MLST extracted from whole genomes of a subset of clustering isolates (proportionally to cluster size) for investigation of potential nosocomial transmission between patients and the surrounding hospital environments.

Results: Concordance between the FTIR and MLST types was overall high for *K. pneumoniae* (Adjusted Rand Index [ARI] of 0.958) and poor for *P. aeruginosa* (ARI of 0.313). FTIR *K. pneumoniae* clusters ($n = 7$) accurately segregated into their respective veterinary facility with evidence of intra-hospital spread of *K. pneumoniae* between patients and environmental surfaces. Notably, *K. pneumoniae* ST147 intensely circulated at one Small Animal Hospital ICU. Conversely, *Pseudomonas aeruginosa* FTIR clusters ($n = 18$) commonly contained isolates of diversified hospital source and heterogeneous genetic background (as also genetically related isolates spread across different clusters); nonetheless,

dissemination of some clones, such as *P. aeruginosa* ST2644 in the equine hospital, was apparent. Importantly, FTIR clustering of clinical, colonisation and/or environmental isolates sharing genomically similar backgrounds was seen for both MDR organisms, highlighting likely cross-contamination events that led to clonal dissemination within settings.

Conclusion: FTIR spectroscopy has high discriminatory power for hospital epidemiological surveillance of veterinary *K. pneumoniae* and could provide sufficient information to support early detection of clonal dissemination, facilitating implementation of appropriate infection control measures. Further work and careful optimisation need to be carried out to improve its performance for typing of *P. aeruginosa* veterinary isolates.

KEYWORDS

veterinary, infection control, Fourier-transform infrared (FTIR) spectroscopy, veterinary settings, companion animals, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*

1 Introduction

Healthcare-associated infections (HCAIs) and the emergence of multi-drug resistant (MDR) nosocomial pathogens in companion animal (dogs, cats and horses) medicine have been increasingly reported in the last two decades, where outbreaks are often associated with, and complicated by, the antimicrobial resistant and zoonotic nature of the pathogens involved (Murphy et al., 2010; Steneroden et al., 2010; Wieler et al., 2011; Walther et al., 2014, 2018; Soza-Ossandón et al., 2020). Advancements in modern veterinary practice, including the progress in patient management, medical procedures and the development of hospital facilities, have given rise to favourable conditions for harbouring veterinary nosocomial MDR opportunistic pathogens, as seen in human hospitals. Despite this, the frequency and nature of HCAIs in veterinary hospitals are not known and the progress made in the field of veterinary infection control (IC) has been slow compared to human medicine (Weese, 2011; Walther et al., 2017). Nowadays, IC is an essential component in the operation of all veterinary hospitals delivering high-quality care; risks associated with lack of biosecurity and infection control programs include outbreaks of HCAIs in hospitalised patients and zoonotic infections of hospital personnel and animal owners, leading to increased morbidity, mortality, medical costs and welfare issues in both animals and people. Screening hospital surfaces in conjunction with cases of clinical infection is advisable as contaminated hospital environments may be an important source of infections in hospitalised human (Weber et al., 2013; Nutman et al., 2016) and animal patients (Weese et al., 2004; Loeffler et al., 2005; Hoet et al., 2011). Furthermore, MDR pathogens can persist in the hospital environment for long periods of time, providing continuous exposure for colonisation and subsequent infection in hospitalised patients (Kramer et al., 2006; Bortolami et al., 2017; Keck et al., 2020).

Rapid typing of bacterial isolates is valuable for outbreak management and hospital surveillance of MDR pathogens by revealing their clonal relationships and possible routes of transmission and by linking clinical isolates to environmental reservoirs (Foxman et al., 2005). These results can guide the implementation of focused interventions to tackle and prevent HCAIs. However, the quick detection of pathogen cross-transmissions in healthcare settings remains challenging despite the broad choice of typing methods available. Molecular typing methods relying on genomic fingerprinting, such as Pulsed-Field Gel Electrophoresis (PFGE), Multi-Locus Sequence Typing (MLST) and Whole Genome Sequencing (WGS) are widely used in hospital epidemiological surveillance and outbreak investigation to discern and track patterns of infection and transmission sources (Boccia et al., 2015; Timofte et al., 2016). However, while molecular tools are the gold standard for diagnostic accuracy and discriminatory power, they are mainly used in retrospective epidemiological studies, thereby lacking clinical applicability. Hence, a quick and reliable typing method detecting pathogen cross-transmissions in the clinical microbiology laboratory is still needed.

Fourier-Transform InfraRed (FTIR) spectroscopy is a phenotypic method generating highly-specific metabolic fingerprint-like signatures (from nucleic acids, proteins, carbohydrates and lipids) that are widely used to differentiate, identify and classify a range of microbial species and strains (Yang et al., 2020). The principle of this technique is that the absorption of the infrared (IR) radiation by bacterial cells causes excitation of the different molecules; different cell components absorb radiation at different wavelengths, originating characteristic spectral peaks that present as a specific fingerprint-like pattern (Novais et al., 2019). The potential of this technique as a quick (results can be available as early as 2–3 h from culture harvest), inexpensive, and high-throughput tool for bacterial typing is widely accepted, which makes it an attractive alternative to current gold standard typing methods for routine diagnostic applications (Finlayson et al., 2019).

Clinical research exploring the performance of FTIR in human hospitals indicated this could be a promising tool for infection control purposes, particularly for fast typing of gram-negative organisms associated with HCAIs' outbreaks (Dinkelacker et al., 2018; Martak et al., 2019; Vogt et al., 2019; Rakovitsky et al., 2020). In our previous work exploring rapid bacterial typing of veterinary hospital-associated MDR bacteria, we reported unexpectedly high circulation of MDR gram-negative organisms belonging to the ESKAPE group of pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella*

pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and *Enterobacter* spp.) within the intensive care units (ICUs) of two veterinary hospitals (one equine and one small animal; Zendri et al., 2023). Here, we consider the integration of this technology into infection control programmes by assessing its value in strain typing of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* from clinical cases, colonised inpatients and clinical environments in veterinary clinics/hospitals.

2 Materials and methods

2.1 Bacterial isolates

Sixty-seven isolates were selected from a previous pilot study (PS) which evaluated the epidemiology of multidrug resistant gram-negative (MDR-GN) bacteria in the ICUs of two veterinary hospitals, one small animal (SAH1) and one equine (EH), at the University of Liverpool (UoL), United Kingdom (Zendri et al., 2023), where $n = 31$ *K. pneumoniae* (Table 1) and $n = 36$ *P. aeruginosa* (Table 2) isolated across both hospitals, were selected for FTIR testing.

In addition, MDR *K. pneumoniae* ($n = 48$) and *P. aeruginosa* ($n = 65$) isolated through routine diagnostics (RD) of clinical (CL) and environmental (ENV) specimens from the same UoL hospitals, were included in the analysis. These isolates covered a broader range of patients and hospital areas than the ICUs, and were collected from 2012 to 2019 for *K. pneumoniae* (Table 1) and from 2016 to 2019 for *P. aeruginosa* (Table 2). Furthermore, several isolates ($n = 13$ *K. pneumoniae* and $n = 6$ *P. aeruginosa*) received in the microbiology laboratory from two external small animal facilities (one hospital [SAH2] and one practice [SAP]) for environmental surveillance ($n = 11$) or for routine clinical diagnostics ($n = 8$) were also included, resulting in $n = 199$ bacterial isolates analysed overall with FTIR spectroscopy, whose summary is presented in Table 3.

2.2 Sample processing

Clinical and environmental specimens processing was performed as previously described (Zendri et al., 2023). In brief, clinical isolates were obtained through routine microbiology diagnostics, processed in accordance with local laboratory protocols for culture and antimicrobial susceptibility testing (AST) using the CLSI methodology and interpretative criteria (Clinical and Laboratory Standards Institute (CLSI), 2018). All isolates were identified using MALDI-TOF MS (MALDI Biotyper 4.1.100 Software, Bruker Daltonics, Bremen, Germany) with a score > 2.0. Hospital environmental bacterial isolates were obtained from routine environmental surveillance samples, collected aseptically by veterinary nurses using sterile gauzes placed individually in Buffered Peptone Water (BPW) and enriched overnight at 37°C aerobically. To screen for MDR isolates, a small BPW inoculum in which faeces and ENV samples were placed, was plated onto eosin methylene blue agar (EMBA; Thermo Scientific) containing 1 mg/mL of cefotaxime (Sigma-Aldrich Ltd., United Kingdom) and incubated for 24 h at 37°C aerobically. EMBA negative morphotypes identified as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* using MALDI-TOF MS were included in the downstream analysis. All environmental surveillance and clinical MDR isolates were continuously stored at-80°C in the local strain collection; inclusion of these isolates in the study was based on phenotypic resistance to extended-spectrum cephalosporins and MDR status upon broth microdilution and/or disc diffusion antimicrobial susceptibility testing (AST) performed at the time of diagnostics.

2.3 Fourier-transform infrared spectroscopy

A total of $n = 199$ MDR bacterial isolates, of which $n = 92$ *K. pneumoniae* and $n = 107$ *P. aeruginosa*, from all hospitals/clinics were typed using FTIR spectroscopy (IR Biotyper, Bruker Daltonics, Bremen, Germany) to determine their clonal relatedness. Bacterial isolates were grown on 5% sheep Blood Agar under strictly controlled temperature, time and humidity conditions (22 h incubation at 37°C under aerobic atmosphere) and harvested in Eppendorf tubes to create bacterial suspensions in equal parts of 70% ethanol and molecular grade water before spotting the target plate. Tests were run with three technical replicates in each of three independent experiments with two bacterial standards for internal quality control included in each run. Spectra were acquired in transmission mode using the OPUS v7.5 software (Bruker Optics GmbH). Spectra were then pre-processed (calculated 2nd derivative, cut to 1,300–800 cm-1, vector-normalised) and further analysed with the IR Biotyper Client Software v1.5 (Bruker Daltonics GmbH). Dendrograms expressing Hierarchical Cluster Analysis (HCA) were generated by the IR Biotyper Client Software v1.5 using the Euclidean distance and average linkage clustering methods using Cut-Off Values (COVs) to define clusters of 0.246 and of 0.287 for *P. aeruginosa* and *K. pneumoniae*, respectively. An optimisation procedure was conducted beforehand to determine optimal COVs for these organisms that were specific to our local laboratory conditions. The isolates used for species-specific optimisation of the COVs included epidemiologically related and non-related (e.g., reference strains) small animal *K. pneumoniae* and *P. aeruginosa* from external sources. The COVs were established according to manufacturer's recommendations on three repetitive experiments each using five technical and/or biological replicates of *K. pneumoniae* ($n = 8$) and *P. aeruginosa* ($n = 9$) isolates, previously typed by MLST or WGS. The automatically generated COVs were adjusted on the dendograms based on prior knowledge of the genetic relatedness of the *K. pneumoniae* and *P. aeruginosa* subsets of isolates used for the optimisation process.

2.4 Whole-genome sequencing and bioinformatics

To evaluate the performance of FTIR spectroscopy against a gold standard typing method, a subset of isolates demonstrating clonal relatedness was further characterised by WGS. Fifty-four *P. aeruginosa* and 27 *K. pneumoniae* isolates were selected, covering most FTIR clusters in a proportional measure to the cluster size. Fragment libraries (NEBNext Ultra II FS Kit; ~300 base pair inserts) were created from purified

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TABLE 1 Sample type and origin of clinical, faecal and environmental MDR *Klebsiella pneumoniae* spp. *pneumoniae* (*n* = 92) from four companion animal veterinary facilities in the UK (2012–2019).

Isolate ID	ICU PS	Hospital	Date collected	Sample type	Animal species	Site	Isolate
K1	Yes	SAH1	09/05/2018	Faecal	Dog	ICU	*Klebsiella pneumoniae*
K2		SAH1	09/05/2018	Environmental	–	ICU Kennel	*Klebsiella pneumoniae*
K3		SAH1	09/05/2018	Environmental	–	ICU Door Plate	*Klebsiella pneumoniae*
K4		SAH1	09/05/2018	Environmental	–	ICU Telephone	*Klebsiella pneumoniae*
K5		SAH1	09/05/2018	Environmental	–	ICU Floor	*Klebsiella pneumoniae*
K6		SAH1	11/05/2018	Environmental	–	ICU Kennel	*Klebsiella pneumoniae*
K7		SAH1	11/05/2018	Environmental	–	ICU Floor	*Klebsiella pneumoniae*
K8		SAH1	11/05/2018	Environmental	–	ICU Door Plate	*Klebsiella pneumoniae*
K9		SAH1	11/05/2018	Environmental	–	ICU Keyboard	*Klebsiella pneumoniae*
K10		SAH1	16/05/2018	Clinical	Dog	ICU Abdominal Fluid	*Klebsiella pneumoniae*
K11		SAH1	16/05/2018	Environmental	–	ICU Keyboard	*Klebsiella pneumoniae*
K12		SAH1	11/05/2018	Faecal	Dog	ICU	*Klebsiella pneumoniae*
K13		SAH1	18/05/2018	Faecal	Dog	ICU	*Klebsiella pneumoniae*
K14		SAH1	18/05/2018	Environmental	–	ICU Kennel	*Klebsiella pneumoniae*
K15		SAH1	18/05/2018	Environmental	–	ICU Floor	*Klebsiella pneumoniae*
K16		SAH1	18/05/2018	Environmental	–	ICU Keyboard	*Klebsiella pneumoniae*
K17		SAH1	18/05/2018	Environmental	–	ICU Telephone	*Klebsiella pneumoniae*
K18		SAH1	18/05/2018	Environmental	–	ICU Door Plate	*Klebsiella pneumoniae*
K19		SAH1	18/05/2018	Environmental	–	ICU Kennel	*Klebsiella pneumoniae*
K20		SAH1	18/05/2018	Environmental	–	ICU Floor	*Klebsiella pneumoniae*
K21		SAH1	18/05/2018	Environmental	–	ICU Telephone	*Klebsiella pneumoniae*
K22		SAH1	18/05/2018	Environmental	–	ICU Keyboard	*Klebsiella pneumoniae*
K23		SAH1	18/05/2018	Environmental	–	ICU Door Plate	*Klebsiella pneumoniae*
K24		SAH1	22/05/2018	Environmental	–	ICU Keyboard	*Klebsiella pneumoniae*
K25		SAH1	22/05/2018	Environmental	–	ICU Kennel	*Klebsiella pneumoniae*
K26		SAH1	22/05/2018	Environmental	–	ICU Door Plate	*Klebsiella pneumoniae*
K27		SAH1	22/05/2018	Environmental	–	ICU Floor	*Klebsiella pneumoniae*
K28		SAH1	22/05/2018	Environmental	–	ICU Telephone	*Klebsiella pneumoniae*
K29		SAH1	22/05/2018	Faecal	Dog	ICU	*Klebsiella pneumoniae*
K30		SAH1	25/05/2018	Faecal	Dog	ICU	*Klebsiella pneumoniae*
K31		SAH1	25/05/2018	Environmental	–	ICU Floor	*Klebsiella pneumoniae*
K32	No	SAH1	09/05/2018	Environmental	–	ICU Door Handle	*Klebsiella pneumoniae*
K33		SAH1	14/08/2017	Environmental	–	Laboratory Fridge	*Klebsiella pneumoniae*
K34		EH	23/06/2016	Environmental	–	Stable A10	*Klebsiella pneumoniae*
K35		SAH1	01/08/2016	Environmental	–	ICU Door Handle	*Klebsiella pneumoniae*
K36		SAH1	18/07/2016	Environmental	–	ICU Keyboard	*Klebsiella pneumoniae*
K37		SAH1	18/07/2016	Environmental	–	Student Locker	*Klebsiella pneumoniae*
K38		SAH1	12/07/2016	Environmental	–	Reception Photocopier	*Klebsiella pneumoniae*
K39		SAH1	09/03/2018	Clinical	Dog	Urine	*Klebsiella pneumoniae*
K40		SAH1	17/01/2018	Clinical	Dog	Faeces	*Klebsiella pneumoniae*
K41		EH	28/06/2013	Environmental	–	Stable A6	*Klebsiella pneumoniae*
K42		EH	17/03/2016	Environmental	–	Student Keyboard	*Klebsiella pneumoniae*
K43		SAH1	01/12/2017	Environmental	–	Dermatology Keyboard	*Klebsiella pneumoniae*

(Continued)

TABLE 1 (Continued)

Isolate ID	ICU PS	Hospital	Date collected	Sample type	Animal species	Site	Isolate
K44		SAH1	07/01/2016	Environmental	–	RT Anaesthetic Machine	*Klebsiella pneumoniae*
K45		EH	17/03/2016	Environmental	–	ICU Stocks	*Klebsiella pneumoniae*
K46		SAH1	29/07/2019	Clinical	Dog	Blood	*Klebsiella pneumoniae*
K47		SAH1	29/07/2019	Clinical	Dog	Abdominal fluid	*Klebsiella pneumoniae*
K48		SAH1	16/07/2019	Clinical	Dog	Skin lesion	*Klebsiella pneumoniae*
K49		SAH1	15/10/2018	Environmental	–	Treatment room tap	*Klebsiella pneumoniae*
K50		SAH1	15/10/2018	Environmental	–	Surgical ward tap	*Klebsiella pneumoniae*
K51		EH	08/08/2018	Clinical	Horse	Surgical site	*Klebsiella pneumoniae*
K52		SAH1	30/07/2018	Environmental	–	ICU Kennel	*Klebsiella pneumoniae*
K53		SAH1	23/07/2018	Clinical	Dog	Surgical site	*Klebsiella pneumoniae*
K54		SAH1	07/11/2017	Clinical	Dog	(Ortho) Surgical site?	*Klebsiella pneumoniae*
K55		SAH1	23/09/2017	Clinical	Dog	Urine	*Klebsiella pneumoniae*
K56		SAH1	14/09/2017	Clinical	Dog	Faeces	*Klebsiella pneumoniae*
K57		SAH1	12/09/2017	Clinical	Dog	Urine	*Klebsiella pneumoniae*
K58		SAP	23/07/2017	Clinical	Dog	Wound	*Klebsiella pneumoniae*
K59		SAH1	09/03/2017	Environmental	–	ICU Bandage Trolley	*Klebsiella pneumoniae*
K60		SAH1	07/03/2017	Clinical	Dog	Urine	*Klebsiella pneumoniae*
K61		EH	04/01/2017	Clinical	Horse	Surgical site	*Klebsiella pneumoniae*
K62		SAH1	07/12/2016	Clinical	Dog	PEG Tube Site	*Klebsiella pneumoniae*
K63		SAH1	05/10/2016	Clinical	Dog	Skin lesion	*Klebsiella pneumoniae*
K64		SAH1	26/01/2016	Environmental	–	Ward shower head	*Klebsiella pneumoniae*
K65		SAP	10/09/2015	Clinical	Dog	Urine	*Klebsiella pneumoniae*
K66		SAP	06/08/2015	Clinical	Cat	Fresh tissue	*Klebsiella pneumoniae*
K67		SAP	01/08/2015	Clinical	Cat	Wound	*Klebsiella pneumoniae*
K68		SAP	25/07/2015	Clinical	Dog	Wound	*Klebsiella pneumoniae*
K69		SAP	16/07/2015	Clinical	Dog	Fresh tissue	*Klebsiella pneumoniae*
K70		SAP	19/06/2015	Clinical	Dog	Skin lesion	*Klebsiella pneumoniae*
K71		SAP	27/05/2015	Clinical	Dog	Urine	*Klebsiella pneumoniae*
K72		EH	24/04/2015	Clinical	Horse	Surgical Site	*Klebsiella pneumoniae*
K73		EH	17/04/2015	Environmental	–	Stable A3	*Klebsiella pneumoniae*
K74		EH	22/12/2014	Clinical	Horse	Surgical site	*Klebsiella pneumoniae*
K75		EH	27/06/2014	Clinical	Horse	Surgical Site	*Klebsiella pneumoniae*
K76		SAH1	17/01/2014	Clinical	Dog	Surgical site	*Klebsiella pneumoniae*
K77		SAH1	14/10/2013	Clinical	Dog	Urine	*Klebsiella pneumoniae*
K78		SAH1	10/10/2013	Clinical	Dog	Urine	*Klebsiella pneumoniae*
K79		SAH1	09/10/2013	Clinical	Dog	Wound	*Klebsiella pneumoniae*
K80		SAH1	31/08/2013	Clinical	Dog	Thoracic fluid	*Klebsiella pneumoniae*
K81		SAH1	15/08/2013	Clinical	Dog	Skin lesion	*Klebsiella pneumoniae*
K82		EH	12/08/2013	Environmental	–	Stable A8	*Klebsiella pneumoniae*
K83		EH	29/06/2013	Environmental	–	Y-piece no. 1	*Klebsiella pneumoniae*
K84		EH	28/06/2013	Environmental	–	ICU Stocks	*Klebsiella pneumoniae*
K85		EH	21/06/2013	Environmental	–	Stable D6 Floor	*Klebsiella pneumoniae*
K86		EH	06/06/2013	Clinical	Horse	Rectal Swab	*Klebsiella pneumoniae*
K87		SAH1	21/06/2012	Clinical	Dog	ET tube swab	*Klebsiella pneumoniae*

(Continued)

TABLE 1 (Continued)

Isolate ID	ICU PS	Hospital	Date collected	Sample type	Animal species	Site	Isolate
K88		SAH2	04/07/2019	Environmental	–	ICU Air con vents pre-clean	*Klebsiella pneumoniae*
K89		SAH2	04/07/2019	Environmental	–	ICU Kennel keys/drawers pre-clean	*Klebsiella pneumoniae*
K90		SAH2	04/07/2019	Environmental	–	ICU Air con vents post-clean	*Klebsiella pneumoniae*
K91		SAH2	04/07/2019	Environmental	–	ICU Kennel keys/drawers post-clean	*Klebsiella pneumoniae*
K92		SAH2	04/07/2019	Environmental	–	ICU Tap & sink post-clean	*Klebsiella pneumoniae*

ICU PS, Intensive Care Unit Pilot Study; SAH1, Small Animal Hospital 1 (University of Liverpool); EH, Equine Hospital (University of Liverpool); SAP, Small Animal Practice (external); SAH2, Small Animal Hospital 2 (external).

genomic DNA (Qiagen QIAmp DNA mini kit) and sequenced using a 2×150 base pair paired-end protocol (Illumina NovaSeq SP; CGR, University of Liverpool). *De novo* assembly was performed using SPAdes 3.16.0 (Bankevich et al., 2012) and genome annotation via Prokka 1.14.5 (Seemann, 2014). MLST profiles and allele sequences were obtained.[1] All allele sequences were aligned to assemblies using Bowtie2 version 2.3.5.1 (Langmead and Salzberg, 2012) in sensitive mode. The allele that aligned best for each locus was selected and the sequence type was determined by comparing perfectly detected alleles against the database profiles. Sequence types were used to infer eBURST groups, using goeBURST,[2] where group members shared at least 2 ST locus alleles. *In silico* identification of virulence and AMR genes was performed using the Virulence Factor Database (VFDB; Chen et al., 2005) and the Comprehensive Antibiotic Resistance Database (CARD; McArthur et al., 2013), respectively. Incompatibility (Inc) group plasmids were identified in *K. pneumoniae* via PlasmidFinder (Carattoli et al., 2014). Using assemblies as input, pangenome reconstruction and concatenated core gene multiple alignment was carried using Panaroo (Tonkin-Hill et al., 2020). Subsequently, the multiple alignment data was used as input to IQ-TREE using the GTR model. The resulting tree was imported to the Interactive Tree of Life (iTOL; Letunic and Bork, 2021). Genomic sequences are deposited on the European Nucleotide Archive (ENA) under the project accession: PRJEB70897.

2.5 Concordance between FTIR and MLST clusters

The clusters defined by MLST extrapolated from genomic sequences were compared with those obtained by FTIR biotyping using the service[3] by calculating the Adjusted Rand index (AR) and Adjusted Wallace coefficient (AW) with 95% confidence (Carriço et al., 2006). AR compares partitions without consideration of the reference method and evaluates the congruence between two typing methods. In comparison, AW provides inter-cluster distances and evaluates the directional agreement between typing methods (Carriço et al., 2006; Pinto et al., 2008; Severiano et al., 2011a,b). In essence, AR and AW equal to 1 indicate a perfect correlation between the two typing methods. The optimal cut-offs for each species were defined by maximising AR between MLST and FTIR results.

1 http://rest.pubmlst.org/db/

2 https://github.com/jacarrico/goeBURST

3 http://www.comparingpartitions.info/

3 Results

3.1 Fourier-transform infrared spectroscopy

After FTIR spectra acquisition, dendrograms were built for each species. FTIR analysis identified several clusters for both organisms tested, suggesting clonal relatedness of the isolates within individual clusters. Overall, $n = 25$ FTIR clusters were identified spanning across both pathogens; the number of isolates within each cluster varied, ranging from two to 43, with an average cluster size of 6.8 isolates. Fourteen of 25 clusters (56%) contained a mixture of environmental and clinical and/or faecal carriage isolates in various combinations, typically several environmental isolates linked to fewer clinical/carriage ones. Wound and skin isolates were the most common clinical specimen type represented within mixed clusters. Environmental isolates only, collected from different hospitals' sites, also accounted for some of the clusters observed. Several isolates collected from the ICUs contributed to cluster formation, either with or without association to isolates collected from the broader hospital patients and departments. Details are provided in the following paragraphs for each organism.

3.1.1 FTIR of *Klebsiella pneumoniae*

Seven *K. pneumoniae* clusters were defined overall totalling 83/92 isolates (Figure 1A); of these, the largest (Cluster 167) contained 43 small animal isolates including environmental, faecal carriage and clinical isolates collected over a 19-month period (March 2017–October 2018); this cluster included isolates predominantly obtained from the SAH1 ICU during the PS (31/43) with fewer non-ICU isolates also found in this cluster. All isolates sequenced from this large cluster ($n = 11$) belonged to ST147. The ICU *K. pneumoniae* isolates from Cluster 167, were identified on numerous ICU surfaces and canine faecal carriage samples within a relatively short time-frame (31 isolates over 16 days). Contaminated environmental sites included ICU telephone receiver, computer keyboards, door handles, equipment trolley, floor and patients' kennels. One clinical *K. pneumoniae* was cultured from a case of hospital-acquired septic peritonitis in a dog enrolled in the ICU study that was previously found to be a faecal carrier of the same strain. Sixteen isolates accounted for the second largest *K. pneumoniae* FTIR cluster (Cluster 168), comprising clinical and environmental isolates collected from the wider SAH1 environments over a 6-year period (from 2012 to 2018). Clinical isolates included in this group consisted of skin and wound isolates ($n = 2$ sequenced, ST11) but also sterile fluid ones

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TABLE 2 Sample type and origin of clinical, faecal and environmental MDR *Pseudomonas aeruginosa* (*n* = 107) from three companion animal veterinary facilities in the UK (2016–2019).

Isolate ID	ICU PS	Hospital	Date collected	Sample type	Animal species	Site	Isolate
P1	Yes	EH	20/03/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P2		SAH1	20/03/2018	Environmental	–	ICU Keyboard	*Pseudomonas aeruginosa*
P3		SAH1	20/03/2018	Environmental	–	ICU Floor	*Pseudomonas aeruginosa*
P4		SAH1	20/03/2018	Environmental	–	ICU Phone	*Pseudomonas aeruginosa*
P5		SAH1	20/03/2018	Environmental	–	ICU Phone	*Pseudomonas aeruginosa*
P6		EH	23/03/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P7		SAH1	04/04/2018	Environmental	–	ICU Floor	*Pseudomonas aeruginosa*
P8		EH	20/04/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P9		EH	30/04/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P10		EH	03/05/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P11		EH	03/05/2018	Environmental	–	ICU Window	*Pseudomonas aeruginosa*
P12		EH	09/05/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P13		EH	09/05/2018	Environmental	–	ICU Window	*Pseudomonas aeruginosa*
P14		EH	09/05/2018	Environmental	–	ICU Water Bucket	*Pseudomonas aeruginosa*
P15		EH	14/05/2018	Environmental	–	ICU Hay Rack	*Pseudomonas aeruginosa*
P16		EH	14/05/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P17		EH	14/05/2018	Environmental	–	ICU Water Bucket	*Pseudomonas aeruginosa*
P18		EH	16/05/2018	Environmental	–	ICU Hay Rack	*Pseudomonas aeruginosa*
P19		EH	16/05/2018	Environmental	–	ICU Water Bucket	*Pseudomonas aeruginosa*
P20		EH	16/05/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P21		EH	16/05/2018	Environmental	–	ICU Window	*Pseudomonas aeruginosa*
P22		EH	16/05/2018	Environmental	–	ICU Front Door Handle	*Pseudomonas aeruginosa*
P23		EH	16/05/2018	Environmental	–	ICU Hay Rack	*Pseudomonas aeruginosa*
P24		EH	17/05/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P25		EH	17/05/2018	Environmental	–	ICU Window	*Pseudomonas aeruginosa*
P26		EH	17/05/2018	Environmental	–	ICU Hay Rack	*Pseudomonas aeruginosa*
P27		EH	17/05/2018	Environmental	–	ICU Tie Ring	*Pseudomonas aeruginosa*
P28		EH	17/05/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P29		EH	17/05/2018	Environmental	–	ICU Window	*Pseudomonas aeruginosa*
P30		EH	17/05/2018	Environmental	–	ICU Water Bucket	*Pseudomonas aeruginosa*
P31		EH	21/05/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P32		EH	21/05/2018	Environmental	–	ICU Water Bucket	*Pseudomonas aeruginosa*
P33		EH	22/05/2018	Faecal	Horse	ICU	*Pseudomonas aeruginosa*
P34		EH	22/05/2018	Environmental	–	ICU Water Bucket	*Pseudomonas aeruginosa*
P35		SAH1	22/05/2018	Environmental	–	ICU Door plate	*Pseudomonas aeruginosa*
P36		SAH1	22/05/2018	Environmental	–	ICU Floor	*Pseudomonas aeruginosa*
P37	No	SAH1	10/10/2016	Environmental	–	Chemotherapy ICU Kennel Handle	*Pseudomonas aeruginosa*
P38		SAH1	26/10/2016	Environmental	–	Wards Drip Pump Holder	*Pseudomonas aeruginosa*
P39		SAH1	01/08/2016	Environmental	–	Wards Keyboard	*Pseudomonas aeruginosa*
P40		SAH1	01/08/2016	Environmental	–	ICU Door Handle	*Pseudomonas aeruginosa*
P41		EH	13/11/2017	Clinical	Horse	Wound	*Pseudomonas aeruginosa*
P42		SAH1	2017	Environmental	–	Congregation water dispenser tray	*Pseudomonas aeruginosa*
P43		EH	23/06/2016	Environmental	–	Stable A1	*Pseudomonas aeruginosa*

(Continued)

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TABLE 2 (Continued)

Isolate ID	ICU PS	Hospital	Date collected	Sample type	Animal species	Site	Isolate
P44		SAH1	15/06/2018	Environmental	–	Sink tap	*Pseudomonas aeruginosa*
P45		SAH1	01/05/2018	Clinical	Dog	Surgical Site	*Pseudomonas aeruginosa*
P46		SAH1	20/03/2018	Clinical	Dog	Wound	*Pseudomonas aeruginosa*
P47		EH	17/03/2016	Environmental	–	Laboratory Worktops	*Pseudomonas aeruginosa*
P48		SAH1	01/12/2017	Environmental	–	Dermatology Keyboard	*Pseudomonas aeruginosa*
P49		SAH1	30/03/2016	Environmental	–	Wards Shower Head	*Pseudomonas aeruginosa*
P50		EH	17/03/2016	Environmental	–	ICU Stocks	*Pseudomonas aeruginosa*
P51		SAH1	11/09/2019	Clinical	Cat	Wound	*Pseudomonas aeruginosa*
P52		SAH1	09/09/2019	Clinical	Dog	O' tube site	*Pseudomonas aeruginosa*
P53		SAH1	06/09/2019	Environmental	–	Anaesthesia breathing system	*Pseudomonas aeruginosa*
P54		SAH1	20/08/2019	Clinical	Dog	Traumatic wound	*Pseudomonas aeruginosa*
P55		SAH1	13/08/2019	Environmental	–	Theatre black crocs	*Pseudomonas aeruginosa*
P56		SAH1	24/06/2019	Clinical	Dog	Surgical wound	*Pseudomonas aeruginosa*
P57		SAH1	24/06/2019	Clinical	Dog	Surgical wound	*Pseudomonas aeruginosa*
P58		SAH1	04/06/2019	Clinical	Dog	Urine	*Pseudomonas aeruginosa*
P59		SAH1	02/04/2019	Environmental	–	Treatment room table	*Pseudomonas aeruginosa*
P60		SAH1	02/04/2019	Environmental	–	Washroom hairdryer	*Pseudomonas aeruginosa*
P61		SAH1	27/03/2019	Environmental	–	Lab keyboard	*Pseudomonas aeruginosa*
P62		SAH1	25/01/2019	Clinical	Dog	External fixator discharge	*Pseudomonas aeruginosa*
P63		SAH1	22/01/2019	Environmental	–	Wards washing machine door	*Pseudomonas aeruginosa*
P64		SAH1	22/01/2019	Environmental	–	Treatment room tap before cleaning	*Pseudomonas aeruginosa*
P65		SAH1	22/01/2019	Environmental	–	Treatment room tap after cleaning	*Pseudomonas aeruginosa*
P66		SAH1	18/01/2019	Clinical	Dog	Interdigital swab post-surgical	*Pseudomonas aeruginosa*
P67		SAH1	16/01/2019	Environmental	–	Wards washing machine	*Pseudomonas aeruginosa*
P68		SAH1	16/01/2019	Environmental	–	Treatment room tap 1	*Pseudomonas aeruginosa*
P69		SAH1	03/01/2019	Clinical	Dog	Synovial fluid post-surgical	*Pseudomonas aeruginosa*
P70		SAH1	30/10/2018	Clinical	Dog	Ortho implants post-TPLO	*Pseudomonas aeruginosa*
P71		SAH1	30/10/2018	Clinical	Dog	Synovial fluid - Stifle	*Pseudomonas aeruginosa*
P72		SAH1	15/10/2018	Environmental	–	Washing machine door (wards)	*Pseudomonas aeruginosa*
P73		SAH1	14/09/2018	Environmental	–	Surgical ward tap	*Pseudomonas aeruginosa*
P74		SAH1	12/10/2018	Clinical	Dog	Synovial fluid post-TPLO	*Pseudomonas aeruginosa*
P75		SAH1	30/08/2018	Environmental	–	ICU Kennel	*Pseudomonas aeruginosa*
P76		SAH1	30/07/2018	Environmental	–	Treatment room	*Pseudomonas aeruginosa*
P77		SAH1	15/06/2018	Environmental	–	Sink tap	*Pseudomonas aeruginosa*
P78		SAH1	31/07/2017	Clinical	Dog	Surgical wound	*Pseudomonas aeruginosa*
P79		SAH1	13/07/2017	Clinical	Dog	External otitis	*Pseudomonas aeruginosa*
P80		SAH1	07/07/2017	Clinical	Dog	External otitis	*Pseudomonas aeruginosa*
P81		SAH1	28/06/2017	Clinical	Dog	Skin disease	*Pseudomonas aeruginosa*
P82		SAH1	21/06/2017	Clinical	Dog	Biopsy (fresh tissue)	*Pseudomonas aeruginosa*
P83		SAH1	09/06/2017	Clinical	Dog	External otitis	*Pseudomonas aeruginosa*
P84		SAH1	08/06/2017	Clinical	Cat	O-tube site	*Pseudomonas aeruginosa*
P85		SAH1	12/05/2017	Clinical	Dog	Skin swab	*Pseudomonas aeruginosa*
P86		SAH1	11/05/2017	Clinical	Cat	R frontal sinus fluid	*Pseudomonas aeruginosa*
P87		SAH1	10/04/2017	Clinical	Dog	Urine	*Pseudomonas aeruginosa*

(Continued)

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TABLE 2 (Continued)

Isolate ID	ICU PS	Hospital	Date collected	Sample type	Animal species	Site	Isolate
P88		SAH1	17/03/2017	Clinical	Dog	Faeces	*Pseudomonas aeruginosa*
P89		SAH1	07/03/2017	Clinical	Dog	Urine	*Pseudomonas aeruginosa*
P90		SAH1	17/02/2017	Clinical	Dog	Tympanic bulla (fresh tissue)	*Pseudomonas aeruginosa*
P91		SAH1	02/02/2017	Clinical	Dog	External otitis	*Pseudomonas aeruginosa*
P92		SAH1	02/02/2017	Clinical	Dog	ET tube swab	*Pseudomonas aeruginosa*
P93		SAH1	13/02/2017	Clinical	Dog	Stifle wound swab	*Pseudomonas aeruginosa*
P94		SAH1	30/01/2017	Clinical	Dog	External otitis	*Pseudomonas aeruginosa*
P95		SAH1	23/01/2017	Clinical	Dog	External otitis	*Pseudomonas aeruginosa*
P96		SAH1	23/01/2017	Clinical	Dog	L tympanic bulla post-flush	*Pseudomonas aeruginosa*
P97		SAH1	11/01/2017	Clinical	Dog	Urine	*Pseudomonas aeruginosa*
P98		SAH1	11/04/2017	Clinical	Dog	Urine	*Pseudomonas aeruginosa*
P99		SAH1	07/02/2017	Environmental	–	Tea room tap	*Pseudomonas aeruginosa*
P100		SAH1	22/01/2017	Environmental	–	Washroom cupboard	*Pseudomonas aeruginosa*
P101		SAH1	2017	Clinical	Dog	Wound swab	*Pseudomonas aeruginosa*
P102		SAH2	11/07/2019	Environmental	–	ICU pre-clean desk area	*Pseudomonas aeruginosa*
P103		SAH2	11/07/2019	Environmental	–	ICU pre-clean cleaning cupboard	*Pseudomonas aeruginosa*
P104		SAH2	11/07/2019	Environmental	–	ICU pre-clean tap & sink	*Pseudomonas aeruginosa*
P105		SAH2	11/07/2019	Environmental	–	ICU pre-clean floor & drain	*Pseudomonas aeruginosa*
P106		SAH2	11/07/2019	Environmental	–	ICU post-clean cleaning cupboard	*Pseudomonas aeruginosa*
P107		SAH2	11/07/2019	Environmental	–	ICU post-clean floor & drain	*Pseudomonas aeruginosa*

ICU PS, Intensive Care Unit Pilot Study; SAH1, Small Animal Hospital 1 (University of Liverpool); EH, Equine Hospital (University of Liverpool); SAH2, Small Animal Hospital 2 (external).

(thoracic fluid and cystocentesis urine) alongside environmental isolates from clinical (e.g., anaesthetic machine and ICU surfaces; $n = 2$ sequenced, ST11) and non-clinical (e.g., reception photocopier, lockers) hospital areas.

Of note, all *K. pneumoniae* from external small animal settings accurately clustered with isolates from their respective hospital source (SAP for Cluster 153 and SAH2 for Cluster 165).

Two small clusters (of 5 and 4 isolates; Clusters 161 and 163) of environmental *K. pneumoniae* (e.g., from stables, ICU) in association with post-surgical infection isolates were identified in the EH; these belonged to ST307 and ST789, respectively.

Importantly, clonal dissemination of *K. pneumoniae* was identified in all veterinary settings; two clones were circulating in each one of the UoL hospitals (ST147 and ST11 in the SAH1 and ST307 and ST789 in the EH). In addition, single clones were identified in the two external facilities included in the study, i.e., Cluster 165 composed of environmental isolates from the SAH2 ICU (ST307) and Cluster 153 of clinical isolates originating from the SAP (ST336).

3.1.2 FTIR of *Pseudomonas aeruginosa*

Of the 18 *P. aeruginosa* FTIR clusters, the two largest contained 16 (Cluster 173) and 13 (Cluster 175) mixed EH and SAH1 isolates collected over two distinct 3-year periods (Figure 1B). Cluster 173 comprised *P. aeruginosa* isolated from a horse faecal samples and multiple ICU environmental sites surrounding the patient ($n = 2$ sequenced, ST2644), alongside few other clinical and environmental isolates from the broader EH one to 2 years prior ($n = 3$ sequenced, ST2644). Small animal *P. aeruginosa* in Cluster 173 were mostly water-borne environmental isolates (mostly typed to ST446, but also ST309 and ST815) cultured from diverse sink taps within the SAH1 (e.g., treatment room taps, wards shower head and tea room tap) over a 3-year interval. Cluster 175 comprised *P. aeruginosa* isolates linked to a single ICU PS horse ($n = 2$ sequenced, ST1714) and the remainder from the SAH1. In this cluster, small animal isolates were mostly clinical *P. aeruginosa* from surgical wounds and otitis cases cultured between 2017 and 2019 ($n = 8$ sequenced, multiple STs). Small ICU clusters (4–6 isolates) of PS isolates (faecal carriage and environmental) linking 2–3 patients over short periods (from 2 days to 7 weeks) were established in the EH (Clusters 161, 162, 164 and 153) but not in the SAH1 (Cluster 154 was linked to a single patient). Some other clusters (2–8 isolates; Clusters 170, 174, 176, 177, 158, 169, 171, 166 and 130) comprised various combinations of environmental and/or clinical isolates within the SAH1; *P. aeruginosa* isolated from a range of clinical specimens featured in these clusters, including both normally contaminated (wound, skin and ear) and sterile (cystocentesis urine and synovial fluid) body sites.

3.2 Whole-genome sequencing and comparison with FTIR typing

In total, 81 ($n = 81$) isolates underwent WGS (at least one isolate per cluster and up to nine) and MLST, eBURST and other genotypic data were extracted from whole genomes. MLST, eBURST groups and AMR genes are summarised in Table 4 (full characterisation including virulence genes and plasmid types is found in Supplementary Table S1).

TABLE 3 Summary of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolates (n = 199) from veterinary settings (small animal and equine) analysed by Fourier-transform Infrared (FTIR) spectroscopy.

	Klebsiella pneumoniae	*Pseudomonas aeruginosa*
Small animal		
ICU-PS	31	7
Faecal	5	0
Environmental	26	7
RD	46	67
Clinical	29	36
Environmental	17	31
Equine		
ICU-PS	0	29
Faecal	–	12
Environmental	–	17
RD	15	4
Clinical	6	1
Environmental	9	3
Total	92	107

ICU-PS, Intensive Care Unit Pilot Study, including faecal colonisation and environmental isolates; RD, Routine diagnostics, including clinical and environmental surveillance isolates from the University of Liverpool (SAH1 and EH) and two external facilities (SAH2 and SAP).

Concordance between the FTIR and WGS findings was overall high for *K. pneumoniae* and poor for *P. aeruginosa* using the established COVs. For *K. pneumoniae*, both typing methods, FTIR and WGS, achieved the same level of discriminatory power, dividing the isolates into seven clusters each. The Simpson's diversity index (SDI; Simpson, 1949; Hunter and Gaston, 1988) for both was 0.789 and 0.781, respectively. FTIR and WGS typing showed very high agreement, resulting in an adjusted Rand index (ARI; Carriço et al., 2006) of 0.958. The adjusted Wallace index (AWI; Severiano et al., 2011b) for FTIR predicting WGS types was 0.983, while in the reverse direction it was 0.934. For *P. aeruginosa*, the congruence of typing results was low. First, WGS was able to distinguish 28 different types (SDI 0.941) while FTIR could only distinguish 18 clusters (SDI 0.243). This was also reflected in quite asymmetrical AWIs, where FTIR predicted WGS types with 0.243, but WGS predicted FTIR clusters with 0.437. Overall congruence was 0.313 using the ARI.

3.2.1 *Klebsiella pneumoniae* WGS

Twenty-seven *K. pneumoniae* across the seven FTIR clusters had WGS data available. Results indicate high level agreement on the identification of related isolates between the spectroscopic and the genomic methods (Figures 1A, 2A); all sequenced *K. pneumoniae* isolates clustered by FTIR spectroscopy belonged to the same or closest ST (one isolate in Cluster 153 was of unknown ST type; however, the nearest ST match aligned with its cluster's ST) with six STs identified overall. Notably, ST307 was detected in two adjacent Clusters, 161 and 165; the former gathering isolates from the EH while the latter from the SAH2, supporting the FTIR Biotyper ability to discriminate between isolates of the same sequence type. One important finding was the association of ST147 harbouring plasmid-encoded quinolone resistance genes *qnr*B and *oqx*A/B, SHV-11 and DHA-1 beta-lactamase types with Cluster 167, the largest FTIR cluster of *K. pneumoniae* intensely circulating in the SAH1 ICU which included the hospital-acquired infection case in a dog. ST11 was associated to *K. pneumoniae* in Cluster 168, linking isolates from the wider SAH1 patients and areas, and additionally carried the fluoroquinolone acetylating aminoglycoside-(6)-N-acetyltransferase (*aac[6′]-Ib-cr*) gene. CTX-M-15-producing *K. pneumoniae* ST307 was identified in the former of two EH clusters (Cluster 161) and *qnrS*-positive ST789 in the latter (Cluster 163). Beyond beta-lactamase resistance genes present in all isolates, multidrug efflux pump genes *oqxA/B* were detected in 100% (27/27) of sequenced *K. pneumoniae*, *qnr* and *aac(6′)-Ib-cr* genes mediating resistance to fluoroquinolones were identified in 81.8% (25/27) and 29.6% (8/27) of the sequenced *K. pneumoniae* isolates, respectively, while CTX-M-15 type enzyme in 22% (6/27) of isolates. IncR type plasmid was common among ST147 and ST11 while IncFIB and IncFII plasmids were both found among ST307 and ST336. Concordance between AMR genes (Clusters 168 and 163) and Inc. types (Clusters 161, 167, 168 and 163) among sequenced isolates was not absolute within some clusters (Table 4), as some but not all (generally ≥50%) isolates carried several certain determinants, such as *qnrS* and IncFIA in 3/4 of Cluster 163 isolates.

3.2.2 *Pseudomonas aeruginosa* WGS

Fifty-four *P. aeruginosa* spanning over 18 FTIR clusters underwent WGS; in contrast to *K. pneumoniae*, the accuracy of FTIR clusters was generally poor when compared to WGS (Figures 1B, 2B). Heterogeneity of MLST types was observed in the vast majority of *P. aeruginosa* clusters where more than one isolate was sequenced, with up to nine STs detected in Cluster 175 (where 10 of 13 were sequenced). Multiple STs were also identified in Clusters 173 (n = 4 STs over 11 sequenced isolates) and 153 (n = 2 STs over 7 sequenced isolates), others combining SAH1 and EH isolates as Cluster 175. In all these mixed clusters, the equine isolates typically accounted for a single ST; notably, the majority of ST2644 isolates from Clusters 173 and 153 were isolated from EH ICU patients (faecal carriage) and surroundings during the pilot study over a short time. In contrast, the SAH1 isolates from any of the mixed clusters were of discordant ST (when more than one small animal isolate was found), apart from ST446 in Cluster 173 (here two thirds of sequenced small animal isolates belonged to this ST). Cluster 162 and 164 (n = 2 and 3 isolates sequenced, respectively), both comprising EH ICU pilot study isolates, were the only examples of genomically highly similar isolates appropriately clustered by the FTIR. SAH1 only clusters where more than one isolate was sequenced displayed substantial within-cluster ST heterogeneity, such as Clusters 166, 174 and 176. In addition to genomically divergent isolates inadequately clustered together by the FTIR, examples of genotypically highly similar isolates inappropriately not clustered were also seen, e.g., ST2644, ST395, ST17 and ST446 isolates from the same facility are seen across more than one cluster (usually 2 or 3). Finally, *P. aeruginosa* STs associated to clusters where single isolates were sequenced differed from one another and from STs in larger clusters, except for ST17. Overall, 28 STs were identified with ST2644, ST446, ST17, ST395, ST27 and ST3016 being most represented (Table 4). Significantly less variability was observed among *P. aeruginosa* with regards to AMR determinants. Sequenced isolates harboured a large arsenal of efflux pumps, particularly of the Mex (A/B, C/D, E/F, X/Y, M/J, and X/Y types amongst others) and Opr

(OprJ, M and N) families and 73.7% contained OXA-50 (42/57) beta-lactamase; *gyrA* mutation and *qnrB* gene conferring fluoroquinolone resistance were detected in 5.3% (3/57) and 1.8% (1/57) of isolates, respectively.

4 Discussion

To the best of the authors' knowledge, this is the first study to evaluate the FTIR potential for bacterial strain typing in a clinically-relevant timeframe within veterinary settings. We assessed the discriminatory power of FTIR spectroscopy by typing two ESKAPE MDR gram-negative bacterial species with potential implication in HCAIs, namely *K. pneumoniae* and *P. aeruginosa,* isolated from cases of clinical infection in hospitalised or vet-visiting companion animals (i.e., dogs, cats and horses), as well as faecal colonisation and environmental sites across veterinary clinics/hospitals. The IR Biotyper could determine clonal relatedness of MDR gram-negative isolates circulating in veterinary hospitals within 2–3 h from fresh culture harvest, making it an attractive tool for rapid strain typing of organisms associated with HCAIs. This technique has the potential to be integrated into the routine diagnostic workflow of a clinical microbiology laboratory for "real-time" monitoring of MDR pathogens' transmission events to support prompt and targeted infection control measures during hospital outbreaks.

In our study, we found that the discriminatory power of FTIR spectroscopy was high for *K. pneumoniae* veterinary isolates using a COV of 0.287, which demonstrated overall concordance (ARI of 0.958) with WGS results for the fraction of isolates that underwent the gold standard technique. This is in agreement with previous studies evaluating the IR Biotyper performance on *K. pneumoniae*-associated human hospital outbreaks (Rakovitsky et al., 2020; Rodrigues et al., 2020; Silva et al., 2020; Hu et al., 2021). In one study assessing the IR Biotyper performance on environmental isolates of *K. pneumoniae* and other gram-negative bacilli from nine human hospitals, however, the instrument displayed limited sensitivity to cluster isolates compared with WGS (Aranega Bou et al., 2023); this contrasts with our findings where both environmental and clinical/carriage *K. pneumoniae* were adequately clustered according to WGS data. In addition, some authors evaluated the performance of the FTIR as a first-line typing tool for the identification of ESBL-producing *Klebsiella pneumoniae* outbreaks in the hospital setting in comparison with conventional epidemiology (CE) investigations and found both AR and AW were significantly higher for FTIR clustering than CE clustering (Wang-Wang et al., 2022). Importantly, the majority of *K. pneumoniae* isolates circulating in veterinary hospitals

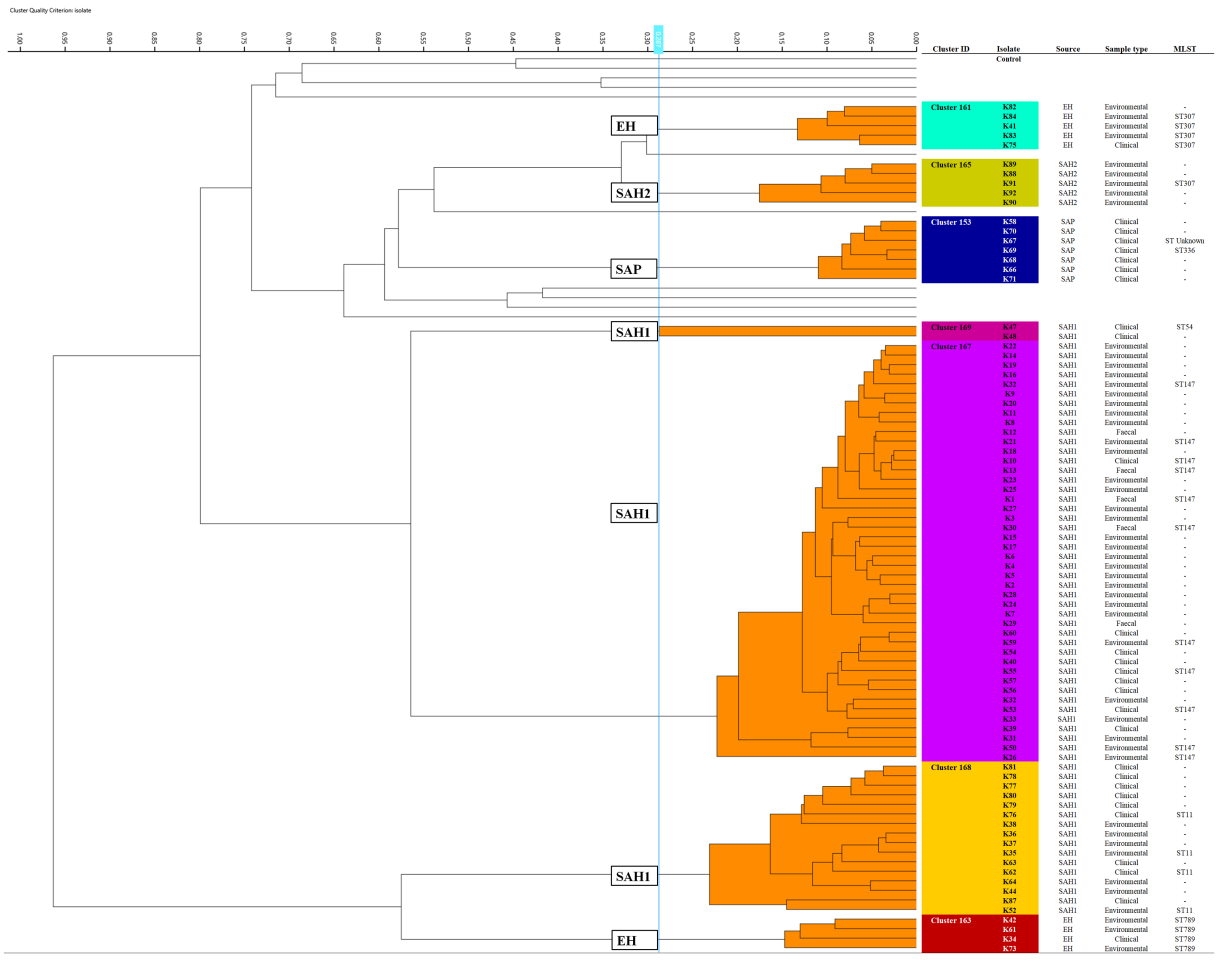

FIGURE 1 (Continued)

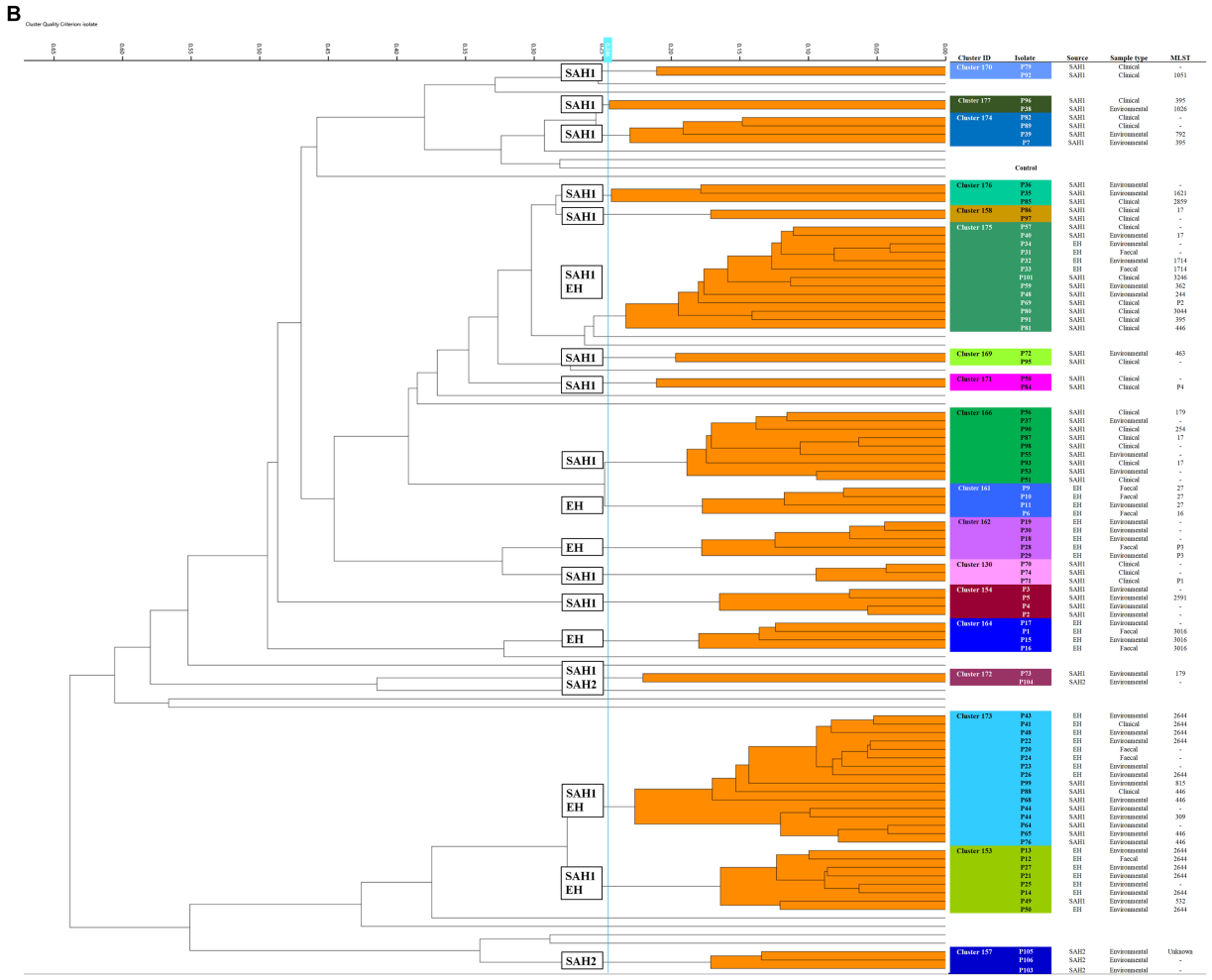

FIGURE 1
Fourier-transform Infrared (FTIR) biotyping of *Klebsiella pneumoniae* subsp. *pneumoniae* **(A)** and *Pseudomonas aeruginosa* **(B)** veterinary isolates. Dendograms were obtained by Euclidean average spectra analysis of *K. pneumoniae* (*n*=92) and *P. aeruginosa* (*n*=107) from clinical, faecal and environmental surveillance specimens collected in small animal and equine veterinary facilities. Analysis was based on three technical replicates of each isolate and included one control strain per each organism; the blue line represents the adjusted cut-off values (COVs) of 0.287 and 0.246 for *K. pneumoniae* and *P. aeruginosa*, respectively. Clusters comprising two or more isolates are shaded in orange. Isolate data is shown for clusters only. SAH1: Small Animal Hospital 1 (University of Liverpool); EH, Equine Hospital (University of Liverpool); SAP, Small Animal Practice (external); SAH2, Small Animal Hospital 2 (external).

in our study were consistent with critically important human lineages and they segregated into well-defined hospital source clusters. Clonally related *K. pneumoniae* ST147 formed the largest SAH1 cluster comprising 72% of isolates collected from the ICU over 16 days. This lineage was identified simultaneously in carriage (faeces), environmental (patients kennels, ICU door handles, keyboards and phone) and one clinical infection case (septic peritonitis) in an ICU hospitalised dog. *K. pneumoniae* ST147 is an emerging high-risk human clone with worldwide distribution (Navon-Venezia et al., 2017) and the potential to become a major public health threat due to its ability to cause serious infections and association with AMR, including pan-resistance. This successful clone consists of multiple clades/clusters, often encoding for ESBLs and carbapenemases such as CTX-M-15, KPC-2 and NDM-1 amongst others (Peirano et al., 2020). To date, *K. pneumoniae* ST147 has been occasionally described in companion and exotic pets' clinical specimens (Ovejero et al., 2017; Marques et al., 2019; Davies et al., 2022) without association to carbapenem resistance. Similar considerations may be formulated for *K. pneumoniae* ST11 identified in the SAH1 and ST307 and ST789 isolated from the EH; these epidemic human-associated clones (except for ST789) have already been described in companion animals more extensively than ST147 (Harada et al., 2016; Loncaric et al., 2020; Garcia-Fierro et al., 2022), with reports of OXA-48-producing ST11 strains causing animal infection in some European countries (Pulss et al., 2018). In contrast, *K. pneumoniae* ST15 broadly reported among dogs, cats and horses (Haenni et al., 2012; Stolle et al., 2013; Ewers et al., 2014; Maeyama et al., 2018), was not detected in the present study. Altogether, these findings point towards the possibility of clonal transmission of *K. pneumoniae* within all veterinary facilities investigated, as outlined by the FTIR association of clusters (and STs) with their hospital sources and, for isolates within some clusters, by the short sample collection window (e.g., the SAH1 ICU isolates in Cluster 167). Furthermore, the tight clustering of clinical isolates from the SAP

TABLE 4 Comparison of Fourier-Transform InfraRed (FTIR) Spectroscopy and Multi-Locus Sequence Typing (MLST) results extracted from Whole-Genome Sequences (WGS).

WGS isolates (n)	WGS Isolate type (n)	Hospital source	WGS Isolate details	FTIR Cluster	Cluster isolation interval	ST Type	eBURST	AMR genes
Klebsiella pneumoniae								
4	CL (1), ENV (3)	EH	Surgical wound, stable, ICU stocks, Y piece	161	12 months	307	C4	*oqx*A/B, aac(6′)-Ib-cr, CTX-M-15, fosA6, SHV-28, TEM-206, *qnr*B
1	ENV	SAH2	Air con vent	165	1 day	307	C4	*oqx*A/B, CTX-M-15, fosA6, SHV-28, TEM-1, *qnr*B
2	CL	SAP	Biopsy, wound	153	26 months	336, UT[1]	C0, Unknown	*oqx*A/B,aac(6′)-Ib-cr, CTX-M-15, DHA-1, FosA6, SHV-11, TEM-1, *qnr*B
1	CL	SAH1	Blood	169	Single time point[2]	54	C1	*oqx*A/B, FosA5, SHV-11
11	ENV (5), *F* (3), CL (3)	SAH1	ICU: door handles, telephones, trolleys, faeces and abdominal fluid. Non-ICU: surgical ward tap, surgical wound, urine.	167	19 months	147	C5	*oqx*A/B, DHA-1, FosA6, SHV-11, *qnr*B
4	CL (2), ENV (2)	SAH1	Surgical wound, PEG tube site, ICU door handle and kennel	168	6 years	11	C2	*oqx*A/B,aac(6′)-Ib-cr, DHA-1, FosA2, FosA6, SHV-11, *qnr*B/S[3]
4	CL (1), ENV (3)	EH	Surgical wound, stables, student keyboard	163	21 months	789	C3	*oqx*A/B, FosA6, SHV-25, TEM-1, *qnr*S[4]
Pseudomonas aeruginosa								
1	CL	SAH1	Endotracheal tube	170	5 months	1,051	C29	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA, gyrA
1	CL	SAH1	Tympanic bulla fluid	177	3 months	395	C11	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	ENV	SAH1	Wards Drip Pump Holder	177	3 months	1,026	C1	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	ENV	SAH1	Wards keyboard	174	20 months	792	C8	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	ENV	SAH1	ICU Floor	174	20 months	395	C11	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	CL	SAH1	Skin	176	12 months	2,859	C7	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-486, FosA
1	ENV	SAH1	ICU Door	176	12 months	1,621	C15	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	CL	SAH1	Frontal sinus exudate	158	4 days	17	C20	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	ENV	SAH1	ICU Door	175	3 years	17	C20	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
2	F, ENV	EH	Faeces, ICU Water bucket	175	3 years	1714	C0	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	CL	SAH1	Wound	175	3 years	3,246	C27	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	ENV	SAH1	Treatment room table	175	3 years	362	C25	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA

(Continued)

TABLE 4 (Continued)

WGS isolates (n)	WGS Isolate type (n)	Hospital source	WGS Isolate details	FTIR Cluster	Cluster isolation interval	ST Type	eBURST	AMR genes
1	ENV	SAH1	Dermatology keyboard	175	3 years	244	C4	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	CL	SAH1	Synovial fluid	175	3 years	P2	C10	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	CL	SAH1	External ear	175	3 years	3,044	C2	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA, gyrA
1	CL	SAH1	External ear	175	3 years	395	C11	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	CL	SAH1	Skin	175	3 years	446	C12	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	ENV	SAH1	Wards washing machine door	169	21 months	463	C9	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, FosA
1	CL	SAH1	PEG tube wound	171	2 years	P4	C30	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA, qnrB
1	CL	SAH1	Wound	166	31 months	179	C23	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	CL	SAH1	Tympanic bulla tissue	166	31 months	254	C22	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
2	CL	SAH1	Urine, wound	166	31 months	17	C20	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
3	*F* (2), ENV	EH	Faeces, ICU Window	161	41 days	27	C18	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	F	EH	Faeces	161	41 days	16	C21	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
2	F, ENV	EH	Faeces, ICU Window	162	2 days	P3	C0	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	CL	SAH1	Synovial fluid	130	18 days	P1	C26	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	ENV	SAH1	ICU phone	154	Single time point	2,591	C14	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
3	F (2), ENV	EH	Faeces, ICU Hay rack	164	56 days	3,016	C6	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	ENV	SAH1	Surgical ward tap	172	Unknown	179	C23	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
4	ENV (4)	EH	ICU Door, ICU Hay rack, stable, laboratory worktop	173	2 years	2,644	C16	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, FosA
1	CL	EH	Wound	173	2 years	2,644	C16	MexAB, MexXY, MexCD, MexEF, FosA
4	CL, ENV (3)	SAH1	Faeces, treatment room tap (3)	173	2 years	446	C12	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	ENV	SAH1	Sink tap	173	2 years	309	C13	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA
1	ENV	SAH1	Tea room tap	173	2 years	815	C17	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, OXA-50, FosA

(Continued)

TABLE 4 (Continued)

WGS isolates (n)	WGS Isolate type (n)	Hospital source	WGS Isolate details	FTIR Cluster	Cluster isolation interval	ST Type	eBURST	AMR genes
6	F, ENV (5)	EH	Faeces, ICU window (2), water bucket, tie ring and stocks	153	26 months	2,644	C16	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, FosA
1	ENV	SAH1	Wards shower head	153	26 months	532	C28	MexAB-OprM, MexXY, MexCD-OprJ, MexEF-OprN, FosA
1	ENV	SAH2	ICU floor and drain	157	Single time point	Unknown	Unknown	–

[1]Nearest ST: 336; [2]Two samples collected from the same patient; [3]aac(6′)-Ib-cr in 75%, FosA2 in 50% and qnrS in 25% of the isolates; [4]qnrS in 75% of the isolates. SAH1, Small Animal Hospital 1 (University of Liverpool); EH, Equine Hospital (University of Liverpool); SAP: Small Animal Practice (external); SAH2, Small Animal Hospital 2 (external).

(Cluster 153) on one hand, and of the environmental isolates from the SAH2 (Cluster 165), also points towards cross-transmission within these facilities.

Compared to *K. pneumoniae*, FTIR-based clustering of *P. aeruginosa* veterinary isolates was much less congruent with WGS results; its ability to correctly detect and cluster isolates of a given ST using a COV of 0.246 was poor, as reflected by an ARI of 0.313, with multiple examples of genomically related isolates spread across different clusters and genomically divergent isolates incorrectly clustered together by the IR Biotyper. Nevertheless, comparable diagnostic resolution to that of current reference methods has been previously reported for this organism by other authors (Martak et al., 2019; Hu et al., 2023). In these studies analysing clinical *P. aeruginosa* isolates from human hospital outbreaks, optimal FTIR spectroscopy COVs varied for this organism; one publication reports the optimal range 0.184 to 0.374 (AR, 0.936; Martak et al., 2019) while the other determined a more stringent COV range of 0.132 to 0.173 (AR, 0.711; Hu et al., 2023). Both studies employed Mueller-Hinton (MH) agar medium to passage *P. aeruginosa* isolates undergoing FTIR Biotyping, and Hu et al., 2023 demonstrated that this resulted in better discriminatory power than 5% Blood Agar medium used in our study, which could have had a negative effect on our results for veterinary *P. aeruginosa* isolates. It may be that our FTIR validation process to optimise the culture scheme and COV determination, which constitutes the main and, perhaps, only complication of applying FTIR to routine laboratory settings, should be revisited and improved for typing veterinary *P. aeruginosa*. No correlation was found between FTIR spectroscopy and molecular methods in another study for *P. aeruginosa* strain classification (Oho et al., 2021). These authors analysed clinical *P. aeruginosa* from multiple institutions and concluded that differences in the measurement principles may account for classification mismatches of *P. aeruginosa* organisms; WGS data could help understanding the genomic variations between isolates that modify carbohydrate composition and therefore impact the FTIR spectrum. As this was a retrospective analysis of veterinary hospital isolates collected over several years, it is plausible that genomic changes which could be reflected in the carbohydrate composition of the bacterial cell wall may have occurred over time, and this may have affected *Pseudomonas aeruginosa* more than *Klebsiella pneumoniae* in light of its larger genome. The genetic background and population structure of *P. aeruginosa* isolates in the present study was greatly diversified; nonetheless a clear association was found between ST2644 isolates and the EH, particularly from the ICU (including colonisation, environmental and clinical isolates). This clone has been previously described in MDR virulent *P. aeruginosa* isolates from small animal hospitals in Japan (Hayashi et al., 2021) and it is herein reported in horses for the first time. Other potential high-risk clones identified in this study included ST395 and ST244, both associated to small animal clinical or environmental isolates. The former ST has been described among carbapenemase-negative carbapenem-nonsusceptible *P. aeruginosa* small animal clinical isolates in France (Haenni et al., 2017) whereas the latter is a global epidemic human clone often expressing acquired carbapenem-resistance genes and MDR/XDR profiles (Chen et al., 2014; del Barrio-Tofiño et al., 2020; Pérez-vázquez et al., 2020) that has also been reported in veterinary infections (Haenni et al., 2015) and hospital environments (Soonthornsit et al., 2023). Finally, *P. aeruginosa* isolated across multiple sink taps across the SAH1 belonged to ST446, a recently identified high-risk clone associated with multidrug resistance (Pincus et al., 2020).

The reasons behind numerous FTIR clusters containing isolates collected at broad time intervals (≥3 years), as herein seen for both organisms, warrants more focused prospective studies. For example, it would be useful to understand whether this is determined by the persistence of MDR-GNs in the hospital environments, their re-introduction or by the highly clonal nature of some isolates within certain bacterial species. Furthermore, larger prospective studies would be beneficial to understand and measure the impact of FTIR real-time hospital surveillance to infection prevention and control. Finally, it is important to bear in mind that, in our study, we compared the concordance of FTIR spectroscopy and WGS-based cluster analyses where only a subset of FTIR clustering isolates underwent the gold standard method (few isolates for each defined cluster, proportional to cluster size) which represents the main limitation of this work.

In conclusion, we found that FTIR spectroscopy accurately clustered veterinary *K. pneumoniae* isolates belonging to the same clone but may be less accurate in discriminating *P. aeruginosa* isolates. The resolution of this method was high for *K. pneumoniae* for both clinical/colonisation and environmental hospital isolates, suggesting that FTIR spectroscopy alone could provide sufficient information to support early and appropriate infection control measures for hospital outbreak and epidemiological surveillance of *K. pneumoniae*. The rapid turnaround time combined with its ease of performance, low cost per sample and lack of requirement for specialised training, bring considerable advantages over the current molecular reference methods (i.e., PFGE, MLST and WGS) for implementation into the routine workflow of a veterinary microbiology laboratory conducting infection

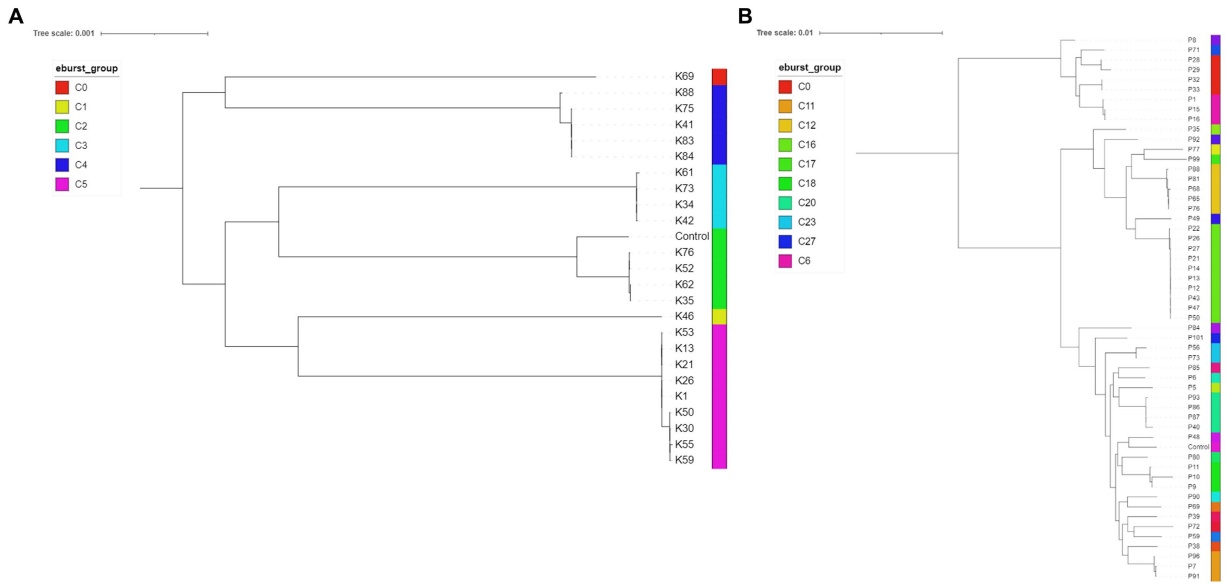

FIGURE 2
Midpoint rooted phylogenetic trees of *Klebsiella pneumoniae* (**A**, *n* = 24; isolates K10, K32 and K67 had incomplete core genome assembly, therefore they are not included in the below figure) and *Pseudomonas aeruginosa* (**B**, *n* = 53; P105 had incomplete genome assembly) isolates. The eBURST groups derived from MLST profiles extracted from the assembled genome sequences are shown. *Klebsiella pneumoniae* HS12286 and *Pseudomonas aeruginosa* PAO1 strains were used as internal controls.

control work. Here, we have shown that FTIR spectroscopy has the potential to become a valuable tool for rapid identification of *Klebsiella pneumoniae* (and potentially other pathogens) transmission events between patients and the veterinary clinical environment, therefore providing real-time surveillance information to aid infection prevention in veterinary settings.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: ENA—PRJEB70897.

Ethics statement

The animal studies were approved by University of Liverpool Veterinary Research Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

FZ: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. VS: Resources, Writing – review & editing. NM: Data curation, Formal analysis, Resources, Writing – review & editing. AL: Resources, Writing – review & editing. RJ: Resources, Writing – review & editing. CI: Resources, Writing – review & editing. GP: Funding acquisition, Writing – review & editing. SH: Data curation, Formal analysis, Resources, Writing – review & editing. DT: Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

NM is employed at Bruker Daltonics GmbH. CI was employed at the School of Veterinary Science, Philip Leverhulme Equine Hospital,

University of Liverpool at the time of commencing the study and moved to Western Counties Equine Hospital Ltd where is currently employed.

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1334268/full#supplementary-material

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Corrigendum: Rapid typing of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* by Fourier-transform Infrared spectroscopy informs infection control in veterinary settings

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In the published article, there was an error in affiliation 3. Instead of "School of Veterinary Science Small Animal Teaching Hospital, University of Liverpool, Neston, United Kingdom", it should be "Bruker Daltonics, Bremen, Germany".

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Comparative microbiome analysis of beef cattle, the feedyard environment, and airborne particulate matter as a function of probiotic and antibiotic use, and change in pen environment

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Introduction: Intensive beef cattle production systems are frequently implicated as a source of bacteria that can be transferred to nearby humans and animals via effluent water, manure used as fertilizer, or airborne particulate matter. It is crucial to understand microbial population dynamics due to manure pack desiccation, antibiotic usage, and antibiotic alternatives within beef cattle and their associated feedyard environment. Understanding how bacterial communities change in the presence of antibiotics can also improve management practices for reducing the spread of foodborne bacteria.

Methods: In this study, we aimed to compare the microbiomes within cattle feces, the feedyard environment and artificially produced airborne particulate matter as a function of pen change and treatment with tylosin or probiotics. We utilized 16S rRNA sequencing to compare bacterial communities among sample types, study days, and treatment groups.

Results: Bacterial community diversity varied as a function of sampling day and pen change (old or new) within fecal and manure pack samples. Manure pack samples from old pens and new pens contained diverse communities of bacteria on days 0 and 84; however, by day 119 of the study these taxonomic differences were less evident. Particulate matter samples exhibited significant differences in community diversity and predominant bacterial taxa compared to the manure pack they originated from. Treatment with tylosin did not meaningfully impact bacterial communities among fecal, environmental, or particulate matter samples; however, minor differences in bacterial community structure were observed in feces from cattle treated with probiotics.

Discussion: This study was the first to characterize and compare microbial communities within feces, manure pack, and airborne particulate matter from the same location and as a function of tylosin and probiotic treatment, and pen change. Although fecal and environmental samples are commonly used

in research studies and other monitoring programs to infer public health risk of bacteria and antimicrobial resistance determinants from feedyard environments, our study suggests that these samples may not be appropriate to infer public health risk associated with airborne particulate matter.

KEYWORDS

environmental microbiome, fecal microbiome, particulate matter, antibiotic alternatives, antimicrobial resistance (AMR)

1 Introduction

The increasing demand to produce meat products for human consumption has led to high volume and fast turnover practices in beef cattle production systems. The finishing period of feedyard cattle consequently puts animals at a higher risk for liver abscesses, reduced weight gain, and poor feed efficiency (Nagaraja and Lechtenberg, 2007; Ban and Guan, 2021). Along with the intense nature of the finishing period, bacterial infectious diseases are also responsible for negative animal performance, health risks, and welfare (Nagaraja and Lechtenberg, 2007; Brown and Lawrence, 2010; Huebner et al., 2019). The use of antimicrobials is a common on-farm practice for combatting both liver abscesses and infectious bacterial disease. For example, tylosin is a bacteriostatic antibiotic within the macrolide class that is frequently administered in feed to reduce the occurrence of liver abscesses in cattle (Huebner et al., 2019; Weinroth et al., 2019; Murray et al., 2020). Although tylosin is shown to be effective in most cases, the mechanism by which liver abscesses are reduced is not fully understood and treatment is not always associated with an absence of liver abscesses within individual cattle (Weinroth et al., 2019). Administration of tylosin has been associated with co-selection for bacterial resistance to the entire macrolide class of 14-, 15-, and 16-membered ring molecules (Marshall and Levy, 2011; Beukers et al., 2015); these include multiple antibiotics that are classified by the World Health Organization (WHO) as critically important for human health (World Health Organization [WHO], 2017). Tylosin administration may promote carriage of antimicrobial resistance (AMR) determinants in cattle, which may increase the risk of human infections associated with AMR pathogens from beef food products and the environment (Noyes et al., 2016; Huebner et al., 2019; Weinroth et al., 2019, 2022). There is a critical need to find alternative therapies that provide the same benefits of antibiotics without the risk of AMR selection and propagation in food animal production systems.

Microbial probiotics and fermentation products have been investigated as alternatives to antibiotics to improve both animal health and performance characteristics in beef cattle (McAllister et al., 2011; Huebner et al., 2019; Ban and Guan, 2021). These products are reported to be associated with reduced shedding of pathogenic bacteria, increased average daily gain, increased feed efficiency, enhanced fiber digestion and an overall enhancement of the gastrointestinal microbiome and animal health (McAllister et al., 2011; Huebner et al., 2019; Ban and Guan, 2021). *Saccharomyces cerevisiae* fermentation products (SCFP) have been shown to reduce the incidence of liver abscesses, and improve growth performance and carcass characteristics in finished cattle (Wagner et al., 2016). Additionally, *Enterococcus* species of gram-positive bacteria are commonly included in commercially available probiotics for beef cattle production (Amachawadi et al., 2018; Shridhar et al., 2022); this is due to the bacteria's favorable metabolism, competitive exclusion, lack of virulence genes in probiotic strains, and ability to survive both within the cattle gastrointestinal tract and the feedyard environment (Amachawadi et al., 2018; Murray et al., 2020, 2022; Shridhar et al., 2022); however, not all studies highlight these findings. In one study by Huebner et al. (2019), there were no positive effects shown in liver abscess reduction when using SCFP. Similarly, researchers did not reveal a positive effect of administering SCFP on performance characteristics in dairy calves (Titi et al., 2008) or finished beef cattle (Geng et al., 2016). Interestingly, one group of researchers administered a probiotic containing *Enterococcus faecium* and *Saccharomyces cerevisiae* in feed and were able to isolate the probiotic strain from the environment over 100 days after the trial began (Murray et al., 2020). Researchers suggest that an *Enterococcus* probiotic may exhibit "faecal-environmental-oral cycling" which may enhance the persistence of the product (Murray et al., 2020). Given these findings, there remains a need for investigating the ability of probiotics to persist and elicit an effect in the feedyard environment. Furthermore, elucidating and characterizing the bacterial communities affected by both antibiotics and antibiotic alternatives is important for both human and animal health.

It is crucial to understand the effects of antimicrobials on the microbiome of beef cattle and their associated environment for best management practices. In one study by Adeyemi et al. (2020), the administration of a *Saccharomyces cerevisiae*-based direct-fed microbial was associated with significant alterations in the bacterial communities within cattle feces. Contrastingly, another research team found no differences in fecal bacterial communities between SCFP treated and untreated cattle (Huebner et al., 2019). Few studies have investigated the effects of an *Enterococcus faecium/Saccharomyces cerevisiae* probiotic on the microbiomes within individual cattle and in their feedyard environment; however, there remains a need to increase our understanding of the effects of concurrent antibiotic and probiotic administration on bacterial communities in finisher beef cattle and the feedyard environment. Multiple researchers have characterized the effects of antibiotic administration on the bacterial communities within individual cattle and the environment (Thomas et al., 2017; Doster et al., 2018; Weinroth et al., 2019). These researchers showed that geographic location of the feedyard was more associated with differing microbial communities than antibiotic administration. These findings suggest that environmental factors and management practices may play a greater role in influencing microbiomes in cattle than commonly administered antimicrobials (Doster et al., 2018; Weinroth et al., 2019). Other studies support this rationale, revealing that animals from different environments

and production facilities exhibit dissimilar microbial communities (Huebner et al., 2019). In this way, improving environmental and management factors may more effectively reduce the spread of bacteria and AMR from food animal production systems.

The cattle feedyard environment has consistently been shown to harbor determinants of AMR and has been implicated as a reservoir capable of spreading AMR to nearby animals and humans via effluent water, airborne particulate matter and manure used as fertilizer, the impacts of which have not been fully elucidated (Guo et al., 2011; Bonifacio et al., 2012; Noyes et al., 2016; Wooten et al., 2019). The generation and emission of particulate matter from the feedyard environment is of particular importance in the High Plains region of Texas where a large proportion of U.S. beef cattle finishing takes place. This region experiences prolonged dry periods and a hot, windy climate, promoting the desiccation of the manure pack into fine particulate matter that can readily become airborne and travel outside of the feedyard production system under certain atmospheric conditions (Bonifacio et al., 2012; McEachran et al., 2015). Airborne particulate matter can be visually appreciated near feedyard production systems especially around dusk when still air, increased animal activity, decreased pen surface moisture, and increasing boundary-layer stability contributes to increased particulate matter concentrations near ground level (Urso et al., 2021). Airborne particulate matter originating from the cattle feedyard environment has been shown to transfer viable bacteria (Zaheer et al., 2019), AMR determinants (McEachran et al., 2015; Zaheer et al., 2019), antibiotics (Wooten et al., 2019; Zaheer et al., 2019), and growth promoting hormones (Blackwell et al., 2015), to surrounding environments; however, little emphasis has been placed to characterize the bacterial communities present within such particulate matter as a function of antibiotic alternatives. Increased resolution of bacterial population dynamics within beef cattle, the feedyard environment, and airborne particulate matter in response to commonly administered therapies is an essential part of assessing their utility and safety in finisher beef production.

The objective of our study was to characterize and compare the microbiomes in cattle feces and the feedyard environment as a function of tylosin and/or commercial probiotic treatment, and due to pen environment change. Additionally, we aimed to artificially model the transition of environmental manure pack desiccation to particulate matter to better characterize and simulate the metagenomic changes experienced in the cattle feedyard environment over time. This objective was aimed at understanding bacterial population dynamics and determining the risk of airborne particulate matter originating from the cattle feedyard environment for human health. While a number of studies have investigated the effects of SCFP and probiotics on the fecal microbiomes in beef cattle, there remains a need to increase our understanding of the effects of concurrent antibiotic and probiotic administration in finisher beef cattle on the bacterial communities present within environmental manure pack and particulate matter samples.

2 Materials and methods

2.1 Experimental design

A 2 × 2 × 2 full factorial and longitudinal controlled trial was conducted at Texas A&M AgriLife Research experimental feedyard in McGregor, TX during the second trial replicate of a longitudinal study published by Murray et al. (2020, 2022), which utilized 96 finisher steers randomly assigned to 8 pens for the first 84 days of the trial. Pen-level treatment groups included: (1) tylosin in feed, (2) probiotic in feed, (3) tylosin and probiotic in feed, or (4) untreated controls. On day 84, pens 1−8 were split in half, and six of the twelve steers in each pen were randomly assigned to newly constructed pens (pens 9−16). Pens 1−8 are hereafter referred to as old pens and pens 9−16 are hereafter referred to as new pens. New pens were manufactured for the purpose of this study and initially had never housed cattle previously administered antibiotics. Free range cows and calves on adjacent pastures were allowed access to the new pens for 4 weeks prior to the start of the study by Murray et al. (2020, 2022) to establish an initial manure pack and microbial composition baseline representative of bovine feces. Animal usage in this study underwent ethical consideration and approval by the Agriculture Animal Care and Use Committee (AACUC AUP #2015-026A).

Steers received tylosin (Tylan, Elanco, Greenfield, IN) in feed at 7.3 g/tonne for the first 84 days of the trial and subsequently went through a voluntary wash out period for the remainder of the study prior to slaughter. A commercially available probiotic containing 1.3×10^7 CFU/g of both *Enterococcus faecium* (ST296) and *Saccharomyces cerevisiae* (Tri-Lution, Agri-King, Fulton, IL) was administered to steers in feed at 824.5 g/tonne for the entire 119 days of the trial. All personnel were blinded to treatment groups, except for feedyard employees who administered the treatments via the feed. Detailed methods including cattle diets, husbandry practices, and pen management have been described previously (Murray et al., 2020, 2022).

2.2 Sample collection and processing

Individual fecal grab sampling per rectum was performed for all steers (n = 96) using a new rectal palpation sleeve for each animal on days 0, 84, and 119 of the study for a total of 288 fecal samples. Manure pack environmental samples were taken at the pen level (n = 16) along a diagonal transect at 6 sites per pen (approximately 25 g per site) on days 0, 84, and 119 of the study using a shovel that was cleaned and sterilized with alcohol between pens for a total of 48 manure pack samples. Day 0 was the first day of the study and sampling occurred prior to cattle entering the study pens. No treatments had been administered to any cattle at that point in time. Manure pack samples from pens 9−16 on day 84 were taken while cattle were being divided into old versus new pens and prior to placement in the new pens. Day 119 was the last day of sampling before steers were sent to slaughter and served as the end point of the study. Therefore, fecal and manure pack samples were collected on days 0, 84, and 119 of the study. Immediately after collection, all samples were transported to a microbiological laboratory for further processing at Texas A&M University in College Station, TX. Upon arrival to the laboratory, fecal and manure pack samples were stored at 4°C overnight, then transferred to 5 ml tubes for preservation at −80°C until further use.

Day 84 manure pack (MP) samples were further processed in series into two additional sample types – dried/milled (DM) samples and particulate matter (PM) samples - to model

the microbiome changes that naturally occur in the cattle feedyard environment over time as a function of desiccation and aerosolization to particulate matter. MP samples from each pen were kept in 102L bins and dried in the Texas A&M AgriLife Research and Extension biomass drying facility in Bushland, TX for 2 weeks. MP was then ground with a 7hp hammer mill using a 3.2 mm screen (Texas A&M AgriLife Research and Extension, Bushland, TX). The mill was cleaned and sterilized with alcohol between samples. An aliquot of each DM sample was taken immediately following milling in 50 ml conical tubes and sent to the laboratory at Texas A&M University in College Station, TX. The remaining DM samples were then used to create aerosolized PM in the downstream section of a 61 cm X 61 cm X 244cm hoof action simulator test chamber (Texas A&M AgriLife Research and Extension). 50 ml of each DM sample was inserted into the airstream from the top of the test chamber and the aerosolized particles were captured on 8 × 10in 1 mm glass fiber filters (Danaher Corp, Washington, D.C.) positioned 1.5 m from the insertion point. Each filter was exposed to aerosolized particles for 1 min in the test chamber at a flow rate of 160CFM to collect the PM sample. The test chamber was cleaned and sterilized with alcohol between samples. Filters containing the PM samples were wrapped in foil, placed in individually labeled bags, and maintained at 4°C until further processing.

Fecal samples were pooled for community DNA extraction to include three fecal samples from the same animals within each pen for days 0, 84, and 119, resulting in a total of 96 pooled fecal samples balanced across pen, day, and treatment. Samples were thoroughly vortexed prior to subsampling for pooling and subsequent DNA extraction such that a representative sub-sample was obtained. Manure pack and DM samples were homogenized using a Tissue Lyser II® (Qiagen, Valencia, CA) with 25 cycles per second for 10 min prior to subsampling for community DNA extraction.

2.3 Molecular methods

Community DNA extraction from pooled fecal, manure pack, and Day 84 samples was performed using the QIAGEN DNeasy® PowerSoil® Pro kit (Qiagen, Valencia, CA) per the manufacturer's protocol within the automated QIAcube robot. Library preparation was conducted using Nextera XT Index Kits (Illumina, Inc., San Diego, CA) and Mastercycler® Thermal Cycler (Eppendorf, Enfield, CT). Amplicon primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5′-GACTACHVGGGTATCTA ATCC-3′) with index adapters were utilized to specifically target and amplify the hypervariable V3/V4 region of the bacterial 16S rRNA gene (Klindworth et al., 2013). Library validation was performed on the Fragment Analyzer System (Agilent Technologies, Inc., Santa Clara, CA) prior to paired-end amplicon sequencing (2 × 300) of the 16S rRNA gene using MiSeq reagent v3 kits and the Illumina MiSeq platform (Illumina, Inc.) per manufacturer's protocol. The 16S rRNA amplicon sequence data can be found in the NCBI database under BioProject accession number: PRJNA595617.

2.4 Sequence data analysis and bioinformatics

Raw fastq files that underwent primer removal and demultiplexing were utilized for 16S rRNA amplicon sequencing analysis using the QIIME 2 pipeline (version qiime2-2022.2) (Bolyen et al., 2019) within the Terra cluster on the Texas A&M High Performance Research Computing (HPRC) system (College Station, TX). Data were divided into three groups: pooled feces (Days 0, 84, and 119), manure pack pen samples (Days 0, 84, and 119) and Day 84 manure pack samples that underwent further serial processing (MP to DM, and then to PM). These sample groups were analyzed independently for greater resolution of variation among study days and sample types. Using the DADA2 plugin (Callahan et al., 2016), single-end reads were assigned amplicon sequence variants (ASVs), noise and chimeras were filtered from all samples and quality trimming was performed at a quality score threshold of 20%. Due to poor quality of reverse reads and inability of sequences to overlap during DADA2 without sacrificing sequence quality, the reverse reads were excluded from further analysis for all samples. The latest version of the SILVA classifier was utilized to assign taxonomic classification to sequences (silva-138-99-nb-classifier.qza) (Quast et al., 2013). ASV data, feature tables, and metadata files from QIIME 2 were transferred to RStudio for further bacterial microbiome characterization and statistical analysis using "metagenomeSeq" (Paulson et al., 2013a), "metagMisc" (Mikryukov, 2023), "phyloseq"(McMurdie and Holmes, 2013), and "vegan" packages within the RStudio platform (Oksanen et al., 2022). Data were filtered to remove any ASV of non-bacterial classification and were normalized using the cumulative sum scaling method as described by Paulson et al. (2013b) to adjust for variation in sampling depth across samples.

Normalized counts were converted to relative abundances and aggregated by taxonomic level. Stacked relative abundance bar plots and heat maps were created at the phylum and class levels using Tableau Desktop (version 2023.2). Bar plots and heat maps were used to visually inspect the dynamics of the predominant phyla and classes within individual samples and among study variables. Taxa with a mean relative abundance less than 1% were collapsed into an "Other" category for ease of visualization. Analysis of the Composition of Microbiomes with Bias Correction II (ANCOM-BC2) was performed to determine whether any of the bacterial classes were differentially abundant among study variables using the R packages "ANCOMBC," "tidyverse," "DT," and "dplyr" (Mandal et al., 2015; Lin and Peddada, 2020, 2023). The ANCOM-BC2 model was run with a formula that included the fixed categorical covariates: day, pen change, and treatments for pooled fecal and manure pack samples. Day was indicated as the "group" variable for downstream pairwise comparisons. Other model parameters included a 10% prevalence inclusion criteria, 95% confidence level, structural zero detection, regularization factor of 5%, and bootstrap level of 100 as described by Lin and Peddada (2023). Additionally, the Holm-Bonferroni method was used to adjust *p*-values and control the false discovery rate among repeated comparisons (Holm, 1979). Model parameters reflect the recommended settings by product developers (Lin and Peddada, 2023). In the model for Day 84 samples, sample type replaced the day variable within the fixed formula and as the group variable, as

previously described for pooled fecal and manure pack samples. All other model parameters were the same across sample groups. Bar charts and heatmaps were created to visualize log fold-changes in differentially abundant taxa using the R package "ggplot2" (Wickham, 2016; Lin and Peddada, 2023).

Alpha diversity was calculated using the Shannon diversity index (Shannon, 1948) and the Wilcoxon rank sum exact test using the *wilcox.test* function in the R package "vegan" was used to investigate whether study variables were associated with variation in intra-sample diversity. A linear regression model was used to investigate the individual and combined effects of day, sample type, probiotic, tylosin, and pen change on the Shannon alpha diversity metric using Stata version 17 (StataCorp, College Station, TX). Marginal mean predictions were calculated in Stata and were used to create bar plots to visually inspect diversity across samples. Weighted and unweighted UniFrac beta diversity matrices were calculated from normalized data using R packages "vegan" and "GUnifrac" to investigate dissimilarity between samples (Oksanen et al., 2022). Distance based permutational analysis of variance (PERMANOVA) function, *adonis,* was used to investigate interactions among study variables and weighted and unweighted UniFrac diversity (Anderson, 2017). Sample dispersion was characterized using the *betadisper* and *permutest* functions to confirm *adonis* significance. Weighted and unweighted UniFrac distances were used to create principal coordinates analysis (PCA) plots, which were visualized using the graphical functions of the R package "ggplot2" (Wickham, 2016).

3 Results

3.1 Description of microbiome data

For pooled fecal samples ($n = 96$), demultiplexed 16S amplicon sequence counts for forward and reverse reads ranged from 73,889 to 268,307 with an average of 142,678 and a total of 13,697,165 reads. Two pooled samples were removed from further analysis due to errors during sequencing leading to sequencing depths less than 200. These samples were both from pen 16 on day 119 of the study. Due to poor quality of reverse reads and inability of sequences to overlap during DADA2, the reverse reads were excluded from further analysis without sacrificing sequence quality. Post DADA2, reads per sample ($n = 94$) ranged from 40,452 to 177,596 with an average of 91,657 reads, which is an average retention rate of 64% (range 55−71%) of reads used for downstream analysis.

For manure pack samples ($n = 48$), demultiplexed 16S amplicon sequence counts for forward and reverse reads ranged from 36,563 to 662,653 with an average of 170,599 and a total of 8,188,750 reads. For the same reason as for pooled fecal samples, reverse reads were excluded from further analysis. Post DADA2, forward reads ranged from 27,985 to 528,372 with an average of 130,659 reads per sample, which is an average retention rate of 76% (range 66−82%) of reads used for downstream analysis.

For Day 84 samples ($n = 48$), which contains MP, DM, and PM samples from day 84 of the study prior to cattle pen change, demultiplexed 16S amplicon sequence counts for forward and reverse reads ranged from 94,495 to 662,653 with an average of 188,281 and a total of 9,037,512 reads. For the same reason as previous samples, reverse reads were excluded from further analysis. Post DADA2, there was a retention rate of 75% (range 66−83%) of reads that were used for downstream analysis. Reads per sample ranged from 68,822 to 527,232 with an average of reads 141,525 per sample.

Alpha rarefaction curves revealed sufficient plateau across all variables of interest (day, sample type, treatment, and pen change) at previously described sampling depths, indicating that sampling depth was sufficient to adequately capture taxa present. Therefore, further sampling would not meaningfully change our interpretation of the bacterial ecology present.

3.2 Taxonomic comparisons of bacterial communities

3.2.1 Pooled fecal samples - Days 0, 84 and 119

Taxonomic profiles indicated modest variation across sampling day (**Figure 1**), however, there were no visual differences as a function of pen change or treatment with antibiotics or probiotics at both the phylum and class levels. The predominant phylum was Firmicutes (69−94% abundance), followed by Bacteroidota, Actinobacteriota and Proteobacteria. The predominant phylum, Firmicutes, was characterized by two classes of bacteria, Bacilli and Clostridia, which exchanged abundance across the study days (**Figure 1**). Other predominant classes included: Bacteroidia, Coriobacteriia, Gammaproteobacteria, and Actinobacteria with the remainder of classes ($n = 30$) comprising less than 1% of the bacterial community (**Figure 1**). Rare taxa are listed in Supplemental Material. There were no obvious visual differences in bacterial taxa among treatment groups or as a function of pen change. ANCOM-BC2 was employed to detect differentially abundant phyla and classes among study variables. ANCOM-BC2 revealed that there were differentially abundant taxa for different sampling days, as a function of pen change, and in probiotic treated cattle ($P < 0.05$); however, most differences involved rare taxa (Alphaproteobacteria, Chloroflexia, Saccharimonadia, Spirochaetia, unclassified Firmicutes, Vampirivibrionia, and Verrucomicrobiae). For predominant classes, there were significant increases in Bacilli and decreases in Gammaproteobacteria between days 0 and 84 of the study. Cattle that received the probiotic treatment exhibited significantly less Gammaproteobacteria in their feces than the control group.

3.2.2 Manure pack samples - Days 0, 84 and 119

There were visible taxonomic changes comparing among sample days and between old and new pens for manure pack relative abundance bar plots (**Figure 2**). Firmicutes, Proteobacteria, Actinobacteriota, Bacteroidota and Chloroflexi were the most abundant phyla across all samples. Actinobacteria, Alphaproteobacteria, Bacilli, Bacteroidia, Chloroflexia, Clostridia, Gammaproteobacteria and Negativicutes were the predominant classes, with the remainder ($n = 104$) making up less than 1% of the bacterial community (**Figure 2**). Rare taxa are listed in Supplemental Material. ANCOM-BC2 revealed that there were significant differentially abundant taxa for different sampling days and in old pens compared to new pens ($P < 0.05$); however, most differences involved rare taxa (Acidimicrobiia, Anaerolineae,

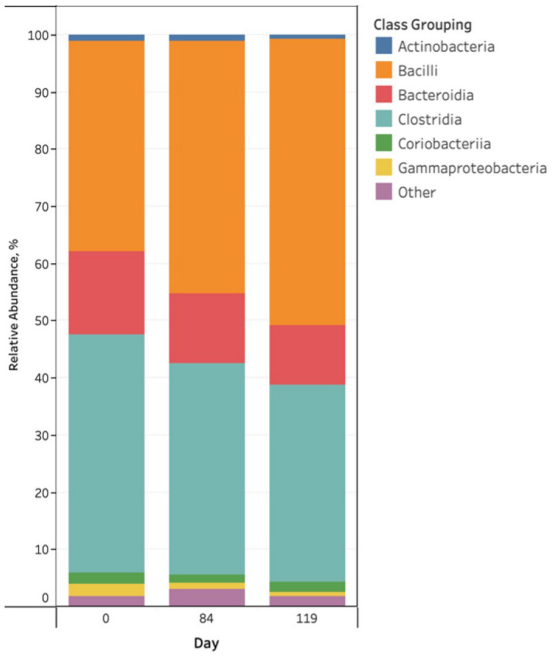

FIGURE 1
Stacked bar chart of the relative abundance of predominant bacterial classes in pooled fecal samples of beef cattle as a function of sampling day.

Armatimonadia, Bdellovibrionia, Blastocatellia, Cyanobacteria, Desulfovibrionia, Gitt-GS-136, Holophagae, KD4-96, Myxococcia, Phycisphaerae, Plantomycetes, Rhodothermia, Rubrobacteria, Saccharimonadia, Spirochaetia, Sumerlaeia, Thermoleophilia, unclassified Bacteria, unclassified Chloroflexi, unclassified Firmicutes, Vampirivibrionia, and Verrucomicrobiae). For predominant bacterial classes, there were significant increases in Actinobacteria and Chloroflexia, and significant decreases in Bacteroidia and Gammaproteobacteria between days 0 and 84 of the study. Between days 84 and 119, there was a significant decrease in Negativicutes. Old pens were shown to contain significantly more Actinobacteria than new pens. Treatments were not associated with differentially abundant taxa.

3.2.3 Day 84 samples – manure pack, dried/milled, and particulate matter

For the three different types of Day 84 samples, sample type and pen change were associated with visual differences in the relative abundance of predominant bacterial taxa. Of note, cattle were not introduced into new pens until after day 84 manure pack samples were taken. Firmicutes, Actinobacteriota, Proteobacteria, Bacteroidota and Chloroflexi were the most abundant phyla across all samples. Actinobacteria, Alphaproteobacteria, Bacilli, Bacteroidia, Chloroflexia, Clostridia, and Gammaproteobacteria were the predominant classes with the remaining classes ($n = 107$) making up less than 1% of the bacterial community (Figure 3). Rare taxa are listed in Supplemental Material. ANCOM-BC2 revealed that sample type and pen change were associated with significant differential abundance among bacterial taxa at the phylum and class levels ($P < 0.05$); however, most of the significant differences were observed in rare taxa (Acidimicrobiia, Blastocatellia, Campylobacteria, Coriobacteriia, Cyanobacteria, Cyanobacteria, Desulfovibrionia, Desulfuromonadia, Elusimicrobia, Fibrobacteria, Gitt-GS-136, Holophagae, KD4-96, Kiritimatiellae, Lentisphaeria, Myxococcia, Phycisphaerae, Plantomycetes, Polyangia, Rhodothermia, Rubrobacteria, S0134_terrestrial_group, Spirochaetia, Sumerlaeia, Syntrophobacteria, Thermoleophilia, unclassified Bacteria, unclassified Chloroflexi, unclassified Firmicutes, Vampirivibrionia, Verrucomicrobiae, and Vicinamibacteria). For predominant classes, there were significant decreases in Actinobacteria, and Chloroflexia once MP samples had been processed to DM samples. PM samples were shown to contain significantly more Actinobacteria, Bacilli, Chloroflexia, and Clostridia, and less Bacteroidia, Gammaproteobacteria and Negativicutes compared to DM samples. Old pens were shown to contain significantly more Actinobacteria than new pens. No taxa were significantly differentially abundant among treatment groups at the phylum and class levels for Day 84 samples.

3.3 Linear regression of Shannon alpha diversity

Linear regression was used to investigate the individual and combined effects of day, sample type, pen change, and treatment with tylosin and/or probiotics on the Shannon alpha diversity metric. Marginal mean predictions were calculated and used to create bar plots to visually inspect diversity across samples.

3.3.1 Pooled fecal samples

In the bivariable linear regression model for pooled fecal samples, day alone accounted for approximately 31% (R-squared = 31.3%) of the variation observed in Shannon diversity. Pen change and treatment with probiotic or tylosin did not significantly influence Shannon diversity when evaluated independently. The final linear regression model for pooled fecal samples was a four-way full factorial model including day, pen change, probiotic treatment, and tylosin treatment, which accounted for approximately 48% (R-squared = 48.8%, adjusted R-squared = 32.0%) of the variation observed in the data (Figure 4). Figure 4 shows separate bar plots for cattle transferred to a new pen on day 84; however, it is important to note that cattle were co-located in their original pens until day 84 of the study. Compared to day 0, Shannon diversity was significantly decreased in the tylosin and probiotic treatment groups on days 84 and 119 ($P < 0.05$) in pooled feces of cattle that did not undergo pen change (Figure 4). Although not statistically significant, cattle transferred to a new pen on day 84 revealed a trend of decreased diversity in their feces from day 0 to 84, which is prior to their movement to new pens (Figure 4). Post pen change, pooled feces from cattle that received the combination probiotic/tylosin treatment resulted in significantly lower Shannon diversity on day 119 ($P < 0.05$), which is similar to the appearance of Shannon diversity in manure pack samples from new pens on day 0 (Figure 5).

3.3.2 Manure pack samples

In the bivariable linear regression models for manure pack samples, day accounted for over 50% (R-squared = 52.5%) of

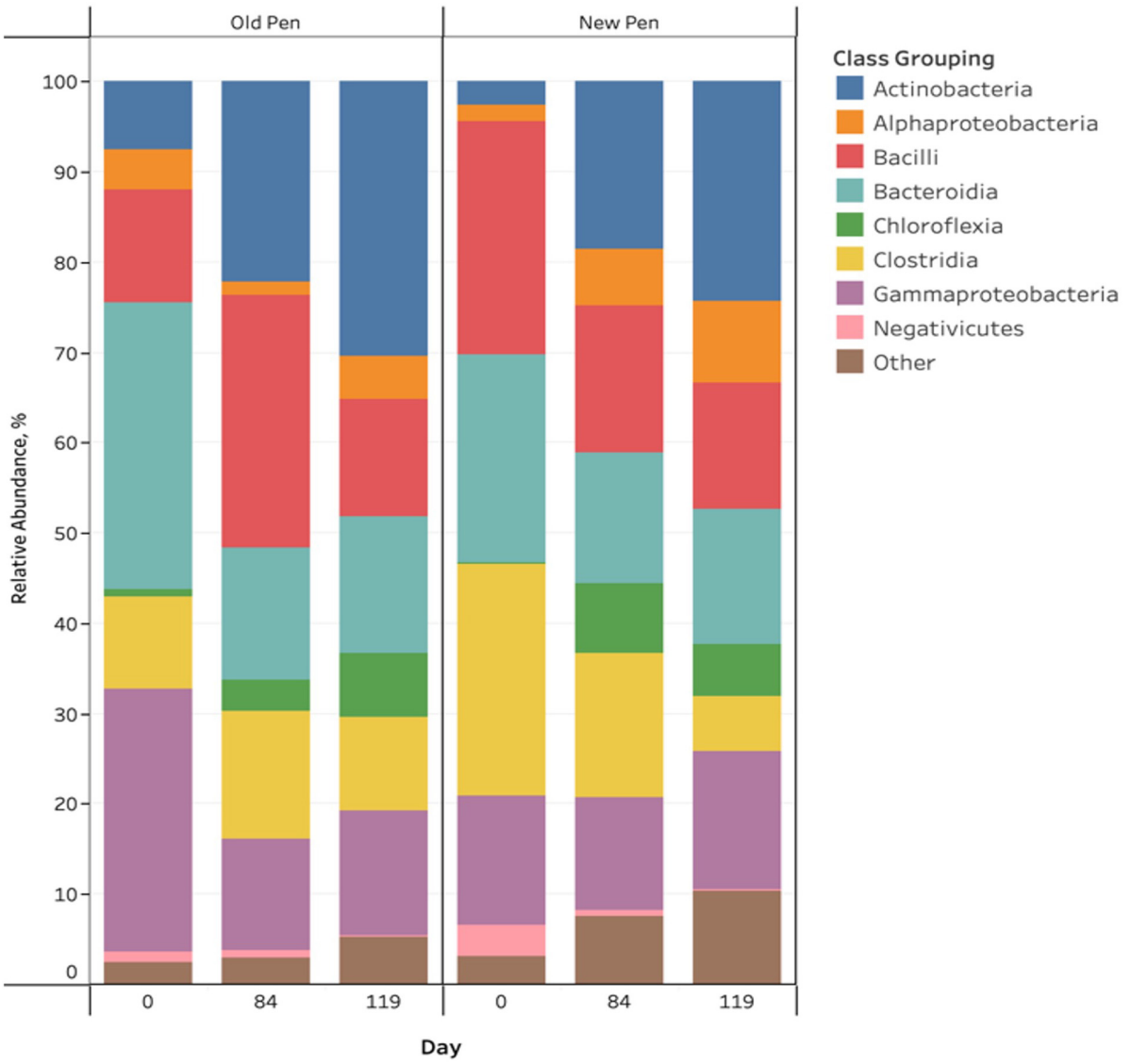

FIGURE 2
Stacked bar chart of the relative abundance of predominant bacterial classes in manure pack samples of beef cattle as a function of sampling day and old/new pen.

the variation observed in the data, while pen change accounted for approximately 9% (R-squared = 9.3%) of the variation observed in the data. Treatment with either the probiotic or tylosin did not significantly influence Shannon diversity. The final linear regression model for manure pack samples was a four-way full factorial model including day, tylosin treatment, and probiotic treatment, which accounted for approximately 84% (R-squared = 84.5%, adjusted R-squared = 69.8%) of the variation observed in the data. Regardless of pen change, Shannon diversity significantly increased from day 0 to 84 ($P < 0.05$) in all treatment groups except the control group, then remained static for the remainder of the study (Figure 5). Day 0 samples revealed significant variation in Shannon diversity as a function of treatment although no cattle had been introduced to the pens yet (Figure 5). More specifically, the control group had significantly higher Shannon diversity than the probiotic or tylosin groups at the beginning of this study (Figure 5). There was an overall upward trend in Shannon diversity in manure pack samples across the study (Figure 5), which was opposite of what was seen in the pooled fecal samples, where diversity decreased across the study (Figure 4).

3.3.3 Day 84 samples

In the bivariable linear regression models for Day 84 samples, sample type and pen change accounted for approximately 62% (R-squared = 62.8%) and 16% (R-squared = 16.8%) of the variation observed in the data, respectively. Treatment with tylosin or the probiotic were not significant. The final linear regression model for Day 84 samples was a four-way full factorial model including sample type, pen change, tylosin treatment and probiotic treatment. This model accounted for approximately 93% (R-squared = 93.9%, adjusted R-squared = 88.0%) of the variation observed in the data. As samples were processed from MP to DM, Shannon diversity remained relatively static, whereas, PM samples were associated

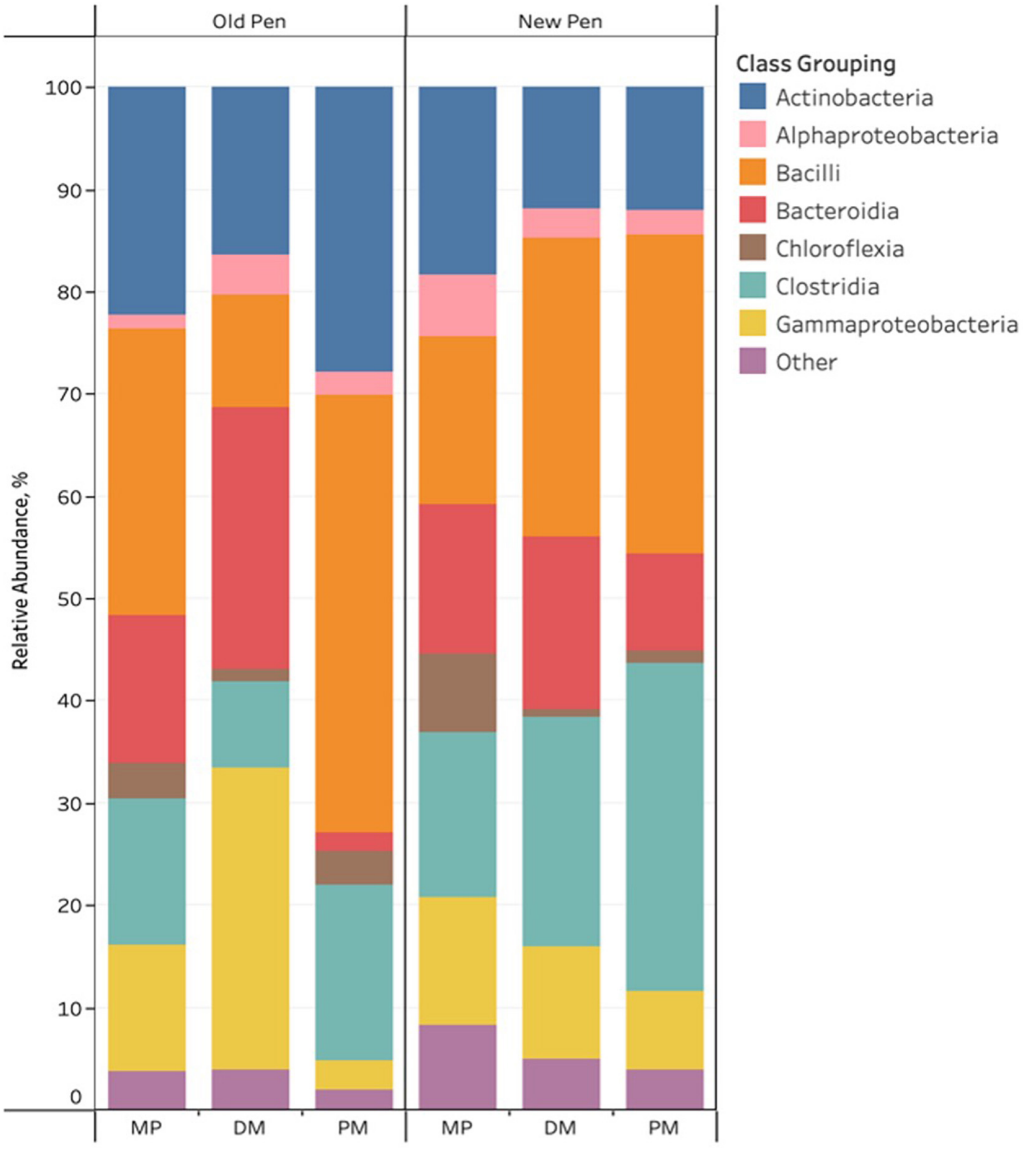

FIGURE 3
Stacked bar chart of the relative abundance of predominant bacterial classes in Day 84 manure pack (MP), dried/milled (DM) and particulate matter (PM) samples as a function of sample type and between old and new pens.

with a significantly lower ($P < 0.05$) Shannon diversity, especially in old pens ($P < 0.05$) (Figure 6).

3.4 Weighted and unweighted UniFrac analysis of beta diversity

Weighted and unweighted UniFrac measures were calculated using the distance based PERMANOVA function to investigate interactions between study variables and weighted and unweighted UniFrac diversity. PCA plots were generated from UniFrac dissimilarity matrices to visually inspect sample distribution.

3.4.1 Pooled fecal samples

For pooled fecal samples, weighted and unweighted UniFrac distances varied significantly by day (Adonis $P = 0.001$ and $P = 0.001$, respectively) and pen change (Adonis $P = 0.039$ and $P = 0.001$, respectively). Both weighted and unweighted UniFrac plots revealed clustering with stronger clustering patterns when using unweighted UniFrac distance (Figure 7). Samples from day 84 and 119 overlap, indicating that the taxa present and their relative abundances are similar between the two days. There were no significant associations between pen change or treatment for beta diversity measures. Based on the top two axes, weighted and unweighted PCA plots accounted for 60 and 24% of the variation in the data, respectively.

3.4.2 Manure pack samples

For manure pack samples, weighted and unweighted UniFrac distances differed significantly by day (Adonis $P = 0.001$ and $P = 0.001$, respectively) and pen change (Adonis $P = 0.016$ and $P = 0.001$, respectively). PCA plots showed a clear separation of

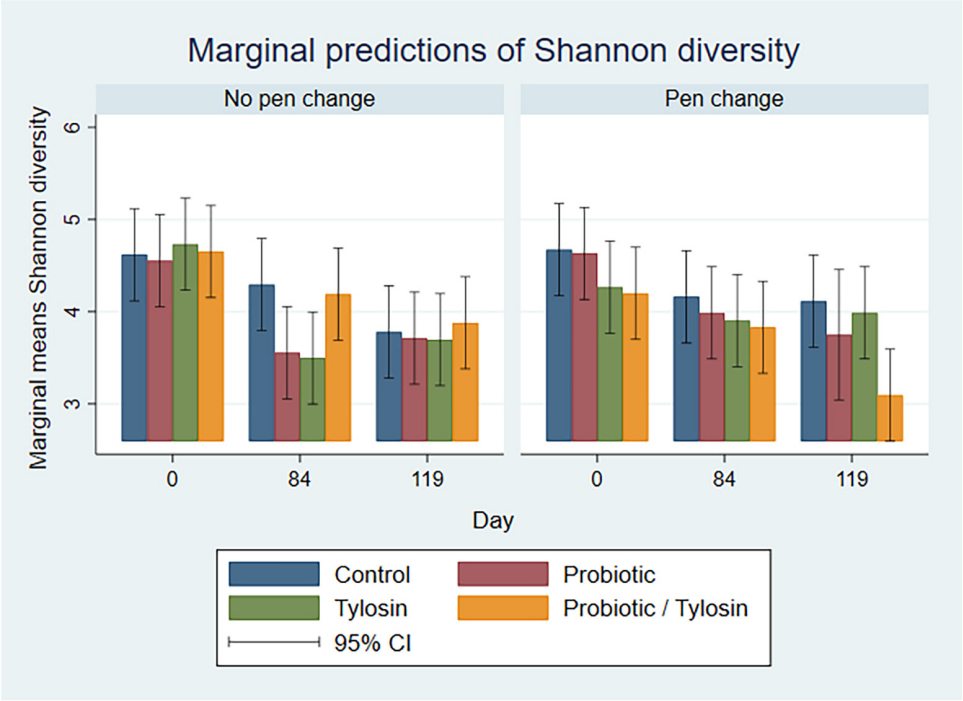

FIGURE 4
Bar plots showing linear regression marginal predictions of Shannon diversity in pooled fecal samples by treatment, sampling day and pen change.

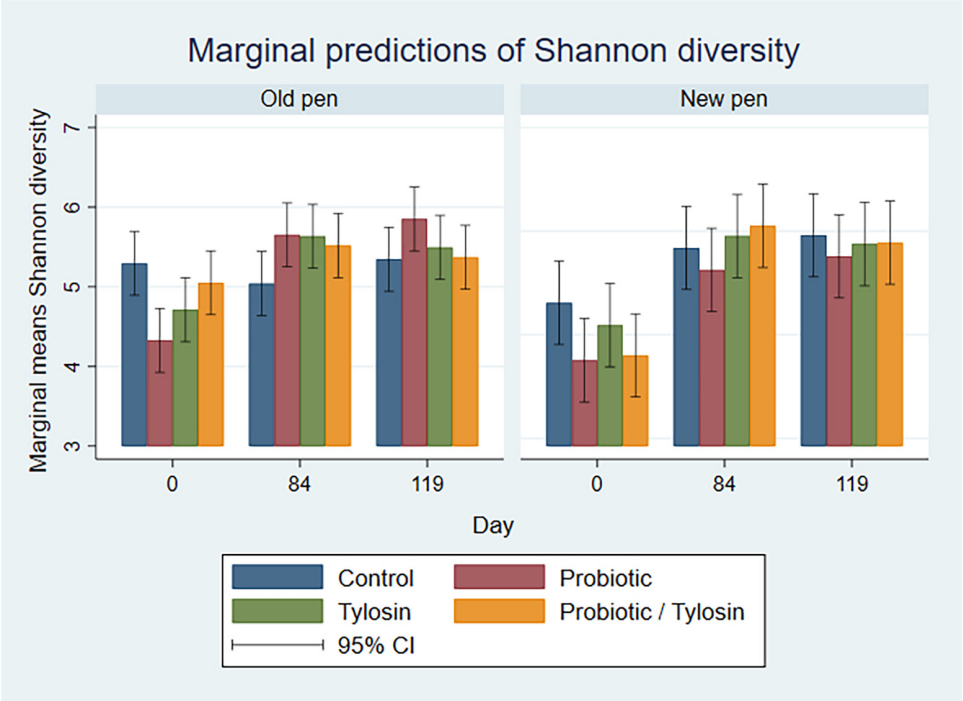

FIGURE 5
Bar plots showing linear regression marginal predictions of Shannon diversity for manure pack samples by treatment, sampling day, and between old and new pens.

day 0 samples from day 84 and 119. Similar to the pooled fecal samples, day 84 and 119 manure pack samples overlap (Figure 8). Within day 0 samples, pen change did not appear to influence UniFrac diversity. On days 84 and 119, samples from old pens clustered separately from new pens, indicating that the taxa present and their relative abundances are distinct between the two groups.

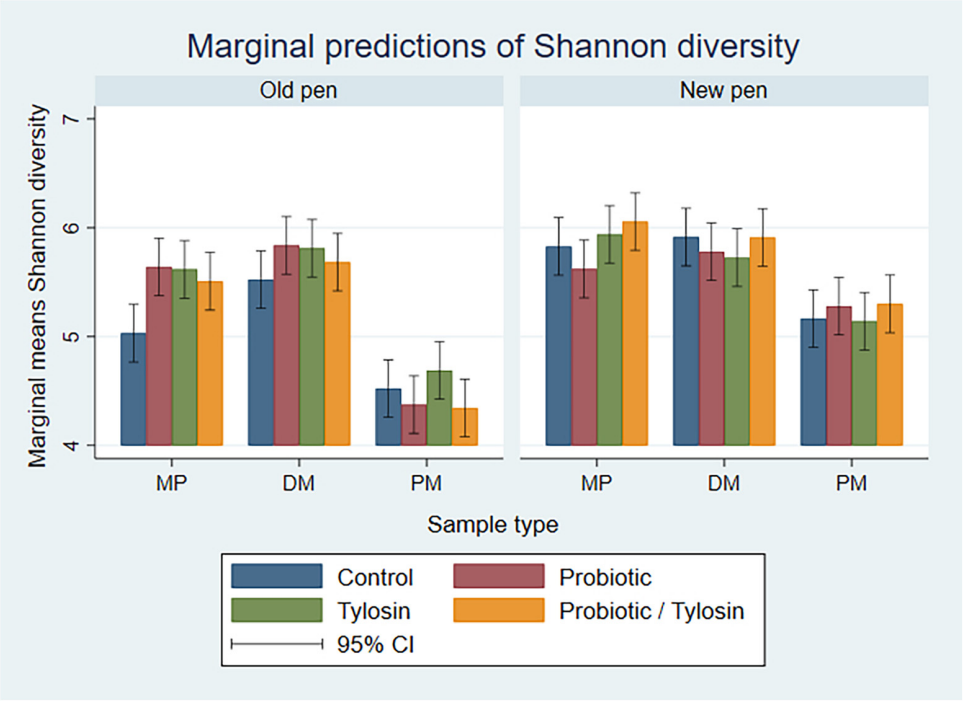

FIGURE 6
Bar plots showing linear regression marginal predictions of Shannon diversity for Day 84 manure pack (MP), dried/milled (DM) and particulate matter (PM) samples by treatment, sample type, and between old and new pens.

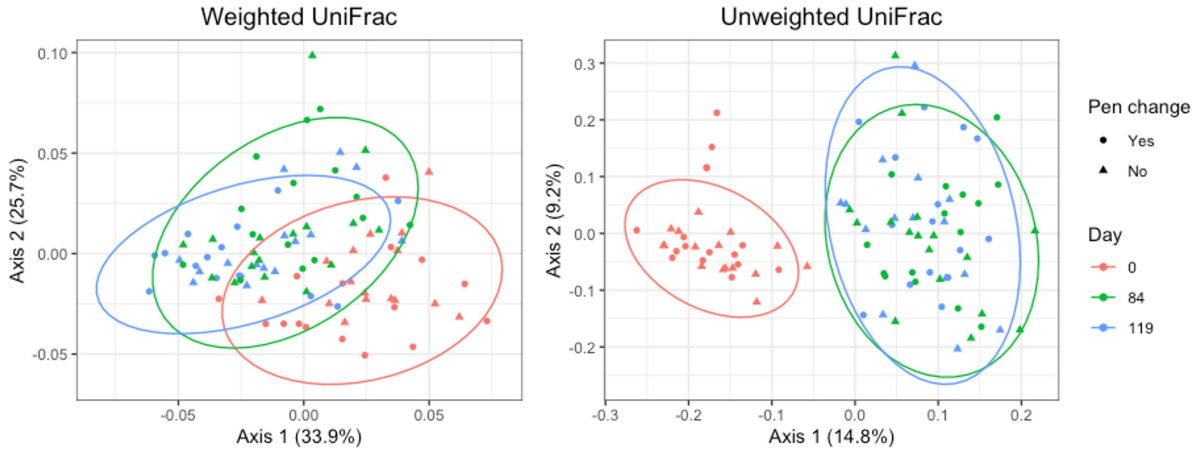

FIGURE 7
Principal coordinates analysis (PCA) plots of pooled fecal samples showing the differences in beta diversity among sampling days and between old and new pens using weighted and unweighted UniFrac dissimilarity matrices.

Treatment group was not associated with differences in weighted or unweighted UniFrac diversity. Based on the top two axes, weighted and unweighted PCA accounted for 70 and 30% of the variation in the data, respectively.

3.4.3 Day 84 samples

For Day 84 samples, weighted and unweighted UniFrac distances varied significantly by sample type (Adonis $P = 0.001$ and $P < 0.014$, respectively) and pen change (Adonis $P = 0.001$ and $P = 0.001$, respectively). PCA plots show independent clustering of samples as a function of sample type (Figure 9) and pen change (Figure 10). MP samples from old and new pens clustered more closely together than to the DM or PM samples from the same old or new pen. Contrastingly, DM and PM samples from old pens were more closely related to each other than to their corresponding sample type in new pens. In this way, PM originating from old pens contained significant differences in the taxa present/absent and their relative abundances than PM originating from new pens. Moreover, PM samples were significantly different than the MP they originated from. Treatment group was not associated with

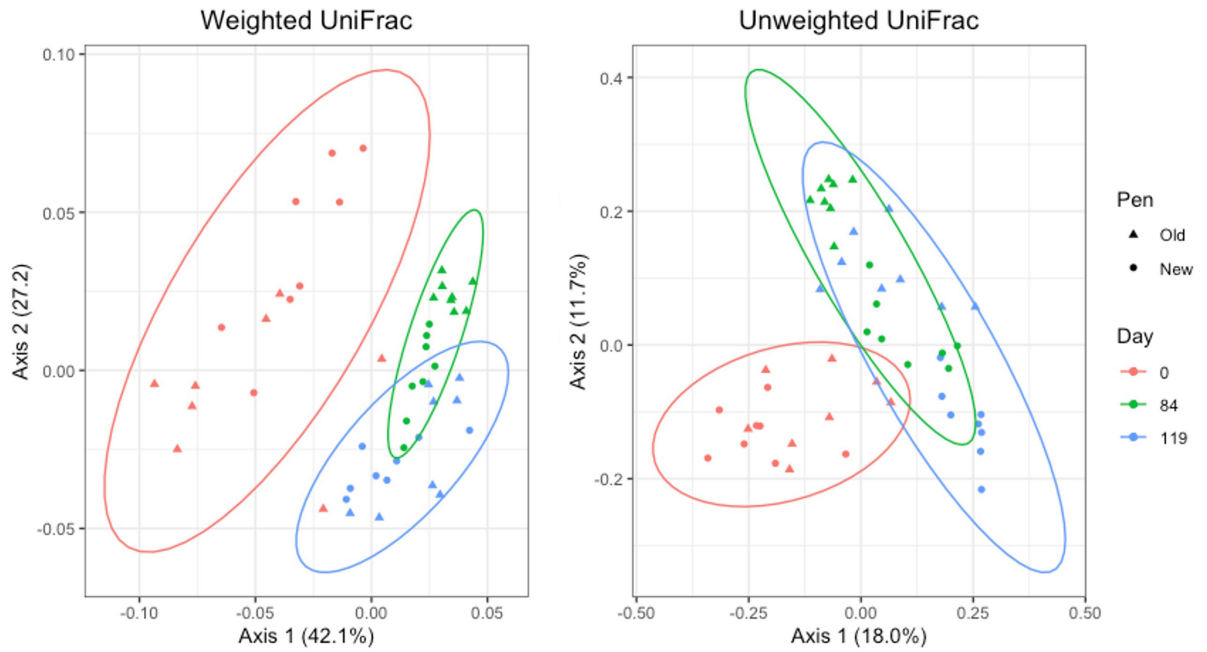

FIGURE 8
Principal coordinates analysis (PCA) plots of manure pack samples showing the differences in beta diversity among sampling days and between old and new pens using weighted and unweighted UniFrac dissimilarity matrices.

variation in beta diversity between samples. Based on the top two axes, weighted and unweighted PCA plots account for 67 and 36% of the variation in the data, respectively (**Figures 9**, **10**).

4 Discussion

This study utilized 16S rRNA amplicon sequencing to characterize the bacterial community structure as a function of antibiotics, probiotics, and pen environment change on the bacterial communities present in cattle feces, the feedyard environment, and airborne particulate matter. It is widely accepted that antibiotic administration to high-risk cattle successfully reduces the occurrence of diseases that impact their performance and health; however, increased scrutiny around the use of antibiotics in food animals has elucidated the need for alternatives that promote animal health without contributing to the emergence and persistence of AMR in animals and their associated feedyard environment. Moreover, as research continues to highlight the public health risk associated with airborne particulate matter originating from food animal production systems (Smit, 2012; Luiken et al., 2020; Urso et al., 2021), it is important to characterize the effects of antibiotics and antibiotic alternatives on the bacteria present in particulate matter that has the potential to transmit to surrounding areas.

Similar to previous findings, our study did not reveal a significant impact on diversity or bacterial communities present within cattle feces or their environment as a function of treatment with antibiotics (Noyes et al., 2016; Doster et al., 2018). Contrastingly, other researchers have observed a significantly lower Shannon diversity in cattle treated with tylosin compared to control animals (Weinroth et al., 2019); however, there were no alterations in bacterial communities observed. Differences in geographic location, study methodologies, sequencing modalities, and bioinformatic analyses likely contribute to the lack of congruency among studies investigating the effects of antibiotics and antibiotic alternatives on animal health, performance characteristics, morbidity, and microbial ecology. To better understand changes in the microbial communities during the finishing period in beef cattle, we evaluated the microbiomes within feces, manure pack and particulate matter within the same feedyard facility.

Day of the study and pen change were associated with significant differences in biodiversity metrics (alpha and beta diversity) across all sample types; however, this was not always reflected by changes in the predominant taxa. Interestingly, day 0 manure pack samples revealed significant differences in Shannon diversity among treatment groups although no cattle were present at that time. These differences were most likely the result of residual effects from the first trial of the study by Murray et al. (2020), and represent the reality of the feedyard infrastructure where previous cohorts of finishing cattle affect subsequent groups of cattle. Additionally, we observed that the Shannon diversity in pooled fecal samples changed to reflect the diversity of the manure pack in the pens to which the cattle were introduced. This is seen in old pens on day 84 and new pens on day 119, both of which were the first fecal sampling day after cattle were introduced into the pens. Additionally, manure pack samples revealed significantly higher Shannon diversity than pooled fecal samples on days 84 and 119 of the study. These findings support previous research which showed that freshly voided feces exhibited significantly lower Shannon diversity than that of cattle feedyard soil (Noyes et al., 2016) and soil collected adjacent to a beef cattle feedyard facility (Zaheer et al., 2019). Further work is needed to understand the

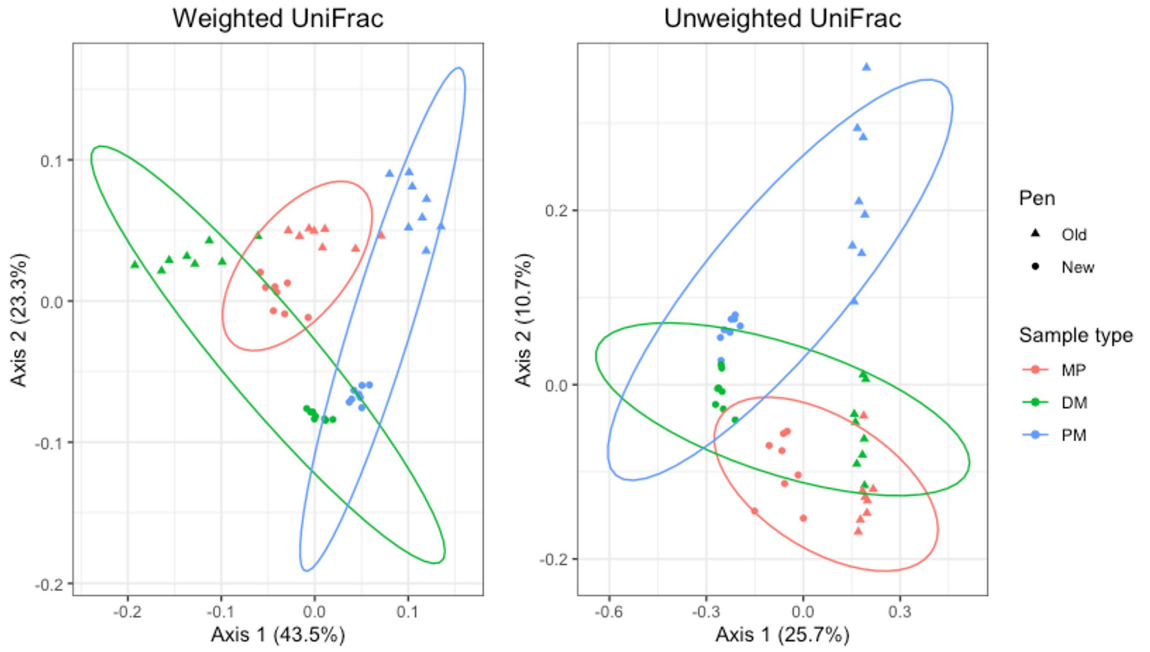

FIGURE 9
Principal coordinates analysis (PCA) plots of Day 84 manure pack (MP), dried/milled (DM) and particulate matter (PM) samples showing the differences in beta diversity among sample types and between old and new pens using weighted and unweighted UniFrac dissimilarity matrices.

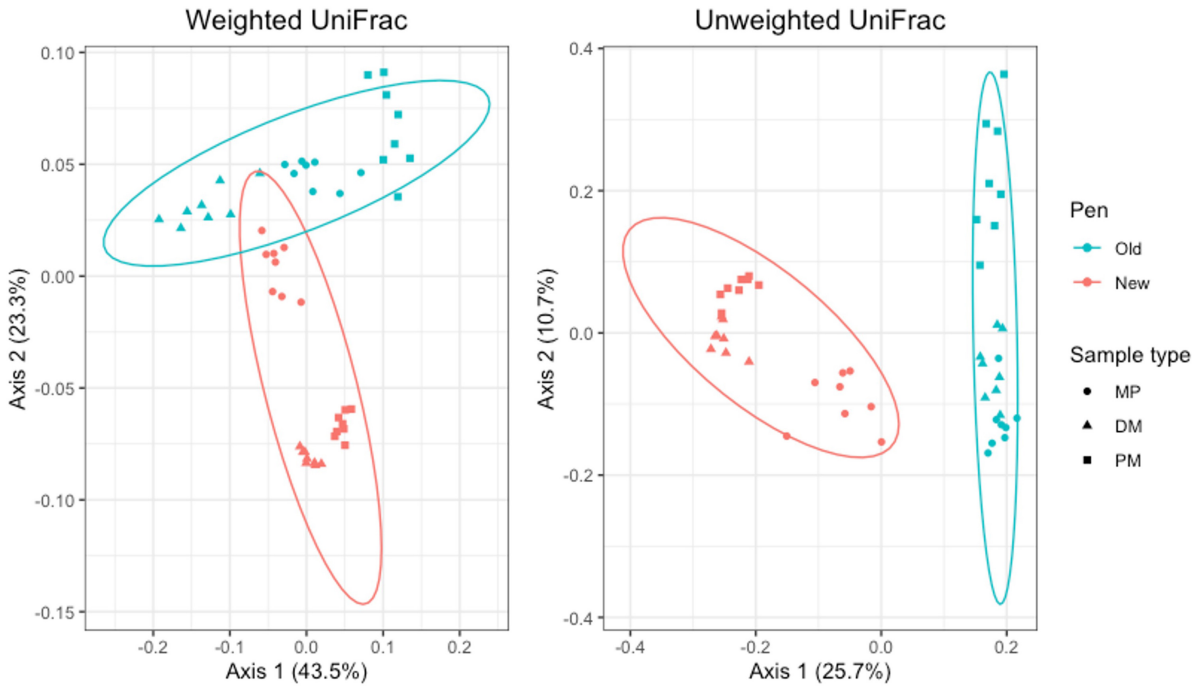

FIGURE 10
Principal coordinates analysis (PCA) plots of Day 84 manure pack (MP), dried/milled (DM) and particulate matter (PM) samples showing the differences in beta diversity between old and new pens and among sample types using weighted and unweighted UniFrac dissimilarity matrices.

complexities and adaptations of microbial community dynamics between cattle and the feedyard environment.

The probiotic used in our study has been shown to exhibit "faecal-environmental-oral cycling" as described by (Murray et al., 2020); however, we did not observe an increase in Bacilli, the class to which the probiotic is classified. Instead, we observed that probiotic treated cattle feces contained significantly less Gammaproteobacteria, which contains many genera of importance to human health including the foodborne bacteria *Escherichia coli* and *Salmonella spp.* We suspect that the administration of the

probiotic may have caused a shift in the microbiome that promoted a decrease in Gammaproteobacteria; however, the implications of this are not fully understood at this time. Increased resolution into the specific orders or genera that are influenced by probiotic administration may shed light into whether aforementioned medically relevant organisms are similarly decreased by this antibiotic alternative. One group of researchers suggest that the phylum Proteobacteria (to which Gammaproteobacteria is classified) may play a role in nitrogen cycling and levels of nitrous oxide emissions from the feedyard environment (Waldrip et al., 2022). In this way, the probiotic used in this study may play a role in the way nitrogen is utilized by the bacterial communities in cattle feces; which may in turn affect animal performance and health, although that was not investigated in this study. Otherwise, there were no significant differences in biodiversity or predominant bacterial taxa within pooled feces, manure pack, or particulate matter as a function of treatment with the probiotic.

We identified differentially abundant classes in each group of samples, however, some of these classes comprised less than 1% of the bacterial community. Rare taxa have been shown to have the potential to rapidly expand and cause meaningful shifts to their associated bacterial communities (Shade et al., 2014); however, in our study there were no meaningful shifts to the community as a function of these rare taxa. Additional work is needed to understand the implications of taxa found to be statistically differentially abundant while remaining less than 1% abundant in the community. Deeper resolution into taxonomic changes using 16S rRNA amplicon sequencing in conjunction with more comprehensive sequencing technologies, such as shotgun sequencing, transcriptomics, and full metagenome characterization may give a more robust understanding of the effects of probiotics and antibiotics within cattle feces, the feedyard environment, and airborne PM.

Multiple studies have quantified bacterial taxa and antimicrobial resistance determinants in airborne particulate matter around cattle feedyards (McEachran et al., 2015; Wooten et al., 2019); however, to our knowledge, this is the first study to compare bacterial communities within fecal, environmental manure pack and airborne particulate matter samples within the same feedyard facility. We modeled MP desiccation to airborne PM to characterize the microbial changes experienced in the cattle feedyard environment over time. Interestingly, the predominant bacterial classes within PM samples more closely resembled the structure of fecal samples than the MP samples from which they originated. This suggests that as desiccation occurs, there may be significant changes to the bacterial communities to a more Firmicutes-dominated population by the time particulate matter is created. The phylum Firmicutes contains many classes of organisms that are known to be drought tolerant, such as Bacilli and Clostridia, both of which were shown to be higher in PM and fecal samples than environmental MP in our study. These findings agree with a previous study that found airborne PM collected downwind from a feedyard facility contained phyla and genera most closely associated with cattle feces; however, they were not able to collect pen level samples for comparison (McEachran et al., 2015). Although not well characterized in beef cattle, researchers investigating the microbiomes within poultry and pig farms revealed that the microbial communities present within PM correlated significantly with the fecal microbiome (Luiken et al., 2020). Further work is needed to determine if the artificially desiccated PM in this study is representative of naturally occurring PM in the feedyard environment. As our study has shown distinct bacterial communities and biodiversity metrics between pooled feces, manure pack, and particulate matter samples, we suggest that it may not be sufficient to use fecal or environmental samples as a proxy to characterize the public health risk associated with particulate matter from food animal production systems.

5 Conclusions

In an effort to combat AMR in food animal production systems, many alternatives to antibiotics have been investigated to promote animal health and performance characteristics of beef cattle during the finishing period. Microbial probiotics, fermentation products and environmental management changes have been suggested as strategies to mitigate AMR in the presence of cattle on antibiotics. Our study revealed varying environmental microbial communities between old and new pens, however, after the introduction of cattle into new pens, these differences were less evident. Although there were changes in cattle feces diversity that reflected the initial environmental conditions, the movement of cattle into new pens did not meaningfully affect the microbial communities within pooled fecal samples, specifically at the end of the finishing period when an effect would be most impactful for public health. Administration of an *Enterococcus faecium/Saccharomyces cerevisiae* probiotic may have promoted a shift in the fecal microbiome leading to a decrease in Gammaproteobacteria; however, treatment was otherwise not associated with changes to the fecal, manure pack or particulate matter bacterial communities. Our study was the first to characterize and compare microbial communities within feces, manure pack and airborne particulate matter from the same location, revealing significantly different bacterial populations. In this way, additional sampling efforts of particulate matter from food animal production systems should be performed in future studies that aim to characterize potential microbial exposures to feedyard personnel and nearby communities. Determining bacterial community dynamics is an essential step in understanding the effects of antibiotics and their alternatives on food animal production systems and inferring public health risk associated with airborne particulate matter.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA595617.

Ethics statement

The animal study was approved by the Agriculture Animal Care and Use Committee (AACUC AUP #2015-026A). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AS: Conceptualization, Data curation, Formal Analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. SM: Data curation, Methodology, Writing – review & editing, Conceptualization. JV: Data curation, Supervision, Writing – review & editing, Conceptualization, Methodology. BA: Data curation, Methodology, Writing – review & editing. KB: Data curation, Methodology, Writing – review & editing. JS: Methodology, Writing – review & editing, Funding acquisition. HS: Data curation, Formal Analysis, Funding acquisition, Project administration, Supervision, Writing – review & editing. KN: Data curation, Formal Analysis, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1348171/full#supplementary-material

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Corrigendum: Comparative microbiome analysis of beef cattle, the feedyard environment, and airborne particulate matter as a function of probiotic and antibiotic use, and change in pen environment

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Major primary bile salts repress *Salmonella enterica* serovar Typhimurium invasiveness partly via the efflux regulatory locus *ramRA*

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Bile represses *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) intestinal cell invasion, but it remains unclear which bile components and mechanisms are implicated. Previous studies reported that bile inhibits the RamR binding to the *ramA* promoter, resulting in *ramA* increased transcription, and that *ramA* overexpression is associated to decreased expression of type III secretion system 1 (TTSS-1) invasion genes and to impaired intestinal cell invasiveness in *S.* Typhimurium. In this study, we assessed the possible involvement of the *ramRA* multidrug efflux regulatory locus and individual bile salts in the bile-mediated repression of *S.* Typhimurium invasion, using Caco-2 intestinal epithelial cells and *S.* Typhimurium strain ATCC 14028s. Our results indicate that (i) major primary bile salts, chenodeoxycholate and its conjugated-derivative salts, cholate, and deoxycholate, activate *ramA* transcription in a RamR-dependent manner, and (ii) it results in repression of *hilA*, encoding the master activator of TTSS-1 genes, and as a consequence in the repression of cellular invasiveness. On the other hand, crude ox bile extract and cholate were also shown to repress the transcription of *hilA* independently of RamR, and to inhibit cell invasion independently of *ramRA*. Altogether, these data suggest that bile-mediated repression of *S.* Typhimurium invasion occurs through pleiotropic effects involving partly *ramRA*, as well as other unknown regulatory pathways. Bile components other than the bile salts used in this study might also participate in this phenomenon.

KEYWORDS

Salmonella*, Typhimurium, invasion, intestinal, bile, regulation, RamR, *ramA

1 Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a Gram-negative enteric pathogen which causes generally localized and self-limiting gastroenteritis in humans, although some severe cases require antimicrobial treatment (Su et al., 2004; Velge et al., 2005; Giraud et al., 2006; Haraga et al., 2008). *S.* Typhimurium, after entering the gastrointestinal tract with contaminated food or water, has to overcome successive stressful environmental conditions, such as the acidic pH of the stomach or the presence of antibacterial compounds,

like bile, in the small intestine (Rychlik and Barrow, 2005; Baumler et al., 2011). At each step of infection, *S.* Typhimurium needs to sense its environment and to coordinate its gene expression in order to survive host defenses and optimize its colonization. Bile, in addition to its antibacterial effect, is thus recognized by *S.* Typhimurium as an important environmental signal, whose sensing leads to important changes in the expression levels of numerous genes involved in pathogenesis (Prouty et al., 2004a; Begley et al., 2005; Rychlik and Barrow, 2005; Antunes et al., 2012).

The *acrAB* and *tolC* multidrug efflux pump genes, which are required for *S.* Typhimurium resistance to bile, are activated by bile itself (Prouty et al., 2004b; Nikaido et al., 2008). We previously reported that this occurs mainly through the transcriptional derepression of the *ramA* gene, whose product is a direct activator of these efflux pump genes (Baucheron et al., 2014). We showed in this study that bile inhibits the binding of the RamR repressor to the *ramA* promoter, however without specifying which particular bile components were involved. More recently, the crystal structure of RamR and its interaction with bile acids have been solved, identifying cholic and chenodeoxycholic acids as the most important to bind RamR (Yamasaki et al., 2019). The interaction between bile acids and RamR occurs through hydrogen bonds (Yamasaki et al., 2019).

Another important feature of the intricate interactions between *S.* Typhimurium and bile is the bile-mediated repression of non-phagocytic intestinal cells invasion (Prouty and Gunn, 2000). This invasion is largely determined by the type III secretion system-1 (TTSS-1), and some of its secreted effectors encoded by the *Salmonella* Pathogenicity Island-1 (SPI-1) (Haraga et al., 2008; Fabrega and Vila, 2013). The transcription of SPI-1 genes is tightly controlled via a complex regulatory network, which ensures that TTSS-1 and its secreted effectors are expressed only when environmental conditions are favorable for invasion (Laughlin et al., 2014). The complex network of interacting transcription factors regulating SPI-1 gene expression results in a bistability pattern (TTSS-1^{ON} and TTSS-1^{OFF} cells) in *Salmonella* populations (Hamed et al., 2019; Sanchez-Romero and Casadesus, 2021; for a review see Lou et al., 2019). This bimodal gene expression of SPI-1 has several phenotypic impacts such as growth impairment, switch in motility and increased antibiotic resistance in subpopulations (Arnoldini et al., 2014; Hamed et al., 2019; Sanchez-Romero and Casadesus, 2021). Moreover, bile was initially pointed out by Prouty et al. (2004a) as one of numerous environmental signals that help *S.* Typhimurium localize and temporally regulate the expression of invasion factors (Prouty and Gunn, 2000). The authors hypothesized that high bile concentrations present in the lumen of the anterior small bowel repressed invasion factors, whereas, for bacteria having reached the distal ileum and crossed the mucous layer of the epithelium, lower bile concentrations allowed the expression of SPI-1 genes to initiate cell invasion. They also showed that a functional BarA/SirA two-component system was required for bile sensing and for the repression of the transcription of SPI-1 invasion genes. However, the sensing of bile components by the BarA sensor kinase was not demonstrated. Other studies have shown that the expression of the SPI-1 *hilA* gene, which encodes the master activator of TTSS-1-related invasion genes, was strongly repressed by bile (Golubeva, 2010; Antunes et al., 2012). More recently, Eade et al. (2016) demonstrated that SPI-1 repression by bile acids is mediated by posttranslational destabilization of HilD, a transcriptional activator acting directly on TTSS-1 genes and indirectly by activating *hilA* transcription.

Interestingly, the *ramRA* locus, besides regulating efflux pump genes, was also suggested to be involved in the regulation of invasion genes of the type III secretion system 1 (TTSS-1) (Bailey et al., 2010; Giraud et al., 2013). Indeed, overexpression of *ramA*, either plasmid-driven or due to mutations in *ramR* or in the RamR DNA-binding site, led to decreased expression of TTSS-1 genes, including *hilA,* and to decreased invasion efficiency in some *S.* Typhimurium strains, depending on their genetic background (Giraud et al., 2013). Altogether, these observations suggested that the *ramRA* regulatory locus may possibly be involved in the bile-mediated repression of intestinal cell invasion.

In the present study, we investigated the roles of (i) individual bile salts, which are the most abundant components of bile (representing about 2/3rd of its organic content) and (ii) the *ramRA* locus in the bile-mediated repression of *S.* Typhimurium invasion. In particular, the major primary bile salts, chenodeoxycholate and cholate, as well as their derivatives conjugated with glycine or taurine, were assessed for their ability to activate *ramA* expression and to repress *hilA* expression, and as a consequence to inhibit the invasion of intestinal epithelial cells. We assessed also whether the expression changes observed with the major bile salts were dependent on *ramR*, and studied the role of the entire regulatory locus *ramRA* on the bile-mediated repression of intestinal cell invasion.

2 Materials and methods

2.1 Bacterial strains and culture conditions

S. Typhimurium wild-type (WT) strain ATCC 14028s and its Δ*ramR,* Δ*ramR*/p*ramR,* and Δ*ramRA*::*kan*/p*ramA* derivatives were used in this study. Deletion mutants were constructed using the Datsenko and Wanner inactivation gene method as previously described (Datsenko and Wanner, 2000; Abouzeed et al., 2008). Complementation plasmids carrying the *ramR* gene (p*ramR*) or the *ramA* gene (p*ramA*) were previously described (Abouzeed et al., 2008; Nikaido et al., 2008). Bacterial strains were grown at 37°C in Luria–Bertani broth (LB, pH 7.5) supplemented with 25.6 g/L bile or with 5 mM of individual bile salts where appropriate. Bile used in this work was a crude ox-bile extract which contains the main bile sodium salts of taurocholic, glycocholic, deoxycholic, and cholic acids purchased under the label "sodium choleate" (Sigma–Aldrich, Steinheim, Germany). Physiological concentrations of bile salts encountered by bacteria in the intestinal lumen are variable with high and low concentrations in the anterior small bowel and distal ileum, respectively, estimated in the millimolar range that is consistent with their critical micellar concentrations (e.g., 6–10 mM for taurocholic acid) (Martinez-Augustin and Sanchez de Medina, 2008). In a previous study, we showed that a bile concentration of 25.6 g/L allowed normal growth (i.e., similar to growth control

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in LB medium) of *S.* Typhimurium isolates (Baucheron et al., 2005). Individual bile salts (Sigma–Alrich, Steinheim, Germany), also allowed normal growth of the tested strains when used at 5 mM.

2.2 Invasion and adhesion assays

Invasions assays were performed as previously described (Rosselin et al., 2010). Caco-2 intestinal epithelial cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% inactivated fetal bovine serum, 1% non-essential amino acids, and 1% antibiotic solution (Gibco, Invitrogen), at 37°C under 5% CO_2. Cells were seeded at a density of 2×10^5 cells/well in a 24-well plate (Falcon) and grown until confluence in the same medium. Antibiotic was removed 24 h before the invasion assays. Bacteria grown to an OD_{600} of 0.6 in LB broth were inoculated on Caco-2 monolayers at a multiplicity of infection (MOI) of 30. After a 30 min incubation, the bacteria-containing medium was removed from the wells, and the cells monolayers were washed with phosphate buffered saline (PBS). For adhesion assays, cells were then lysed for 30 min with sterile ultrapure water and serial dilutions of lysates were plated on LB agar. For invasion assays, cells were further incubated for 1.5 h with DMEM supplemented with gentamicin at 100 μg/mL. After washing with PBS, cells were lysed with sterile ultrapure water and serial dilutions of lysates were plated on LB agar. The percentage of penetrating bacteria was calculated as the ratio of the counted colony forming units (cfu) to the bacterial inoculum. All assays were repeated at least twice, with three replicates for each tested condition. Data presented correspond to mean values of at least six replicates for invasion and adhesion assays.

2.3 Gene expression analysis by qRT-PCR

Bacteria were grown in 20 ml liquid cultures (standard LB, 1% NaCl), in 125 mL Erlenmeyer flasks, under shaking at 180 RPM, for about 150 min, until they reached an OD_{600} value of 0.6. Culture samples were pelleted by centrifugation, stabilized with RNAprotect Bacteria Reagent (Qiagen) and stored at −80°C until use. Total RNA was extracted using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Residual genomic DNA was removed using the Turbo DNA-free kit (Ambion). Total RNA (1.5 μg) was reverse-transcribed using random hexamers and the Superscript III First Strand Synthesis System (Applied Biosystems). The expression level of each gene was calculated from three independent cDNA samples. For each cDNA sample and each gene, qRT-PCR runs were performed in duplicated wells. Primers and cycling conditions used for qRT-PCR were previously described (Giraud et al., 2013). The relative quantities of transcripts were normalized against the geometric mean of three reference genes (*gmk*, *gyrB*, *rrs*). Statistical significance was assessed at a *P*-value of <0.05 using a two-tailed Student's *t*-test.

3 Results and discussion

3.1 Differential effects of individual bile salts on the expression of *S.* Typhimurium ATCC 14028s invasion regulatory genes and on invasion of intestinal epithelial cells

The qRT-PCR assays of this study confirmed that crude ox bile extract at 25.6 g/L increased *ramA* transcript levels about 20-fold, as previously described (Figure 1A) (Baucheron et al., 2014). To address the effects of individual bile salts on *ramA* expression, primary bile salts and their derivatives (dehydroxylated and glycine- or taurine-conjugated) were tested at the concentration of 5 mM. Chenodeoxycholate showed the most important effect, by increasing *ramA* transcript level ~7- vs. 4-5-fold for its taurine- and glycine- conjugated derivatives in the *S.* Typhimurium strain ATCC 14028s (Figure 1A). Cholate also increased *ramA* mRNA levels by a 4-fold factor, i.e., notably more than its dehydroxylated derivative, deoxycholate (2.5-fold). In contrast, no *ramA*-inducing activity could be detected for both cholate conjugates, taurocholate and glycocholate (Figure 1A).

In contrast to their effects on *ramA* expression, bile and individual bile salts globally showed a repressive effect on *hilA* expression in *S.* Typhimurium ATCC 14028s (Figure 1B). Furthermore, this repressive effect on *hilA* expression appeared correlated to the *ramA*-inducing effect. Indeed, crude bile extract, which showed the strongest *ramA*-inducing effect, decreased *hilA* transcripts to very low levels (about 1.5% that of the control) (Figure 1B). By contrast, bile salts with low (deoxycholate) or no (taurocholate, glycocholate) *ramA*-inducing activity, showed no significant repressive effects on *hilA* expression. Other bile salts, which induced *ramA* between 4- and 7-fold (see above), decreased *hilA* transcript levels 3–4-fold (chenodeoxycholate and taurochenodeoxycholate), 2.3-fold (cholate), and 1.6-fold (glycochenodeoxycholate) (Figure 1B). The repressive effects exerted on *hilA* by crude bile extract and by chenodeoxycholate and its conjugates were also observed for *invA*, which encodes another SPI-1 positive regulatory protein (Supplementary Figure S1). However, no significant effects of cholate and its derived salts were observed on *invA* expression (Supplementary Figure S1).

Further to above data, we hypothesized that the repression exerted by some bile salts on *hilA* expression could result into decreased cell invasion efficiency of *S.* Typhimurium ATCC 14028s. Therefore, gentamicin protection assays were performed to address the effects of bile and individual bile salts on adhesion to and invasion of Caco-2 intestinal epithelial cells (Figures 1C, D). Crude bile extract, chenodeoxycholate and its two conjugates, taurochenodeoxycholate and glycochenodeoxycholate, decreased adhesion to the Caco-2 cells by about 20% (Figure 1C). Crude bile extract decreased *S.* Typhimurium ATCC 14028s invasion about 5-fold, and chenodeoxycholate and taurochenodeoxycholate (i.e., the bile salts which most efficiently repressed *hilA* expression), decreased its invasion by about 2.5-fold (Figure 1D). These decreased invasions may be explained partly by the decreased adhesion of the strain mentioned above. It is also possible that the

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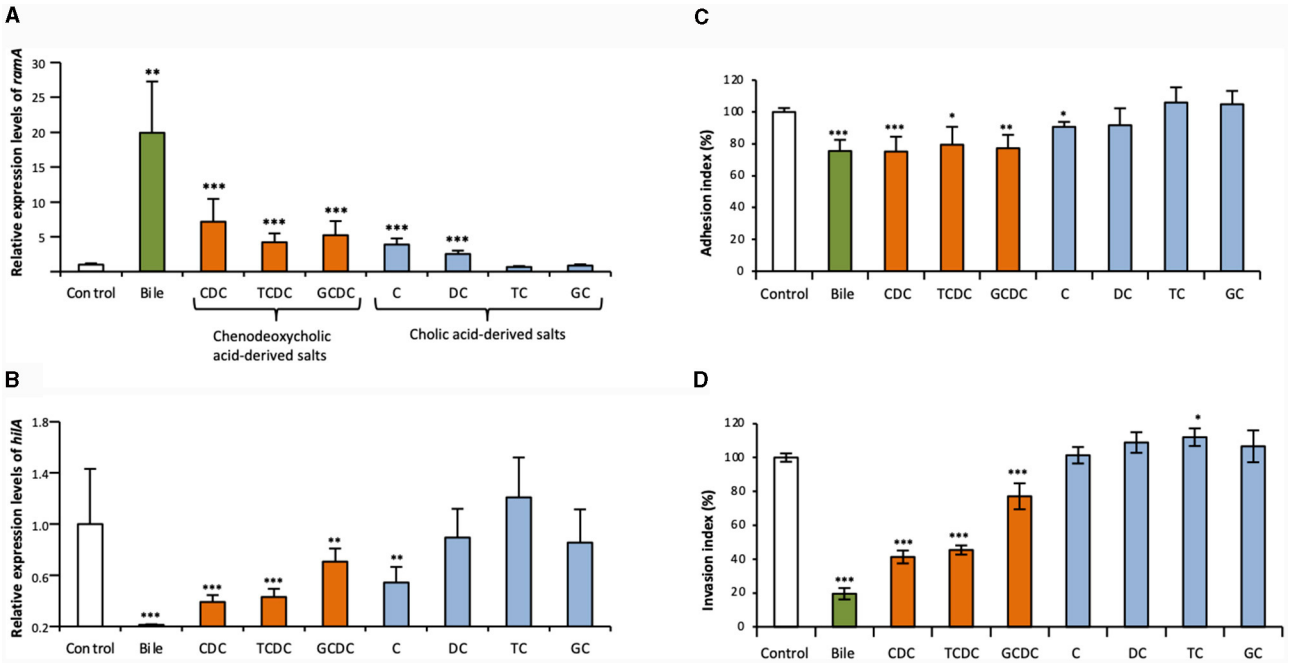

FIGURE 1
Effects of crude bile extract at 25.6 g/L or of individual bile salts at 5 mM on *ramA* and *hilA* expression and on adhesion to/invasion of Caco-2 intestinal epithelial cells. Transcript levels of *ramA* **(A)** and *hilA* **(B)** were determined using qRT-PCR for the WT *S.* Typhimurium strain 14028s. Values were normalized by those obtained for control (LB alone). Bars represent the standard deviation from three independent replicates. **(C)** Adhesion to and **(D)** invasion of Caco-2 cells by the WT strain 14028s. Results are expressed relative to values obtained for control (LB alone), arbitrarily set at 100%. Bars indicate the percentage of attached/internalized bacteria ± standard error of the mean. In all panels, asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). □ Controls (LB alone), ■ crude bile extract, ■ chenodeoxycholic acid-derived salts (CDC, chenodeoxycholate; TCDC, taurochenodeoxycholate; GCDC, glycochenodeoxycholate) and ■ cholic acid-derived salts (C, cholate; DC, deoxycholate; TC, taurocholate; GC, glycocholate).

20% decrease of invasion observed with glycochenodeoxycholate was mostly due to defective adhesion of the strain. Bile salts that had no significant effect on *hilA* expression did not repress invasion of the *S.* Typhimurium strain ATCC 14028s.

These data suggest that some individual bile salts actively participate to the repression of *S.* Typhimurium cell invasion by bile. They also reveal that the specific structure of bile salts may determine their activity as environmental signals to regulate gene expression and cell invasion of *S.* Typhimurium. Nevertheless, since only one *S.* Typhimurium strain was investigated, these data must be taken with caution, to avoid any overinterpretation regarding *S.* Typhimurium as a pathogenic serovar, or more generally *Salmonella* as a pathogen, since as previously published distinct genetic lineages of serovar Typhimurium may behave differently regarding cell invasion and its regulation (Giraud et al., 2013).

In our experimental conditions, chenodeoxycholate seemed to be the most active bile salt and its conjugation to notably glycine, appeared to decrease its activity. Although they do not establish any causality relationship between *ramA* activation and *hilA* repression, our results indicate that *ramA* overexpression is associated to decreased TTSS-1 genes expression and to decreased invasion of the *S.* Typhimurium strain studied. These different correlations led us to examine further the actual involvement of the *ramRA* regulatory locus in the bile-mediated repression of *S.* Typhimurium ATCC 14028s cell invasion.

3.2 Bile salts effects on *ramA* and *hilA* expression are dependent on *ramR* in *S.* Typhimurium ATCC 14028s

A previous study suggested that, whereas *ramA* is activated by bile mainly depending on *ramR*, another undetermined *ramR*-independent pathway also contributes to the up-regulation of *ramA* by bile in *S.* Typhimurium (Baucheron et al., 2014). Here, we assessed whether the major individual bile salts (chenodeoxycholate, cholate and deoxycholate) could induce *ramA* expression in *S.* Typhimurium ATCC 14028s by different, *ramR*-dependent and/or *ramR*-independent pathways.

The increase of *ramA* expression in the WT *S.* Typhimurium ATCC 14028s strain, in the presence of bile or of the three tested salts, were similar to those reported above (Figure 2A). As expected, in control cultures (LB alone), the Δ*ramR* mutant expressed *ramA* transcript levels about 10-fold higher than those of the WT strain, and complementation with a functional *ramR* gene (using p*ramR*) restored WT *ramA* expression levels. Chenodeoxycholate increased *ramA* transcript levels by 7.2-fold in the WT strain, probably in a fully *ramR*-dependent manner, since no increase was observed in the Δ*ramR* mutant. Cholate increased *ramA* transcript level 5.6-fold in the WT strain. However, in contrast to chenodeoxycholate, cholate also increased *ramA* expression about 1.6-fold in the Δ*ramR* mutant compared to LB medium alone, indicating that it might also use a minor *ramR*-independent

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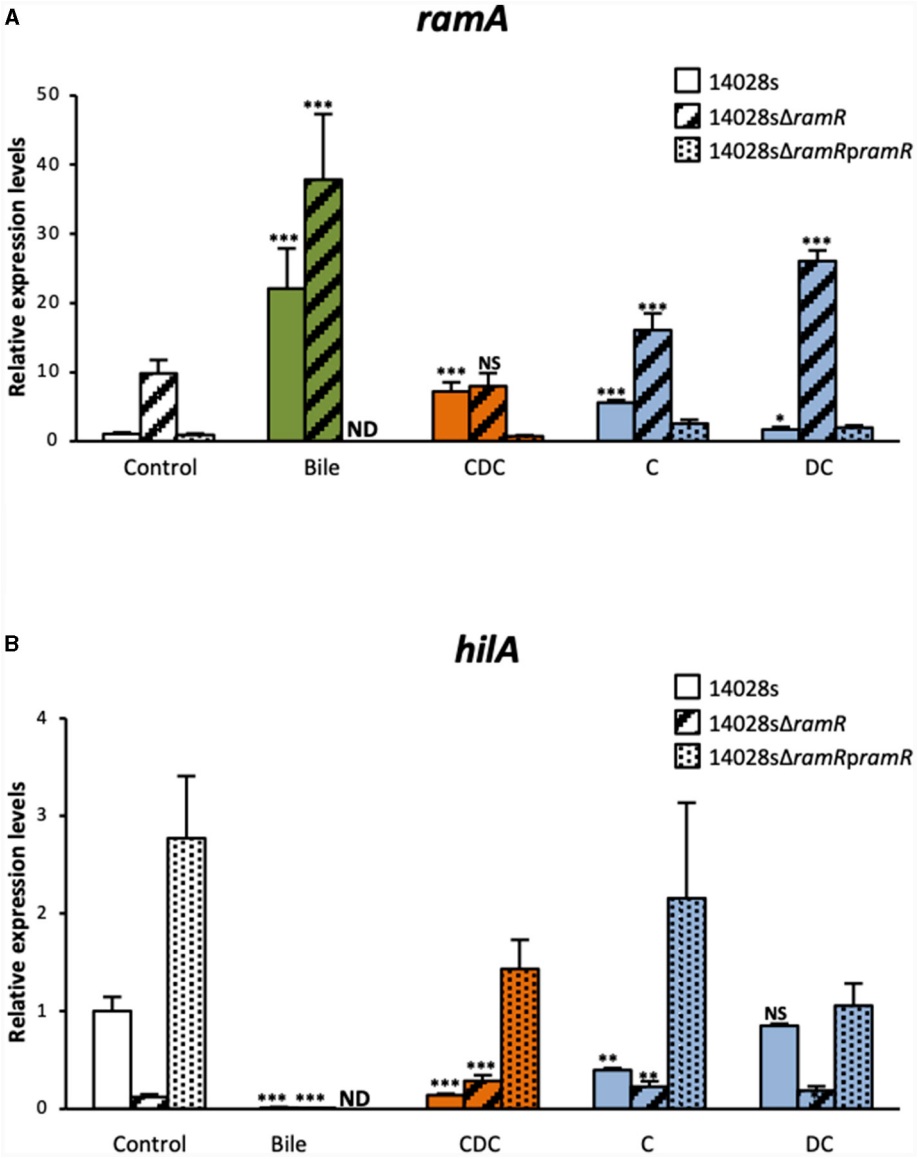

FIGURE 2
qRT-PCR analysis of the dependence on *ramR* of bile and individual bile salts effects on *ramA* and *hilA* expression. Transcript levels of *ramA* **(A)** and *hilA* **(B)** were determined for the WT *S*. Typhimurium strain 14028s strain and for its *ramR* deletion mutant, complemented or not with a p*ramR* plasmid, after growth in the presence of crude bile extract at 25.6 g/L or of individual bile salts at 5 mM. Bars represent the standard deviation from three independent replicates. □ Controls (LB alone), ■ crude bile extract, ■ chenodeoxycholate (CDC), ■ cholate (C), and ■ deoxycholate (DC). Asterisks indicate significant differences (NS, non-significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). ND, not determined.

pathway to activate *ramA* expression. Lastly, the slight effects of deoxycholate on *ramA* transcript level appeared similar in the WT and in the Δ*ramR* background (1.7- and 2.7-fold increase, respectively), indicating that deoxycholate likely uses mainly a *ramR*-independent pathway to achieve this effect. Irrespective of the tested bile salt, complementation using the p*ramR* multicopy plasmid resulted in *ramA* transcript levels similar, or even lower, than those observed in the WT strain. These results indicate that the individual bile salts tested differ not only in the magnitude of their effects on *ramA* expression, but also in their relative use of the *ramR*-dependent and *ramR*-independent pathways to achieve this effect. Considering these results, the bile-mediated increase of *ramA* expression that we observed, here and before, can be interpreted as being, at least partly, the intricate result of the individual effects of bile salts, which would explain the implication of both *ramR*-dependent and *ramR*-independent mechanisms. It remains also possible that bile components other than the tested bile salts also participate to *ramA* up-regulation, depending or not on *ramR*.

In view of the negative correlation between *ramA* and *hilA* expression levels, we also tested to what extent *hilA* repression by chenodeoxycholate, cholate and deoxycholate depended on *ramR*. In control cultures (LB alone), *hilA* expression levels in the Δ*ramR* mutant were 8-fold lower than in the WT strain, in

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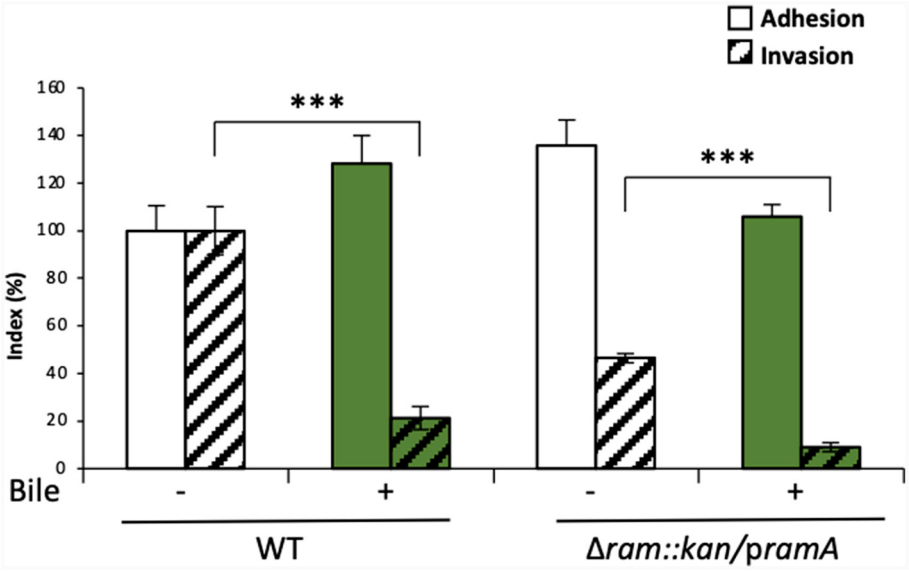

FIGURE 3
In vitro analysis of the dependence on the *ramRA* locus of bile effects on adhesion to/invasion of intestinal epithelial cells. Adhesion to and invasion of Caco-2 cells was analyzed after growth, in the absence (□, −) or presence (■, +) of crude bile extract at 25.6 g/L, of the WT *S.* Typhimurium strain 14028s and its *ramRA*::kan deletion mutant complemented with a p*ramA* plasmid. Bars indicate the percentage of attached/internalized bacteria ± standard error of the mean. Asterisks indicate significant differences (***$P < 0.001$).

agreement with previously reported results (Giraud et al., 2013). In the WT strain, the *hilA* transcript levels were significantly decreased in the presence of chenodeoxycholate (6.8-fold) or cholate (2.5-fold), whereas deoxycholate had no effect. In the Δ*ramR* mutant (where *ramA* is overexpressed by 4–8-fold, Figure 2A), the tested bile salts did not further decrease the *hilA* transcript levels (Figure 2B). Possibly the *ramA*-mediated repression of *hilA* is saturated in the Δ*ramR* background, explaining why further increase of *ramA* expression does not result in further *hilA* repression.

Irrespective of the tested bile salt, complementation using a p*ramR* multicopy plasmid resulted in *hilA* transcript levels higher (for chenodeoxycholate and cholate) or similar (for deoxycholate) than those observed in the WT strain (Figure 2B). These results suggest that *hilA* downregulation by chenodeoxycholate and cholate is dependent on *ramR*, and via the upregulation of *ramA*. This observation is congruent with our previous study on the crystal structure of RamR and its interaction with bile acids (Yamasaki et al., 2019). Both cholic and chenodeoxycholic acids, but not deoxycholic acid, were indeed shown to bind to RamR, and more precisely through four hydrogen bonds with RamR, and to induce *ramA* expression.

Of note, crude bile extract decreased *hilA* expression to undetectable levels as well in the Δ*ramR* mutant as in the WT strain (Figure 2B). Although it is below the detection level in our experimental conditions, it may suggest that another *ramR*-independent mechanism, possibly induced by bile components other than bile salts, acts also in the bile-mediated repression of *hilA*.

3.3 Influence of RamA overexpression on the bile-mediated repression of cell invasion of *S.* Typhimurium ATCC 14028s

Altogether, the results described above suggested a possible involvement of the *ramRA* locus in the bile-mediated repression of invasion, partly through RamA overexpression, resulting in repression of the master regulator *hilA* of TTSS-1 genes. To further confirm this hypothesis, the role of RamA in bile-mediated repression of invasion was tested in gentamicin protection assays, using a *S.* Typhimurium ATCC 14028s mutant overexpressing RamA (Δ*ramRA*/p*ramA*). First, invasion efficiency was confirmed to be decreased about 5-fold by bile in the WT strain (Figure 3, WT). In absence of bile (LB medium alone), ectopic overexpression of RamA in the mutant Δ*ramRA*/p*ramA* resulted in a slight increase of adhesion and in a 2-fold decrease of invasion. Using this mutant, bile further decreased invasion efficiency of an additional 5-fold (Figure 3, Δ*ramRA*/p*ramA*). These results strengthened the hypothesis that the repression of invasion occurs via different pathways dependent or not on RamA overexpression.

In sum, these results suggest that the bile-induced repression of invasion may be driven by a *ramRA*-dependent mechanism, via *ramA* overexpression, as well as through other pathways independent of the *ramRA* regulators which can alternatively have an additive effect to repress invasion. Nevertheless, the observed results must be taken with caution, because of the experimental conditions (e.g., ectopic overexpression) and the use of a single *S.* Typhimurium strain. At least, *ramRA* alone seems not entirely responsible for bile-mediated repression of *S.* Typhimurium cell invasion, and thus other genetic factors need to be further investigated.

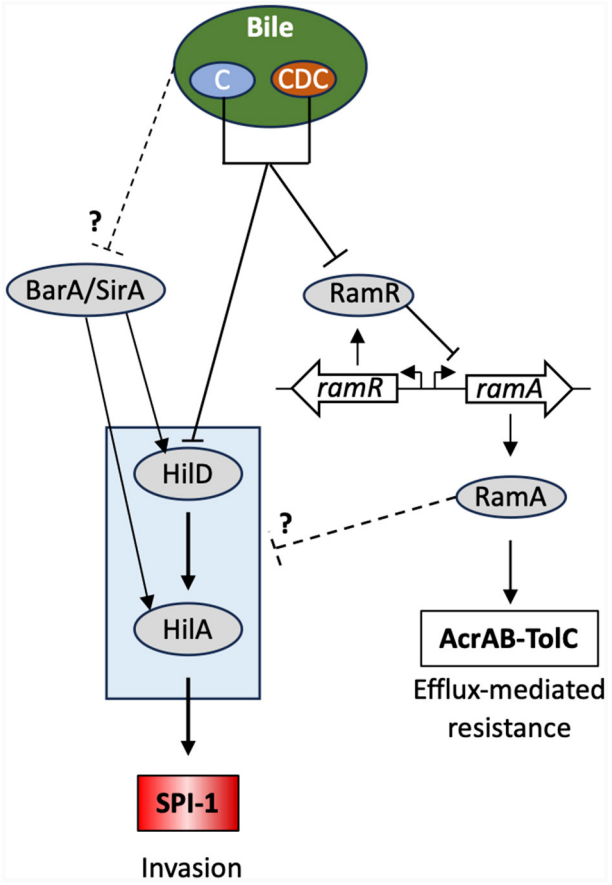

FIGURE 4
Bile-mediated regulatory network controlling SPI-1 gene expression. Only the key regulators and relevant proteins in this context are shown. Bile acids (C, cholic acid; CDC, chenodeoxycholic acid) directly bind to RamR alleviating its transcriptional repression of *ramA*. This leads to *ramA* overexpression and concomitantly repression of *hilA* and overexpression of the AcrAB-TolC efflux pump. The precise regulatory pathway between *ramA* n and SPI-1 remains unknown. See main text for further details and Lou et al. (2019) for a review.

4 Conclusion

In summary, two contrasting situations were observed, depending on whether individual bile salts or crude bile extract were used in the transcription and intestinal cellular invasion assays. On the one hand, the individual bile salts tested, mainly chenodeoxycholate or derived bile salts, were shown to activate *ramA* and to repress *hilA* in the *S.* Typhimurium strain ATCC 14028s (Figure 4). Evidence is also provided that this activation is dependent on *ramR*, and that this activation seems to vary depending on the considered bile salt, although *ramA* overexpression in any case appears to repress cellular invasion of the *S.* Typhimurium strain. In our conditions, those bile salts also repress *hilA* expression in the strain studied and its invasion of intestinal cells, likely also via the *ramRA* locus. On the other hand, crude bile extract and cholate seem also to repress *hilA* expression and intestinal cell invasion independently of *ramR* (higher repressions with the addition of bile in the Δ*ramR* genetic background), at least in our experimental conditions and for the *S.* Typhimurium strain assessed (Figure 4). This discrepancy on the dependency of the *ramRA* locus is not explained yet, but we may consider that whole bile is a complex mixture, not only by its bile salts content, but also by the presence of other molecules. Some of them may possibly counteract the activity of the one or the other individual bile salt and have pleiotropic effects at other regulatory loci than *ramRA*. In line with this, a study of Antunes et al. (2012), reported that repression by physiological bile of *phoP*, another major virulence regulator of *S.* Typhimurium, is not caused by bile salts, but rather by still unidentified small molecules present in bile. In addition, many other intestinal factors than the bile content participate in the complex regulatory network of *S.* Typhimurium intestinal cell invasion, such as intestinal fatty acid and many other small molecules found in the intestine, and interplay with the intestinal microbiota as well (Lou et al., 2019; Rogers et al., 2021; Chodhury et al., 2021a,b, 2023). Thus, further studies are needed to clarify the possible role of other bile molecules and their possible interaction in the invasion process of *S.* Typhimurium. The possible linkage(s) of the *ramRA* regulatory locus with other cell invasion regulatory loci need also to be further investigated.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

EG: Conceptualization, Data curation, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. SB: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – review & editing. IF: Investigation, Methodology, Validation, Writing – review & editing. BD: Data curation, Supervision, Validation, Writing – review & editing. KN: Conceptualization, Data curation, Supervision, Validation, Writing – review & editing. AC: Conceptualization, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1338261/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Effects of bile and individual bile salts on *invA* expression. Transcript levels of *invA* were determined using qRT-PCR, for the WT *S.* Typhimurium 14028s strain grown in the presence of bile at 25.6 g/L or of individual bile salts at 5 mM. Values were normalized by those obtained for control samples (LB alone). Bars represent the standard deviation from three independent replicates.

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Diversity of antimicrobial-resistant bacteria isolated from Australian chicken and pork meat

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Antimicrobial-resistant bacteria are frequently isolated from retail meat and may infect humans. To determine the diversity of antimicrobial-resistant bacteria in Australian retail meat, bacteria were cultured on selective media from raw chicken ($n = 244$) and pork ($n = 160$) meat samples obtained from all four major supermarket chains in the ACT/NSW, Australia, between March and June 2021. Antimicrobial susceptibility testing (AST) was performed for 13 critically and 4 highly important antibiotics as categorised by the World Health Organization (WHO) for a wide range of species detected in the meat samples. A total of 288 isolates underwent whole-genome sequencing (WGS) to identify the presence of antimicrobial resistance (AMR) genes, virulence genes, and plasmids. AST testing revealed that 35/288 (12%) of the isolates were found to be multidrug-resistant (MDR). Using WGS data, 232/288 (81%) of the isolates were found to harbour resistance genes for critically or highly important antibiotics. This study reveals a greater diversity of AMR genes in bacteria isolated from retail meat in Australia than previous studies have shown, emphasising the importance of monitoring AMR in not only foodborne pathogenic bacteria, but other species that are capable of transferring AMR genes to pathogenic bacteria.

KEYWORDS

antibiotic, antibiotic resistance, antimicrobial resistance, antimicrobial susceptibility testing, multidrug-resistance, resistance genes, whole-genome sequencing

Introduction

The prevalence of antimicrobial-resistant (AMR) bacteria and resistance to traditional antibiotics is increasing globally and is therefore a significant global health issue (Collignon, 2015). Antimicrobials are used to prevent and control bacterial infections in food and animal production systems; however, their overuse in the agri-food industry has expedited the spread of AMR bacteria worldwide. The use of antimicrobials in food animal production selects for AMR bacteria, which may be transmitted to humans via zoonotic bacteria in the food chain (Barlow et al., 2015). The continued prophylactic use of antimicrobials in the Australian meat industry no doubt contributes to the acquisition and maintenance of AMR (Landers et al., 2012; Kirchhelle, 2018).

European Union legislation imposed in 2022 prohibits the routine use and prophylactic use of antimicrobial medicinal products in farming, including the use of medicated feeds.[1] The United States has followed a similar path; in 2019, approximately 60% of broilers were raised in no antibiotics ever (NAE) conditions.[2] Australian government regulations do not go as far, as the prophylactic use of antimicrobials is still allowed. In 2015, Australia was reported to have relatively low rates of antibiotic resistance to third-generation cephalosporins, fluoroquinolones, aminoglycosides, and carbapenems (Collignon, 2015). However, a recent study showed that AMR rates are variable across Australia, with some areas showing high rates of AMR in hospital-acquired pathogens. It was estimated that 1,031 human deaths were attributed to five hospital-associated AMR pathogens in 2020 (Wozniak et al., 2022). This estimate is four times higher than an estimate provided by the OECD in 2018 (Dunachie et al., 2020).

Carbapenems are useful antibiotics because of their broad spectrum of activity and effectiveness against both Gram-positive and Gram-negative bacteria (Papp-Wallace et al., 2011). Colistin, a last resort antimicrobial, is used to treat carbapenem-resistant *Enterobacteriaceae* (CRE) infections in many countries; however, colistin resistance has emerged in CRE, producing conditions for which no effective antibiotic treatment is now available (antimicrobial resistance, El-Sayed Ahmed et al., 2020; WHO, 2020). Colistin is also used to treat infections caused by other MDR bacteria, including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*; however, colistin resistance has emerged in these species as well. Some bacteria, such as *Serratia* spp., *Proteus* spp., and *Burkholderia* spp., are naturally resistant to colistin (Aghapour et al., 2019); however, they may still acquire plasmids with colistin resistance genes and therefore still participate in the spread of colistin resistance via horizontal gene transfer (Zhong et al., 2022). Very few studies have assessed the presence of colistin resistance genes in non-pathogenic species of bacteria; however, such species may act as reservoirs for colistin resistance.

Bacterial species (spp.), such as *Campylobacter* spp. (Habib et al., 2020), *Escherichia coli* (Vangchhia et al., 2018;Touchon et al., 2020; Abraham et al., 2020), *Enterococcus* spp. (Lee et al., 2021), and *Salmonella* spp. (Abraham et al., 2020), are known to be pathogenic. These species are frequently used as AMR "indicators" in surveillance studies of production animals because they are important in human disease, are relatively easy to culture and identify, and have known AMR minimum inhibitory concentrations (MIC) (Cameron and McAllister, 2016). While pathogenic bacteria typically contain AMR genes, other species of bacteria and bacteriophages are capable of transferring MGEs to pathogenic bacteria, but are often overlooked in surveillance studies because they are not pathogenic.

Many AMR studies have revealed *Campylobacter* spp., *Escherichia* spp., *Salmonella* spp., and *Enterococcus* spp. to be widespread in meat samples. *E. coli* is a common member of the enteric community of poultry and other birds (Blyton et al., 2015). The poultry sector has been identified as a likely source of extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria that can infect people who consume or handle contaminated meat (Leverstein-van Hall et al., 2011). According to Overdevest et al. (2011), 80% of ESBL genes found in chickens are mostly identical to ESBL genes found in human rectal swabs, and *E. coli* typing confirmed the similarity between chicken and human strains, albeit using low-resolution typing methods (Kluytmans et al., 2013).

AMR bacteria are of serious concern because they pose a direct threat to humans. Screening for the presence of AMR bacteria in meat produced for human consumption, beyond the most common foodborne pathogens, may provide important information about the diversity of AMR genes and the bacteria that carry them in food-producing animals. Additionally, it is important to know the extent to which AMR genes are encoded on MGEs, as they may be transferred to pathogenic bacteria from bacteria not commonly screened in surveillance studies. The potential virulence of strains isolated from retail meat is also not commonly assessed. Therefore, the goals of this study were to isolate and identify bacterial species beyond the commonly surveyed food pathogens in Australian retail chicken and pork meat using selective media and whole-genome sequencing; to assess the extent of phenotypic AMR; and to identify MGEs and virulence genes present in the bacteria to understand their ability to disseminate AMR genes and cause disease.

Materials and methods

Sample acquisition and processing

A total of 404 meat samples (244 chicken and 160 pork) were purchased by a third-party contractor from Aldi (39 chicken, 39 pork), Coles (85 chicken, 41 pork), IGA (41 chicken, 33 pork), and Woolworths (79 chicken, 47 pork) supermarkets across 39.5/50 ACT/NSW electorates in Australia between March and June 2021. All chicken and pork meat samples available at each supermarket were purchased, provided they met the inclusion criteria: raw, unprocessed, unmarinated, unseasoned, and not labelled either "free range" or "organic." Once purchased, all meat samples were transported, stored at 4°C, and processed within 24 h, before their expiration date. All sample packaging was disinfected with 80% ethanol before being processed aseptically in a Class II Biosafety Cabinet. Approximately 10 g of meat was taken from four locations of each sample and added to both 25 mL pre-warmed peptone buffered water and 25 mL Bolton broth (for *Campylobacter* isolation) and homogenised using a stomacher. Approximately 20 mL of homogenate for chicken samples obtained from a single supermarket were combined in a single tube. The same was done to combine pork samples from a single supermarket. This resulted in a total of 302 pooled samples (152 chicken, 150 pork). Of the pooled samples, 211 (70%) samples comprised a single brand product, 82 (27%) comprised two, seven (2%) comprised three, and two comprised four (1%). These pooled samples were grown in selective media.

Selection of isolates

The selective media used to grow bacteria from the meat samples included Brilliance™ ESBL agar, used for the detection of

1 https://eur-lex.europa.eu/eli/reg/2019/6/oj

2 https://poultryhealthtoday.com/nearly-60-of-us-broilers-now-raised-without-antibiotics-but-that-number-may-have-peaked/

ESBL-producing bacteria; Brilliance™ CRE agar, used for the detection of carbapenem-resistant Enterobacteriaceae (CRE); Brilliance™ VRE agar, used for the detection of vancomycin-resistant enterococci (VRE); *Campylobacter* selective agar (CAMPY), used for the selection of *Campylobacter* spp.; MacConkey (MAC) agar, used for the identification and differentiation of *Enterobacteriaceae* spp., including *E. coli*; and xylose lysine deoxycholate (XLD) agar, used for the identification of *Salmonella* spp. A 1 mL aliquot of the PBW homogenate sample was added to selenite broth at 41°C for 18 h with shaking to select for *Salmonella*. Plating on XLD agar at 37°C overnight followed. A representative of each different colony, based on colony morphology and colour, was selected for each media type, regardless of whether they appeared to be a target organism for the selective agar or not. A freezer stock containing 30% glycerol was made for each isolate. Whole-genome sequencing (WGS) was performed for 288 isolates, with all isolates that grew on Brilliance™ ESBL, Brilliance™ CRE, Brilliance™ VRE, and CAMPY agar being prioritised, and the remainder being made up of isolates that grew on MAC or XLD agar. A single isolate of *E. coli* was randomly chosen from each electorate, despite having identified multiple different isolates of *E. coli* for each electorate. Due to the small number of isolates grown on XLD, MAC and XLD results are presented together.

Antimicrobial susceptibility testing

Antimicrobial sensitivity testing was performed for the 288 isolates using an automated MIC broth microdilution method and commercially prepared Gram-negative (CMV3AGNF™) and *Campylobacter* spp. (EUCAMP2™) Sensititre™ antibiotic plates (Thermo Scientific™). All bacterial isolates, apart from *Campylobacter* spp., were grown from glycerol freezer stocks on their respective agar (Brilliance™ ESBL/Brilliance™ CRE/Brilliance™ VRE, MAC, and XLD) and incubated overnight at 37°C. The *Campylobacter* isolates were grown on CAMPY agar and incubated at 41°C for 48 h in anaerobic jars with CampyGen sachets (Oxoid™).

After incubation, a few colonies from each agar plate were transferred to 5 mL Sensititre™ demineralised sterile water (Thermo Scientific™) to achieve a density equivalent to the 0.5 McFarland standard. A 10 µL aliquot of each 0.5 density dilution was transferred to a 5 mL Sensititre™ Mueller Hinton Broth and mixed well. A Sensititre™ 96-well plate was then inoculated with 50 µL volume per well of the suspension using the Sensititre™ AIM™ (Automated Inoculation Delivery) system. The Gram-negative CMV3AGNF™ plates were sealed and incubated at 37°C in a non-CO_2 incubator for 24 h, and at 41°C for 48 h for the EUCAMP2™ plates. Following incubation, plates were placed inside a Sensititre™ Vizion™ Digital MIC viewing system, and results were recorded and interpreted using Sensititre™ SWIN™ software, based on the Clinical & Laboratory Standards Institute (CLSI) breakpoints for MIC determination.

To determine whether or not an isolate was MDR, we used the definitions as set out by Magiorakos et al. (2012). If a species was not included in this definition, then we used the same definition as a species from the same genus; if no species or genus encountered was included in their definition, then we searched the literature to determine if the genus/species was intrinsically resistant to the antibiotics tested. As with the Magiorakos et al. (2012) definition, intrinsic resistance was not taken into account.

Whole-genome sequencing and analysis

DNA from the 288 prioritised isolates was extracted from a 1 mL aliquot of an overnight broth culture using Bioline® ISOLATE II Genomic DNA Kits according to the manufacturer's protocol. Quantification of DNA was performed using a TapeStation system (Agilent Technologies, Inc.). plexWell™ 96 Kits (seqWell™) were used for library preparation, and sequencing was performed on an Illumina® NovaSeq™ platform (Illumina®, Inc.) in a 150 bp paired-end format.

The raw paired-read data of each isolate were assembled using the St. Petersburg genome assembler (SPAdes) (Bankevich et al., 2012) tool from the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (Olson et al., 2023). The assembled sequences were annotated using the Rapid Annotations utilising Subsystems Technology (RASTtk) (Brettin et al., 2015) tool kit based on genus/species identification. Each assembled sequence was given a taxonomy-based annotation (genus or species) using the NCBI's BLAST tool. The acquired antibiotic resistance genes, plasmids, and virulence genes were identified using the Mobile Genetic Element (MGE) finder tool from the Center for Genomic Epidemiology (CGE). The MGE tool identifies mobile genetic elements and their relation to AMR genes and virulence factors (Johansson et al., 2021). The PathogenFinder 1.1 tool, also from the CGE, was used to predict the likelihood of isolates being pathogenic to humans (Cosentino et al., 2013). Multilocus sequence typing was performed using the MLST tool from CGE, which can identify the sequence types (ST) of 66 bacterial species (Larsen et al., 2012).

Results

The breakdown of bacterial genera detected according to the selective media used for and the supermarket chain from which the meat samples were purchased for pooled chicken and pork samples is presented in Figure 1. For the pooled chicken samples, *Serratia* spp. were most commonly isolated (67/206, 32%), followed by *E. coli* (47/206, 23%), *Pseudomonas* spp. (29/206, 14%), and *Acinetobacter* spp. (13/206, 6%). For the pooled pork samples, *Serratia* spp. were most commonly isolated (35/82, 43%), followed by *Hafnia* spp. (14/82, 17%), *Acinetobacter* spp. (8/82, 9%), and *E. coli* (6/82, 7%). Overall, the 288 isolates represented 17 different genera (Table 1). A total of 41 isolates produced colonies on Brilliance™ CRE agar (30 chicken, 11 pork), 17 on Brilliance™ VRE agar (13 chicken, 4 pork), 132 on Brilliance™ ESBL agar (91 chicken, 41 pork), 7 on CAMPY agar (7 chicken, 0 pork), and 91 on MAC/XLD agar (65 chicken, 26 pork). None of the isolates that produced colonies on Brilliance™ VRE agar and were presumed to be *Enterococcus*, according to WGS identification, were indeed *Enterococcus*. All isolates from Brilliance™ VRE were Gram-negative bacteria, which vancomycin is not active against. None of the isolates that grew in selenite broth, and later on XLD, were *Salmonella*. All isolates from XLD belonged to the closely related genus *Hafnia*.

Antimicrobial resistance phenotyping

The 288 isolates that underwent WGS were tested for antibiotic sensitivity using an automated minimum inhibitory concentration (MIC) broth microdilution method and commercially available

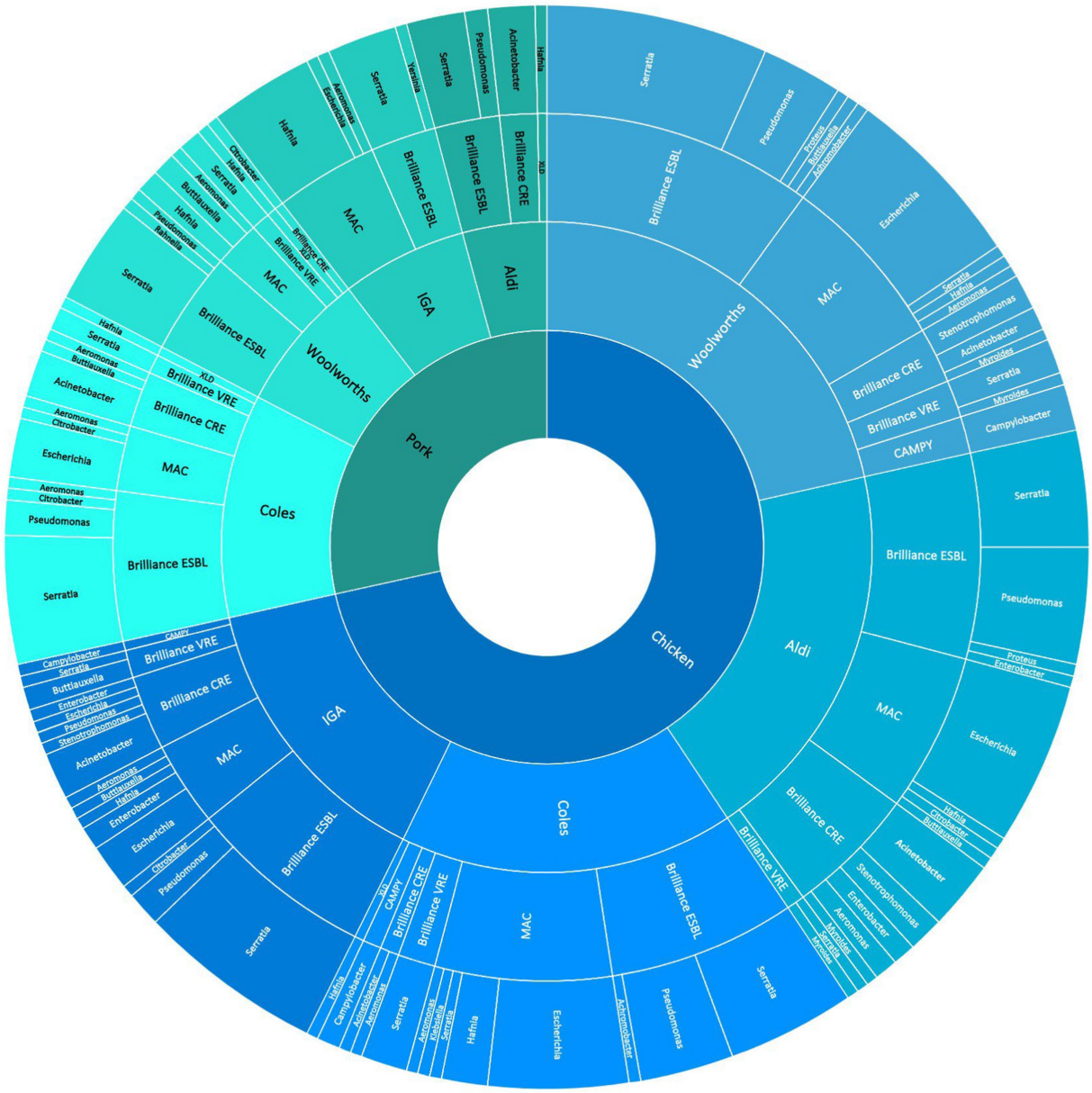

FIGURE 1
Sunburst diagram shows the breakdown of meat samples (innermost ring) across supermarket chains from 39.5 electorates in the ACT/NSW regions of Australia (second innermost ring), the abundance of isolates grown on various selective media (third innermost ring), and the abundance of bacterial genera grown on the media (outermost ring).

Gram-negative (CMV3AGNF) and *Campylobacter* (EUCAMP2) Sensititre™ antibiotic plates (Thermo Scientific™). According to World Health Organization (2022), each antibiotic on the list is either a critically important antibiotic (CIA) or a highly important antibiotic (HIA) for human health. Based on the chosen antibiotics, each isolate was evaluated to determine whether it was MDR, XDR, or PDR.

The AMR phenotype and MDR results for all 288 isolates that underwent WGS are provided in Table 2, for all pooled chicken and pork samples across all selective media used in the study. Of the 288 isolates, 35 (12%) were MDR, and of these, 17 were *Serratia* spp. that grew on Brilliance™ ESBL (15 chicken, 2 pork). The MDR criteria did not include antibiotics for which *Serratia* spp. are intrinsically resistant. The remaining 18 MDR isolates belonged to a variety of bacterial genera, including *Proteus* spp. (2/18, 11%), *Rahnella* spp. (1/18, 6%), *Yersinia* spp. (1/18, 6%), *Buttiauxella* spp. (2/18, 11%), *Citrobacter* spp. (2/18, 11%), *Aeromonas* spp. (3/18, 17%), *Acinetobacter* spp. (1/18, 6%), *Enterobacter* spp. (4/18, 22%), *Pseudomonas* spp. (1/18, 6%), and *Escherichia* spp. (1/18, 6%). All of the MDR bacteria were isolated from Brilliance™ ESBL (25/35, 71%), Brilliance™ CRE (9/35, 26%), or Brilliance™ VRE agar (1/35, 3%). No MDR isolate was cultured from either MAC or XLD. The frequency of MDR varied across bacterial isolates from chicken and pork samples and across supermarkets, with 28% (11/39), 1% (1/85), 15% (6/41), and 13% (10/79) of chicken isolates; and 3% (1/39), 10% (4/41), 0% (0/33), and 4% (2/47) of pork isolates being MDR from Aldi, Coles, IGA, and Woolworths, respectively.

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TABLE 1 Frequency of the 17 bacterial genera isolated from all pooled chicken and pork samples.

Organisms	Chicken	Pork
	Observed (n = 206, 71.53%)	Observed (n = 82, 28.47%)
Achromobacter spp.	2 (1.0%)	0 (0.0%)
Acinetobacter spp.	13 (6.3%)	8 (9.8%)
Aeromonas spp.	6 (2.9%)	5 (6.1%)
Buttiauxella spp.	5 (2.4%)	3 (3.7%)
Campylobacter spp.	7 (3.4%)	0 (0.0%)
Citrobacter spp.	2 (1.0%)	3 (3.7%)
Enterobacter spp.	6 (2.9%)	0 (0.0%)
Escherichia spp.	47 (22.8%)	6 (7.3%)
Hafnia spp.	8 (3.9%)	14 (17.1%)
Klebsiella spp.	1 (0.5%)	0 (0.0%)
Myroides spp.	4 (1.9%)	0 (0.0%)
Proteus spp.	2 (1.0%)	0 (0.0%)
Pseudomonas spp.	29 (14.1%)	6 (7.3%)
Rahnella spp.	0 (0.0%)	1 (1.2%)
Serratia spp.	67 (32.5%)	35 (42.7)
Stenotrophomonas spp.	7 (3.4%)	0 (0.0%)
Yersinia spp.	0 (0.0%)	1 (1.2%)

Of the MDR isolates cultured on Brillance™ ESBL agar, *Serratia* spp. from chicken meat had the highest rate of MDR (15/56, 27%), which was significantly higher than *Serratia* spp. isolated from pork samples (2/30, 6.67%), although pooled chicken samples were more likely to be comprised of more than one product. Ciprofloxacin resistance was found in one of the MDR pork strains. One *Buttiauxella* spp. isolate from the chicken was resistant to four CIAs (AMP, AUG2, AXO, and FOX) and one HIA (CHL, SXT, and TET). One *Citrobacter* spp. isolate from the chicken was resistant to four CIAs (AMP, AUG2, AXO, and FOX) and one HIA (CHL), while the *Citrobacter* spp. isolate from pork was resistant to three CIAs (AMP, AUG2, and FOX) and two HIAs (SXT and TET). One chicken meat-derived *Enterobacter* spp. isolate was resistant to four CIAs (AMP, AUG2, AXO, and FOX) and three HIAs (CHL, SXT, and TET). One of the two MDR *Proteus* isolates from the chicken was resistant to two CIAs (AMP and AXO) and two HIAs (CHL, TET), while the other was resistant to four CIAs (AUG2, AXO, GEN, and STR) and one HIA (CHL). One *Rahnella* spp. and one *Yersinia* spp. isolate, both from pork meat, were resistant to three CIAs (AUG2, AMP, and AXO). One *Pseudomonas* spp. isolate from chicken meat and two strains from pork meat were resistant to multiple antibiotics; however, when the Magiorakos et al. (2012) definition of MDR was applied to *P. aeruginosa*, none of them were classified as MDR.

A total of nine isolates that grew on Brilliance™ CRE were MDR; eight were from chicken samples and one from a pork sample. Strains isolated from chicken samples belonged to the following genera: *Acinetobacter* (1/13, 7.7%), *Aeromonas* (2/3, 66.7%), *Enterobacter* (3/3, 100%), *E. coli* (1/1, 100%), and *Pseudomonas* (1/1, 100%). The MDR pork isolate was from *Aeromonas* spp. Several MDR isolates from Brilliance™ CRE were resistant to ciprofloxacin. One *Enterobacter* spp. isolate was highly MDR, as it was resistant to six CIAs (AUG2, AMP, FOX, AXO, CIP, and GEN) and one HIA (SXT), including ciprofloxacin. A strain of *E. coli* was resistant to five CIAs (AZI, AXO, CIP, NAL, and STR) and two HIAs (XNL and FIS), including ciprofloxacin. In addition, two *Acinetobacter* spp. isolates and one *Pseudomonas* spp. isolate were ciprofloxacin-resistant and MDR. Of the three *Enterobacter* spp. isolates from chicken meat that were MDR, one was resistant to ciprofloxacin. One *Aeromonas* spp. isolate from a pork sample was ciprofloxacin-resistant and MDR.

Of the 17 isolates that grew on Brilliance™ VRE agar, one *Buttiauxella* spp. isolate from a chicken sample displayed MDR. No isolates grown on MAC or XLD were MDR. Of the seven *Campylobacter* spp. isolates that grew on CAMPY agar, one displayed tetracycline resistance, but none were deemed MDR. No *Campylobacter* spp. were isolated from pork samples.

Distribution of antimicrobial resistance genes

AMR genes were detected in the genomes of the 288 isolates using MobileElementFinder,[3] a database for the identification of horizontally acquired AMR genes, virulence genes, and mobile genetic elements. Using a detection threshold of 95%, we found that 232/288 (81%) of the isolates carried at least one resistance gene (Table 3). AMR genes detected in these 232 isolates confer resistance to aminoglycosides, amphenicols, β-lactams, colistin, fosfomycin, hydrogen peroxide, olaquindox, quinolones, sulphonamides, tetracyclines, and trimethoprim. A full outline of the AMR genes for each strain is provided in Supplementary Table S1.

Aminoglycoside resistance genes

A total of 6/91 (7%) isolates from Brilliance™ ESBL agar from chicken meat and 1/41 (2%) from pork were found to contain aminoglycoside resistance genes. Among the chicken isolates, one *Proteus* spp. isolate (1/2, 50%) carried the *aadA1* gene, and 5/28 (18%) of *Pseudomonas* spp. carried the *aph(3′)-IIb* gene. One (17%) pork *Pseudomonas* spp. isolate carried the *aph(3′)-Ib* gene. A total of four (13%) chicken isolates from Brilliance™ CRE agar harboured aminoglycoside resistance genes: two *Stenotrophomonas* spp. isolates carried *aph(3′)-IIC*, one carried *aph(3′)-IIC* and *aac(6′)-Iz*, and another carried *aph(3′)-IIC* and *aadA5*. None of the pork isolates from Brilliance™ CRE agar nor any isolates from Brilliance™ VRE agar (both chicken and pork) carried aminoglycoside resistance genes. Of the isolates that grew on MAC, 3/46 (7%) *Escherichia* spp. isolates from chicken carried both *aph(3″)-Ib* and *aph(6)-Id*, and one isolate from pork carried the *aadA1* gene.

β-lactamase resistance genes

Of the isolates obtained from Brilliance™ ESBL agar, 59/91 (65%) from chicken and 26/41 (63%) from pork harboured genes

3 https://cge.food.dtu.dk/services/MobileElementFinder/

TABLE 2 Antibiotic resistance phenotype and multiple drug resistance results for bacterial genera isolated from various media for all pooled chicken and pork samples.

Media	Meat sample	Bacterial genus	AMP	AUG2	AXO	AZI	CHL	CIP	ERY	FIS	FOX	GEN	NAL	STR	SXT	TET	XNL	MDR *n* (%)
			1–32mg/mL	0.5/1.16–32 mg/mL	0.25–64 mg/mL	0.12–16 mg/mL	2–32 mg/mL	0.015–4mg/mL	1–128mg/mL	16–256 mg/mL	0.5–32 mg/mL	0.25–16 mg/mL	0.5–32 mg/mL	2–64mg/mL	2–64mg/mL	4–32 mg/mL	0.12–8mg/mL	
Brilliance™ ESBL	Chicken (*n* = 91)	*Achromobacter* spp., *n* = 2 (%)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Buttiauxella spp., *n* = 1 (%)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	**1 (100)**
		Citrobacter spp., *n* = 1 (%)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**1 (100)**
		Enterobacter spp., *n* = 1 (%)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	**1 (100)**
		Proteus spp., *n* = 2 (%)	1 (100)	1 (50)	2 (100)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	**2 (100)**
		Pseudomonas spp., *n* = 28 (%)	0 (0)	0 (0)	3 (10.7)	0 (0)	25 (89.3)	1 (3.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.5)	1 (3.5)	16 (57.4)	0 (0)	1 (3.5)	**0 (0)**
		Serratia spp., *n* = 56 (%)	52 (92.8)	42 (75)	46 (82.1)	0 (0)	12 (21.4)	0 (0)	0 (0)	0 (0)	32 (57.1)	1 (1.8)	5 (8.9)	4 (7.1)	6 (10.7)	9 (16.1)	1 (1.8)	**15 (27)**
	Pork (*n* = 41)	*Aeromonas* spp., *n* = 1 (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	**0 (0)**
		Citrobacter spp., *n* = 1 (%)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	**1 (100)**
		Pseudomonas spp., *n* = 6 (%)	0 (0)	0 (0)	2 (33.3)	0 (0)	2 (33.3)	2 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (66.6)	2 (33.3)	0 (0)	**0 (0)**
		Rahnella spp., *n* = 1 (%)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**1 (100)**
		Serratia spp., *n* = 31 (%)	25 (80.6)	21 (67.7)	25 (80.6)	0 (0)	0 (0)	1 (3.2)	0 (0)	0 (0)	18 (58.1)	0 (0)	2 (6.4)	2 (6.4)	0 (0)	2 (6.4)	2 (6.4)	**2 (6.5)**
		Yersinia spp., *n* = 1 (%)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**1 (100)**
Brilliance™ CRE	Chicken (*n* = 30)	*Acinetobacter* spp., *n* = 13 (%)	0 (0)	0 (0)	5 (38.5)	0 (0)	0 (0)	2 (15.4)	0 (0)	0 (0)	1 (7.7)	3 (23.1)	0 (0)	2 (15.4)	1 (7.7)	0 (0)	1 (7.7)	**1 (7.7)**
		Aeromonas spp., *n* = 3 (%)	0 (0)	0 (0)	1 (33.3)	1 (33.3)	1 (33.3)	0 (0)	0 (0)	0 (0)	1 (33.3)	2 (66.7)	0 (0)	1 (33.3)	0 (0)	3 (100)	1 (33.3)	**2 (66.7)**
		Enterobacter spp., *n* = 3 (%)	3 (100)	3 (100)	1 (33.3)	0 (0)	0 (0)	1 (33.3)	0 (0)	0 (0)	3 (100)	1 (33.3)	0 (0)	1 (33.3)	1 (33.3)	1 (33.3)	0 (0)	**3 (100)**
		Escherichia spp., *n* = 1 (%)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	**1 (100)**
		Myroides spp., *n* = 2 (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Pseudomonas spp., *n* = 1 (%)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	**1 (100)**
		Stenotrophomonas spp., *n* = 7 (%)	0 (0)	0 (0)	0 (0)	0 (0)	3 (42.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (42.9)	0 (0)	0 (0)	**0 (0)**
	Pork (*n* = 11)	*Acinetobacter* spp., *n* = 8 (%)	0 (0)	0 (0)	1 (12.5)	0 (0)	1 (14.3)	4 (62.5)	0 (0)	0 (0)	0 (0)	2 (37.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Aeromonas spp., *n* = 1 (%)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	**1 (100)**
		Buttiauxella spp., *n* = 1 (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Citrobacter spp., *n* = 1 (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
Brilliance™ VRE	Chicken (*n* = 13)	*Buttiauxella* spp., *n* = 2 (%)	1 (50)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	**1 (50)**
		Myroides spp., *n* = 2 (%)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	2 (100)	0 (0)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Serratia spp., *n* = 9 (%)	6 (66.7)	5 (55.5)	3 (33.3)	1 (11.1)	1 (11.1)	0 (0)	0 (0)	0 (0)	3 (33.3)	0 (0)	0 (0)	0 (0)	1 (11.1)	1 (11.1)	1 (16.7)	**0 (0)**
	Pork (*n* = 4)	Serratia sp., *n* = 4 (%)	2 (50)	2 (50)	2 (50)	0 (0)	3 (75)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**

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TABLE 2 (Continued)

Media	Meat sample	Bacterial genus	AMP	AUG2	AXO	AZI	CHL	CIP	ERY	FIS	FOX	GEN	NAL	STR	SXT	TET	XNL	MDR *n* (%)
			1–32mg/mL	0.5/1.16–32 mg/mL	0.25–64 mg/mL	0.12–16 mg/mL	2–32 mg/mL	0.015–4mg/mL	1–128mg/mL	16–256 mg/mL	0.5–32 mg/mL	0.25–16 mg/mL	0.5–32 mg/mL	2–64mg/mL	2–64mg/mL	4–32 mg/mL	0.12–8mg/mL	
MAC/XLD	Chicken (*n* = 65)	*Aeromonas* spp., *n* = 3 (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (33.3)	0 (0)	0 (0)	**0 (0)**
		Buttiauxella spp., *n* = 2 (%)	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Citrobacter spp., *n* = 1 (%)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Enterobacter spp., *n* = 2 (%)	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Escherichia spp., *n* = 46 (%)	2 (4.3)	3 (6.5)	46 (100)	0 (0)	46 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.2)	6 (13)	0 (0)	**0 (0)**
		Hafnia spp., *n* = 8 (%)	2 (25)	0 (0)	6 (75)	0 (0)	6 (75)	6 (75)	0 (0)	0 (0)	6 (75)	6 (75)	0 (0)	0 (0)	6 (75)	6 (75)	0 (0)	**0 (0)**
		Klebsiella spp., *n* = 1 (%)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Serratia spp., *n* = 2 (%)	1 (50)	0 (0)	0 (0)	1 (50)	1 (50)	1 (50)	0 (0)	1 (50)	1 (50)	1 (50)	1 (50)	0 (0)	2 (100)	2 (100)	0 (0)	**0 (0)**
	Pork (*n* = 26)	*Aeromonas* spp., *n* = 3 (%)	1 (33.3)	1 (33.3)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Buttiauxella spp., *n* = 2 (%)	2 (100)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Citrobacter spp., *n* = 1 (%)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Escherichia spp., *n* = 6 (%)	1 (16.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
		Hafnia spp., *n* = 14 (%)	3 (21.4)	4 (28.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**0 (0)**
CAM	Chicken (*n* = 7)	*Campylobacter* spp., *n* = 7 (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (16.7)	0 (0)	**0 (0)**

These data are pivotal for comprehending the antibiotic resistance profiles exhibited by bacterial isolates derived from the pooled chicken and pork samples. The table represents the AMR phenotype and MDR (35/288, 12%) for each isolate at the genus level. These isolates were cultured on selective media, including Brilliance™ ESBL, Brilliance™ CRE, Brilliance™ VRE agar, CAMPY, MAC, and XLD, from pooled chicken and pork samples. Specifically, for Gram-negative bacteria (CMV3AGNF Sensititre, Thermo Scientific™) 14 antibiotics comprising 9 CIAs and 5 HIAs, and for Campylobacter (EUCAMP2 Sensititre, Thermo Scientific™), 6 antibiotics comprising 5 CIAs and 1 HIA were tested against antibiotics at concentrations recommended by World Health Organization (2022). Additional details regarding selected antibiotics are provided in Supplementary Table S1.

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TABLE 3 Distribution of antimicrobial resistance genes found in bacterial isolates from pooled chicken and pork samples.

Antibiotics	Media	Genus	Resistance genes	Chicken (*n* = 206) Observed/total (%)	Pork (*n* = 82) Observed/total (%)
Aminoglycoside	Brilliance™ ESBL	*Proteus* spp.	*aadA1*	1/2 (50.0)	0/0 (0.0)
		Pseudomonas spp.	*aph(3′)-Ib, aph(6)-Id*	0/0 (0.0)	1/6 (16.7)
			aph(3′)-llb	5/28 (17.9)	1/6 (16.7)
	Brilliance™ CRE	*Stenotrophomonas* spp.	*aac(6′)-lz, aadA5*	1/7 (14.3)	0/0 (0.0)
			aph(3′)-IIC	3/7 (42.9)	0/0 (0.0)
	MAC/XLD	*Escherichia* spp.	*aadA1*	0/0 (0.0)	1/6 (16.7)
			aph(3″)-lb, aph(6)-Id	3/46 (6.5)	0/0 (0.0)
Amphenicols	Brilliance™ ESBL	*Pseudomonas* spp.	*catB7*	5/28 (17.9)	1/6 (16.7)
	MAC/XLD	*Escherichia* spp.	*catB7*	0/0 (0.0)	1/6 (16.7)
β-lactamase	Brilliance™ ESBL	*Achromobacter* spp.	*blaL1*	1/2 (50.0)	0/0 (0.0)
		Aeromonas spp.	*ampS, cphA4*	0/0 (0.0)	1/1 (100.0)
		Buttiauxella spp.	*qacE*	1/1 (100.0)	0/0 (0.0)
		Citrobacter spp.	*blaCMY-101*	0/0 (0.0)	1/1 (100.0)
			blaCMY-82	1/1 (100.0)	0/0 (0.0)
		Pseudomonas spp.	*blaOXA-485, blaOXA-488*	0/0 (0.0)	1/6 (16.7)
			blaOXA-494	2/28 (7.15)	0/0 (0.0)
			POM-1	2/28 (7.15)	3/6 (50.0)
			qacE	3/28 (10.7)	1/31 (3.2)
			blaOXA-50, blaOXA-396	5/28 (17.9)	0/0 (0.0)
			blaPAO	5/28 (17.9)	1/6 (16.7)
		Rahnella spp.	*blaRAHN-2*	0/0 (0.0)	1/1 (100.0)
		Serratia spp.	*blaFONA-1*	0/0 (0.0)	2/31 (6.4)
			blaFONA-4	1/56 (1.8)	1/31 (3.2)
			blaFONA-2	1/56 (1.8)	3/31 (9.7)
			blaFONA-5	3/56 (5.3)	5/31 (16.1)
			blaFONA-6	50/56 (89.3)	19/31 (61.3)
		Yersinia spp.	*blaFONA-6*	0/0 (0.0)	1/1 (100.0)
	Brilliance™ CRE	*Acinetobacter* spp.	*blaOXA-67*	0/0 (0.0)	4/8 (50)
			blaMUS-1, blaOXA-117, blaOXA-120, blaOXA-355, blaOXA-98	1/13 (7.7)	0/0 (0.0)
			blaOXA-64	2/13 (15.4)	0/0 (0.0)
			blaOXA-51	4/13 (30.8)	0/0 (0.0)
			blaADC-25	9/13 (69.2)	4/8 (50)
		Aeromonas spp.	*blaFONA-1*	0/0 (0.0)	1/1 (100)
			ampS, blaCEPH-A3, blaFONA-2, blaRAHN-2	1/3 (33.3)	0/0 (0.0)
			cphA5	1/3 (33.3)	1/1 (100)
		Citrobacter spp.	*blaACC-3*	0/0 (0.0)	1/1 (100)
		Enterobacter spp.	*blaACT-4*	2/3 (66.7)	0/0 (0.0)
		Myroides spp.	*blaMUS-1*	2/2 (100)	0/0 (0.0)
		Stenotrophomonas spp.	*blaL1, qacE*	1/7 (14.3)	0/0 (0.0)

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TABLE 3 (Continued)

Antibiotics	Media	Genus	Resistance genes	Chicken (*n* = 206) Observed/total (%)	Pork (*n* = 82) Observed/total (%)
	Brilliance™ VRE	*Buttiauxella* spp.	*blaFONA-6*	1/2 (50)	0/0 (0.0)
		Serratia spp.	*blaFONA-5*	2/9 (22.2)	0/0 (0.0)
			blaFONA-6	9/9 (100)	1/4 (25)
	MAC/XLD	*Aeromonas* spp.	*ampS*, *blaTEM-1B*	0/0 (0.0)	1/3 (33.3)
			cphA4	2/3 (66.7)	0/0 (0.0)
		Citrobacter spp.	*blaCMY-89*	1/1 (100)	0/0 (0.0)
		Escherichia spp.	*blaSHV-56*, *qacE*, *blaTEM-1B*	0/0 (0.0)	1/6 (16.7)
			cphA5	1/46 (2.2)	0/0 (0.0)
		Hafnia spp.	*blaACC-3*	0/0 (0.0)	1/14 (7.1)
			blaACC-1	1/8 (12.5)	0/0 (0.0)
			blaACC-1a	1/8 (12.5)	11/14 (78.6)
			blaACC-5, *blaCMY-105*	2/8 (25)	0/0 (0.0)
			blaACC-1b	6/8 (75.0)	1/14 (7.1)
		Klebsiella spp.	*blaSHV-40*, *blaSHV-56*, *blaSHV-79*, *blaSHV-85*, *blaSHV-89*	1/1 (100)	0/0 (0.0)
		Serratia spp.	*blaFONA-6*	2/2 (100)	0/0 (0.0)
	CAMPY	*Campylobacter* spp.	*blaOXA-193*, *blaOXA-450*, *blaOXA-451*, *blaOXA-452*, *blaOXA-453*, *blaOXA-489*, *blaOXA-61*, *blaTEM-116*	4/7 (66.7)	0/0 (0.0)
Colistin	Brilliance™ ESBL	*Achromobacter* spp.	*mcr-5.1*	1/1 (100.0)	0/0 (0.0)
		Serratia spp.	*mcr-9*	0/0 (0.0)	1/31 (3.2)
	MAC/XLD	*Aeromonas* spp.	*mcr-3.15*	1/3 (33.3)	0/0 (0.0)
Formaldehyde	MAC/XLD	*Escherichia* spp.	*formA*	1/46 (2.2)	0/0 (0.0)
Fosfomycin	Brilliance™ ESBL	*Pseudomonas* spp.	*fosA*	5/28 (17.9)	1/6 (16.7)
	Brilliance™ CRE	*Enterobacter* spp.	*fosA*	2/3 (66.6)	0/0 (0.0)
	MAC/XLD	*Klebsiella* spp.	*fosA*	1/1 (100)	0/0 (0.0)
Hydrogen peroxide	Brilliance™ CRE	*Escherichia* spp.	*sitABCD*	1/1 (100)	0/0 (0.0)
	Brilliance™ VRE	*Myroides* spp.	*sitABCD*	2/2 (100)	0/0 (0.0)
	MAC/XLD	*Escherichia* spp.	sitABCD	28/46 (60.9)	2/6 (33.3)
Olaquindox	MAC/XLD	*Klebsiella* spp.	*OqxA*, *OqxB*	1/1 (100)	0/0 (0.0)
Quinolones	Brilliance™ ESBL	*Citrobacter* spp.	*qnrB72*	1/1 (100.0)	0/0 (0.0)
	MAC/XLD	*Citrobacter* spp.	*qnrB60*	1/1 (100)	0/0 (0.0)
Sulphonamide	Brilliance™ ESBL	*Pseudomonas* spp.	*sul1*	0/0 (0.0)	1/6 (16.7)
			crpP	3/28 (10.7)	1/6 (16.7)
	Brilliance™ CRE	*Stenotrophomonas* spp.	*sul1*	1/7 (14.3)	0/0 (0.0)
	MAC/XLD	*Aeromonas* spp.	*sul1*	1/3 (33.3)	0/0 (0.0)
		Escherichia spp.	*sul2*	3/46 (6.5)	0/0 (0.0)

(Continued)

TABLE 3 (Continued)

Antibiotics	Media	Genus	Resistance genes	Chicken (*n* = 206) Observed/total (%)	Pork (*n* = 82) Observed/total (%)
Tetracycline	Brilliance™ ESBL	*Aeromonas* spp.	*tet(E)*	0/0 (0.0)	1/6 (16.7)
	Brilliance™ CRE	*Aeromonas* spp.	*tet(E)*	1/3 (33.3)	0/0 (0.0)
		Stenotrophomonas spp.	*tet(A)*	1/7 (14.3)	0/0 (0.0)
	Brilliance™ VRE	*Serratia* spp.	*tet(A)*	1/9 (11.1)	2/4 (50)
	MAC/XLD	*Aeromonas* spp.	*tet(E)*	1/3 (33.3)	1/3 (33.3)
		Escherichia spp.	*tet(A)*	7/46 (15.2)	2/6 (33.3)
	CAMPY	*Campylobacter* spp.	*tet(O)*	1/7 (16.6)	0/0 (0.0)
Trimethoprim	MAC/XLD	*Escherichia* spp.	*dfrA5*, *dfrA14*	1/46 (2.2)	0/0 (0.0)

These data represent the antibiotic resistance genes detected in bacterial isolates, categorising them across 12 distinct antibiotic classes. Notably, during AST, five of these classes were scrutinised. Among these, three were CIAs: aminoglycoside, β-lactamase, and quinolone, while two were HIAs: sulphonamide and tetracycline.

conferring resistance to β-lactam antibiotics. Of the chicken isolates, one *Achromobacter* spp. carried *blaL1*, one *Buttiauxella* spp. carried *qacE*, and one *Citrobacter* spp. carried *blaCMY-82*. A total of 11/28 (39%) *Pseudomonas* spp. carried β-lactamase resistance genes; two carried multiple genes (*blaOXA-494*, *blaOXA-50*, *blaOXA-396*, and *blaPAO*); another two carried only *POM-1*; three carried *blaOXA-50* and *blaPAO*; and four isolates carried only *qacE*. Of the *Serratia* spp. isolates, 55/56 (98%) carried a *blaFONA* gene variant, with variant *blaFONA-6* being the most common ($n = 50$). Among the pork isolates, one *Aeromonas* spp. carried *ampS* and *cphA4*, one *Citrobacter* spp. carried *blaCMY-101*, three *Pseudomonas* spp. carried *POM-1* only, and another *Pseudomonas* spp. isolate carried *blaPAO*, *blaOXA-485*, and *blaOXA-488*. One *Rahnella* spp. carried *blaRAHN-2*, and 30/31 (97%) *Serratia* spp. carried a *blaFONA* variant.

Of the isolates that grew on Brilliance™ CRE agar, 15/30 (50%) of the chicken isolates and 6/11 (55%) of the pork isolates carried a β-lactamase resistance gene. Among the chicken isolates, 11/13 (85%) *Acinetobacter* spp. carried various genes (*blaMUS-1*, *blaADC-25*, and *blaOXA* variants). Three *Aeromonas* spp., two *Enterobacter* spp., two *Myroides* spp., and one *Stenotrophomonas* spp. also carried various genes (*ampS*, *blaCEPH*, *balRAHN*, *cph*, *blaACT*, *blaMUS*, *blaL1*, *qacE*, and *blaFONA* variants). Among the pork isolates, four *Acinetobacter* spp., one *Aeromonas* spp., and one *Citrobacter* spp. carried β-lactamase resistance genes (*blaOXA*, *blaADC*, *cph*, *blaACC*, and *blaFONA-1*). Of the isolates that grew on Brilliance™ VRE agar, 12/13 (92%) of chicken and 3/4 (75%) of pork isolates carried β-lactamase resistance genes, with *blaFONA* variants being most common, particularly in *Serratia* spp.

Notably, isolates from MAC and XLD agar did not grow on Brilliance™ ESBL agar but were found to harbour β-lactamase resistance genes. These genes were identified in various species, including *Aeromonas* spp., *Citrobacter* spp., *Escherichia* spp., *Hafnia* spp., *Klebsiella* spp., and *Serratia* spp., in both chicken and pork samples. Of the *Campylobacter* isolates grown on CAMPY agar from chicken samples, 6/7 (86%, one *E. coli*, five *C. jejuni*) carried β-lactamase resistance genes, mostly *blaOXA* variants. One *C. jejuni* isolate harboured *blaTEM-116*.

Quinolone resistance genes

A single *Citrobacter* spp. chicken isolate from Brilliance™ ESBL harboured *qnrB72*. Two *Enterobacter* spp. chicken isolates from Brilliance™ CRE carried *qnrE1*. No pork samples carried quinolone resistance genes. One *Citrobacter* spp. chicken isolate from MAC carried *qnrB60*. No pork isolates harboured a quinolone resistance gene.

Sulphonamide resistance genes

Three chicken and one pork isolate from Brilliance™ ESBL agar harboured a sulphonamide resistance gene. All of these isolates were *Pseudomonas* spp., with chicken isolates carrying the *crpP* gene and the pork isolate carrying both *crpP* and *sul1*. One *Stenotrophomonas* spp. chicken isolate from Brilliance™ CRE agar carried *sul1*. Of the MAC isolates, one *Aeromonas* spp. carried *sul1*, and three *Escherichia* spp. carried *sul2*.

Tetracycline resistance genes

A single *Aeromonas* spp. pork isolate from Brilliance™ ESBL carried *tet(E)*. Of the isolates obtained from Brilliance™ CRE agar, one *Aeromonas* spp. chicken isolate carried *tet(E)* and one *Stenotrophomonas* spp. carried *tet(A)*. Of the Brilliance™ VRE isolates, one *Serratia* spp. chicken isolate carried *tet(A)*, and two *Serratia* spp. pork isolates also harboured *tet(A)*. The majority of tetracycline resistance genes were detected in isolates from MAC/XLD agar. This included 7/46 (15%) *Escherichia* spp. from chicken samples and 2/6 (33%) *Escherichia* spp. from pork, which carried *tet(A)*. One *Aeromonas* spp. isolate from chicken and one from pork carried the *tet(E)* gene. A single strain of *C. jejuni* from CAMPY agar carried the *tet(O)*.

Polymixin (colistin) resistance genes

Three isolates in this study were found to harbour a mobile colistin resistance (*mcr*) gene. One was a *Serratia* strain that was isolated on *Brilliance*™ ESBL agar and harboured the *mcr-9* variant. This strain also harboured the IncHI2 plasmid, which is known to be capable of

carrying *mcr* genes, as well as a β-lactam resistance gene (*bla*FONA-6) and a gene conferring resistance to antiseptics (*qacE*). An *Aeromonas* strain, isolated from MacConkey agar, harboured the *mcr-3.15* variant and was detected in the same section of DNA (contig) as transposon Tn4671 and insertion sequence ISAs17, suggesting that these genetic elements may have played a role in the acquisition of *mcr-3.15*. This strain also harboured the tetracycline resistance gene, *tet(E)*. The third strain harbouring an *mcr* gene grew on *Brilliance*™ ESBL agar and was classified as *Achromobacter*. It harboured *mcr-5.1*, a contig containing the insertion sequence ISRme15, and *blaL1*.

Resistance genes associated with other antimicrobials

Resistance genes associated with various other antimicrobials were identified. A fosfomycin resistance gene, *fosA*, was detected in eight different isolates, including five *Pseudomonas* spp., two *Enterobacter* spp., and one *Klebsiella* spp. A single *Escherichia* spp. isolate from MAC/XLD agar was identified as carrying the formaldehyde resistance gene, *formA*, and the hydrogen peroxide resistance and metal transporter gene *sitABCD* was present in 28/46 (61%) of *Escherichia* spp. A small number of strains from MAC/XLD carried genes conferring resistance to amphenicols, olaquindox, and trimethoprim.

Distribution of plasmids

A large number of plasmids were detected across the 288 isolates (Supplementary Table S2). A list of plasmids (and resistance genes) associated with the MDR isolates is provided in Table 4.

Of the MDR isolates, 13/35 (37%) carried one or more plasmids. Nine of these isolates were *Serratia* spp., and all except one strain carried the ColE10 plasmid. Other plasmids in these strains included IncHI1A, IncHI1B, IncN2, Col4401, ColRAAI, and ColYe4449. One *Citrobacter* spp. carried the IncFIB(pB171) plasmid, and another *Citrobacter* spp. carried the Col(Ye4449) plasmid. One *Escherichia* spp. carried the IncFIB(AP001918) and IncFII plasmids.

Distribution of virulence factors

One or more virulence genes were present in 61/288 (21%) of the isolates (Table 5). The vast majority of virulence factors were detected in *E. coli*, likely owing to the large number of virulence factors associated with this species in the VirulenceFinder database. All of the *E. coli* isolates in the study, apart from one isolate from pork, were found to harbour multiple virulence genes, ranging from 3 to 31 genes. Of note was the MDR *E. coli* isolate from Brilliance™ CRE agar, which had 17 virulence genes, nine of which were encoded on a plasmid (*etsC*, *iroN*, *cia*, *hlyF*, *cvaC*, *mchF*, *traT*, *ompT*, and *iss*), and the remainder elsewhere in the genome (*sitA*, *gad*, *terC*, *eilA*, *air*, *hlyE*, and *chuA*). All of the *E. coli* isolates (53/53, 100%) carried *terC*, consistent with other studies (Byarugaba et al., 2023). The next most frequent virulence factor was *traT* (37/46, 80%). Other common virulence factors included *chuA*, *cia*, *cvaC*, *etsC*, *fyuA*, *gad*, *hlyE*, *hlyF*, *hra*, *ipfA*, *ireA*, *iroN*, *irp2*, *iss*, *iucC*, *iutA*, *ompT*, and *sitA*.

Pathogenicity of bacterial isolates

To determine the likelihood that an isolate is pathogenic to humans, the PathogenFinder tool from the CGE database was employed.[4] Of the 288 bacterial isolates, 233 (81%) were determined to be pathogenic to humans, with probabilities ranging from 0.563 to 0.92. *Campylobacter* spp. and *Escherichia coli* exhibited notably high pathogenicity, with an average pathogenicity score of 0.91 and 0.87, respectively. In contrast, *Hafnia* spp. had the lowest pathogenicity, averaging 0.59. *Acinetobacter* spp. possessed the highest abundance of pathogenic protein families, totalling 402.2 on average. In contrast, *Hafnia* spp. exhibited the lowest number of pathogenic protein families, containing only 22.8 on average.

Discussion

In this study, we describe the phenotypic and sequence-based AMR profiles of bacteria isolated from chicken and pork meat. Since only one representative for each colony morphology and colour was chosen from a limited number of selective agars, the total number and diversity of bacterial isolates present in the chicken and pork meat samples are likely to have been underestimated. Preferential selection of 288 isolates resulted in the detection of 17 bacterial genera or 33 bacterial species. Of the 288 isolates, 12% were MDR. By assessing the AMR phenotypic and genotypic profiles of species other than those typically used in surveillance studies, we were able to show that the reservoir of antimicrobial resistance to critically and highly important antibiotics in bacteria isolated from retail meat in Australia is more diverse than previously demonstrated.

In this study, we detected three mobilised colistin resistance (*mcr*) genes, conferring resistance to a last-line antimicrobial (colistin) for multidrug-resistant Gram-negative infections. These genes are capable of transmitting to different genera and species of bacteria that colonise humans and animals or are present in the environment. While Australia is thought to have a low abundance of colistin resistance, a recent analysis of host-derived and environmental metagenomes revealed that the highest log-ratio abundances of *mcr* fragments could be found in metagenomes from Australia when compared to countries all over the world (Martiny et al., 2022). This study showed that the *mcr*-9 variant is most common in Australia and that *mcr* variants are not equally distributed among different countries or bacterial genus/species. Surveillance studies of common foodborne pathogens would not have detected the presence of *mcr* variants in this study, as they were carried by bacteria not normally included in such studies, such as *Aeromonas*, *Achromobacter*, and *Serratia*.

The detection of multiple variants of *mcr* (variants −3.15, −5.1, and − 9) across different species of bacteria, harbouring other AMR genes on MGEs known to transmit *mcr* genes suggests that the distribution of *mcr* genes in Australia is likely to increase. Co-selection of *mcr* genes with AMR genes conferring resistance to antibiotics that are permitted for use in the agri-food industry in Australia, such as tetracycline, may accelerate the spread of colistin resistance. Although *Serratia* are known to be intrinsically resistant

4 https://cge.food.dtu.dk/services/PathogenFinder/

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TABLE 4 A list of resistance genes and plasmids detected in the multidrug-resistant isolates.

Bacterial genus/sp.	Meat type	Media	Antibiotic resistance gene(s)	Plasmid(s)
Enterobacter sp.	Chicken	CRE		
Escherichia coli	Chicken	CRE	*sit*ABCD	IncFIB(AP001918), IncFII
Pseudomonas sp.	Chicken	CRE		
Acinetobacter baumanii	Chicken	CRE	*bla*ADC-25, *bla*OXA-51	
Enterobacter asburiae	Chicken	CRE	*bla*ACT-4, *qnrE1*, *fosA*	
Enterobacter asburiae	Chicken	CRE	*bla*ACT-4, *qnrE1*, *fosA*	
Aeromonas veronii	Chicken	CRE	*ampS*, *bla*CEPH-A3, *tet(E)*	
Aeromonas sp.	Chicken	CRE	*ampS*, *bla*CEPH-A3, *tet(E)*	
Aeromonas veronii	Pork	CRE	*ampS*, *bla*CEPH-A4, *tet(E)*	
Serratia sp.	Pork	ESBL	*bla*FONA-6	IncHI1A, IncHI1B, ColE10
Serratia sp.	Pork	ESBL	*bla*FONA-6	ColE10
Serratia sp.	Chicken	ESBL	*bla*FONA-6	ColE10
Serratia sp.	Chicken	ESBL	*bla*FONA-6	
Serratia sp.	Chicken	ESBL	*bla*FONA-5	Col4401, ColE10
Serratia sp.	Chicken	ESBL	*bla*FONA-6	
Serratia sp.	Chicken	ESBL	*bla*FONA-2, *qacE*	
Serratia sp.	Chicken	ESBL	*bla*FONA-6	ColE10, ColE10
Serratia sp.	Chicken	ESBL	*bla*FONA-6	
Serratia sp.	Chicken	ESBL	*bla*FONA-6	ColE10, ColE10
Serratia sp.	Chicken	ESBL	*bla*FONA-6	ColE10, Col44011, ColRAAI, ColE10, IncN2
Serratia sp.	Chicken	ESBL		
Serratia sp.	Chicken	ESBL	*bla*FONA-6	
Serratia sp.	Chicken	ESBL	*bla*FONA-6	ColE10, ColE10
Serratia sp.	Chicken	ESBL	*bla*FONA-6	ColE10
Serratia sp.	Chicken	ESBL	*bla*FONA-6	
Serratia sp.	Chicken	ESBL	*bla*FONA-6	ColYe4449
Buttiauxella sp.	Chicken	ESBL	*qac*E	
Buttiauxella sp.	Pork	ESBL		
Citrobacter braakii	Pork	ESBL	*bla*CMY-101	Col(Ye4449)
Citrobacter sp.	Chicken	ESBL	*bla*CMY-82, *qnrB72*	IncFIB(pB171)
Citrobacter sp.	Chicken	ESBL	*bla*CMY-105, *bla*CMY-89, *qnrB60*	
Enterobacter	Chicken	ESBL		
Proteus sp.	Chicken	ESBL	*hug*A	
Rahnella sp.	Pork	ESBL	*bla*RAHN-2	
Yersinia sp.	Pork	ESBL	*bla*FONA-6	

to colistin (Bean et al., 2020), we showed that *Serratia* are capable of acquiring *mcr* genes on plasmids that can be transferred to other species of bacteria. A ban on the prophylactic use of antibiotics, similar to that adopted by EU countries, may provide protection against the spread of colistin resistance. It is known that removing an antibiotic for use in the agri-food industry in Australia can result in a dramatic decline in resistance to that antibiotic. For example, a steady and significant reduction in erythromycin resistance in *Campylobacter* and *Enterococcus* isolated from food-producing chickens has been observed since the reduction in the use of macrolides in the 1990s, owing to the introduction of *Mycoplasma* vaccines (Australian Chicken Meat Federation, 2022). Similarly, the frequency of resistance to quinupristin-dalfopristin in *Enterococcus faecium* also significantly declined from 54.5 to 6.1% following a ban on the use of virginamycin in chickens in Australia. Removal of all antibiotics for prophylactic use in Australia would provide the best strategy for halting the spread of resistance to CIAs that arises due to the co-selection of genes conferring antimicrobial resistance to antibiotics currently used for "prevention" and genes conferring resistance to CIAs on MGEs.

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TABLE 5 Distribution of virulence genes found in bacterial isolates from pooled chicken and pork samples.

Genus	Virulence genes	Chicken (*n* = 206) Observed/total (%)	Pork (*n* = 82) Observed/total (%)
Brilliance™ ESBL			
Citrobacter spp.	*traT*	1/1 (100)	1/1 (100)
Serratia spp.	*terC*	0/0 (0.0)	2/31 (6.45)
Brilliance™ CRE			
Escherichia spp.	*Air*, *chuA*, *cia*, *cvaC*, *eilA*, *etsC*, *gad*, *hylE*, *hlyF*, *iroN*, *iss*, *mchF*, *ompT*, *sitA*, *terC*, *traT*	1/1 (100)	
MAC/XLD			
Citrobacter spp.	*terC*	1/1 (100)	6/6 (100)
Enterobacter spp.	*terC*	2/2 (100)	0/0 (0.0)
Escherichia spp.	*afaA*, *afaB*, *afaC*, *afaE8*, *efa1*, *espJ*, *etpD*, *f17A*, *f17G*, *ibeA*, *mcbA*, *nleC*	1/46 (2.2)	0/0 (0.0)
	papC	1/46 (2.2)	1/6 (16.7)
	Cib, *kpsMII*, *papA_F48*	2/46 (4.3)	0/0 (0.0)
	Cba, *cif*, *eae*, *espA*, *espB*, *espF*, *nleB*, *papA_F11*, *tir*	3/46 (6.5)	0/0 (0.0)
	afaD, *celb*, *papA_F19*	4/46 (8.7)	0/0 (0.0)
	nleA	4/46 (8.7)	2/6 (33.3)
	kpsMIII_K96	5/46 (10.9)	0/0 (0.0)
	Air	7/46 (15.2)	2/6 (33.3)
	eilA, *iha*	8/46 (17.4)	2/6 (33.3)
	pic	9/46 (19.6)	0/0 (0.0)
	astA, *kpsMII_K1*	9/46 (19.6)	2/6 (33.3)
	usp	10/46 (21.7)	1/6 (16.7)
	vat	11/46 (23.9)	1/6 (16.7)
	cma	11/46 (23.9)	2/6 (33.3)
	yfcV, *cea*	12/46 (26.1)	1/6 (16.7)
	tsh	13/46 (28.3)	1/6 (16.7)
	mchF	14/46 (30.4)	3/6 (50)
	neuC	16/46 (34.8)	3/6 (50)
	PacC	17/46 (36.9)	3/6 (50)
	kpsE	18/46 (39.1)	1/6 (16.7)
	fyuA	20/46 (43.5)	0/0 (0.0)
	irp2	20/46 (43.5)	1/6 (16.7)
	cia, *ireA*, *ompT*	20/46 (43.5)	3/6 (50)
	ipfA	22/46 (47.8)	1/6 (16.7)
	hra	22/46 (47.8)	3/6 (50)
	cvaC	23/46 (50)	3/6 (50)
	chuA	26/46 (56.5)	3/6 (50)
	iroN	27/46 (58.7)	2/6 (33.3)
	hlyF, *iucC*, *iutA*	30/46 (65.2)	3/6 (50)
	etsC	31/46 (67.4)	1/6 (16.7)
	gad	33/46 (71.7)	6/6 (100)
	sitA	34/46 (73.9)	3/6 (50)
	hlyE	35/46 (76.1)	1/6 (16.7)
	traT	37/46 (80.4)	4/6 (66.7)
	iss	39/46 (84.8)	3/6 (50)
	terC	46/46 (100)	6/6 (100)
Klebsiella spp.	*iutA*, *traT*	1/1 (100)	0/0 (0.0)

The majority of the *Enterobacteriaceae* identified in this study belonged to the genus *Serratia*. Members of this genus were found to have acquired resistance genes for aminoglycosides, β-lactamases, fosfomycin, quinolones, amphenicols, and polypeptides, in a study by Sandner-Miranda et al. (2018). *Serratia* spp. isolates in this study harboured acquired resistance genes for colistin, β-lactamases, fosfomycin, and quinolones. *Serratia marcesensi*, which was previously thought to be a non-pathogenic environmental species, is responsible for a number of hospital-acquired infections, and MDR is already making infections with this species difficult to treat (Moradigaravand et al., 2016). It is largely unknown if other species of *Serratia* are contributing to AMR in *S. marcesens* and if AMR in the wider species poses a threat to human health as opportunistic pathogens.

The vast majority of virulence genes identified in this study were in *E. coli* isolates. However, genomic data for *E. coli* are more common than some of the lesser-known species identified, and therefore the virulence database is likely biased towards detecting virulence genes associated with *E. coli*. Several studies have shown that APEC and ExPEC virulence genes share similarities. Virulence genes such as *iss*, *iuA*, *ompT*, *papGII*, and *sfa* have been detected in zoonotic pathogens (Najafi et al., 2019). In this study, *papA*, *papC*, *usp.*, *kpsMII*, and *ibeA* were frequently detected in *E. coli*, similar to APEC isolates harbouring *pap*, *sfa*, *usp.*, *cnf1*, *kpsMTII*, *hlyA*, and *ibeA* virulence genes (Cunha et al., 2017). ExPEC-related virulence genes include *astA*, *cvaC*, *hra*, *hlyF*, *fyuA*, *ibeA*, *ireA*, *iss*, *ompT*, *papA*, *papC*, *papE*, *papF*, *tsh*, and *traT*, all of which were detected in this study and were previously found to be prevalent in *E. coli* isolated from chicken meat samples (Mitchell et al., 2015). One study performed a cluster analysis of *E. coli* isolated from UTIs, community-dwelling humans, meat, and meat production animals, which included data on the presence of eight ExPEC-related virulence genes (*kpsM II*, *papA*, *papC*, *iutA*, *sfaS*, *focG*, *afa*, *hlyD*) and AMR and found a strong association between the isolates from the various sources, suggesting that strains isolated from meat and meat production animals may be zoonotic pathogens (Jakobsen et al., 2010). The findings of the current study suggest that *E. coli* isolates from chicken meat may pose a zoonotic risk to humans given that most of the isolates (98%) were predicted to be pathogenic towards humans and that 47/53 (89%) of the *E. coli* isolates were isolated from chicken meat.

The level of MDR in this study was not particularly high relative to other countries (Collignon, 2015). However, given that we used selective media containing antibiotics, this figure does not accurately reflect the level of MDR in bacteria isolated from chicken and pork meat in Australia. This approach did allow us, however, to observe the diversity of MDR bacteria that grew on the various selective media. While few species identified in this study are commonly associated with infections in humans, most have been associated with infections in humans. For the most part, the AMR genes detected using WGS did not explain the resistance phenotypes observed on the selective agars or in the commercial antibiotic plates. This may be due to not all resistance genes being discovered, particularly for the less-well-studied species identified in this study, or the resistance mechanisms being chromosomally encoded, rather than acquired. Nonetheless, we identified a variety and diversity of bacteria that harboured horizontally acquired genes conferring resistance to either critically or highly important antibiotics. Some of these were MDR and are likely to be pathogenic to humans.

AMR is a worldwide issue, and its management calls for a "One Health" approach. The use of antibiotics in food-producing animals maintains antibiotic resistance mechanisms that make treatment of resistant bacterial infections in humans and animals difficult. Future surveillance studies should include analysis of a greater diversity of bacteria, to ensure the full diversity of AMR genes is revealed.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The article presents research on animals that do not require ethical approval for their study.

Author contributions

OD: Data curation, Formal analysis, Methodology, Writing – original draft, Investigation. MB: Methodology, Writing – review & editing. AP: Methodology, Writing – review & editing, Investigation. CO'B: Methodology, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1347597/full#supplementary-material

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Development of an *in vitro* biofilm model for the study of the impact of fluoroquinolones on sewer biofilm microbiota

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Sewer biofilms are likely to constitute hotspots for selecting and accumulating antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). This study aimed to optimize culture conditions to obtain *in vitro* biofilms, mimicking the biofilm collected in sewers, to study the impact of fluoroquinolones (FQs) on sewer biofilm microbiota. Biofilms were grown on coupons in CDC Biofilm Reactors®, continuously fed with nutrients and inoculum (1/100 diluted wastewater). Different culture conditions were tested: (i) initial inoculum: diluted wastewater with or without sewer biofilm, (ii) coupon material: concrete vs. polycarbonate, and (iii) time of culture: 7 versus 14days. This study found that the biomass was highest when *in vitro* biofilms were formed on concrete coupons. The biofilm taxonomic diversity was not affected by adding sewer biofilm to the initial inoculum nor by the coupon material. *Pseudomonadales, Burkholderiales* and *Enterobacterales* dominated in the sewer biofilm composition, whereas *in vitro* biofilms were mainly composed of *Enterobacterales*. The relative abundance of *qnrA, B, D* and *S* genes was higher in *in vitro* biofilms than sewer biofilm. The resistome of sewer biofilm showed the highest Shannon diversity index compared to wastewater and *in vitro* biofilms. A PCoA analysis showed differentiation of samples according to the nature of the sample, and a Procrustes analysis showed that the ARG changes observed were linked to changes in the microbial community. The following growing conditions were selected for *in vitro* biofilms: concrete coupons, initial inoculation with sewer biofilm, and a culture duration of 14days. Then, biofilms were established under high and low concentrations of FQs to validate our *in vitro* biofilm model. Fluoroquinolone exposure had no significant impact on the abundance of *qnr* genes, but high concentration exposure increased the proportion of mutations in *gyr*A (codons S83L and D87N) and *par*C (codon S80I). In conclusion, this study allowed the determination of the culture conditions to develop an *in vitro* model of sewer biofilm; and was successfully used to investigate the impact of FQs on sewer microbiota. In the future, this setup could be used to clarify the role of sewer biofilms in disseminating resistance to FQs in the environment.

KEYWORDS

wastewater, sewer, biofilm, fluoroquinolone, bioreactor, antibiotic resistance

1 Introduction

The first global impact analysis of antibiotic resistance estimated that antibiotic resistance caused 1.27 million deaths worldwide in 2019 (Murray et al., 2022). This study revealed the magnitude of antibiotic resistance's global threat to human health and proposed measures to save lives. Measures to combat antibiotic resistance are not limited to clinical and community settings. This global ecological problem is addressed through a "One Health" approach, which considers the interconnections between the human, animal and environmental spheres (Hernando-Amado et al., 2019).

The environment is a reservoir of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), either naturally occurring or introduced by the discharge of human and animal effluents. The environment is thought to be both a place of emergence and a route of dissemination of antibiotic resistance (Wellington et al., 2013; Bengtsson-Palme et al., 2018). For example, the quinolone resistance gene *qnrA*, commonly found in clinical enterobacteria isolates, would have been acquired by horizontal transfer from the aquatic species *Shewanella algae* (Poirel et al., 2005). In connection with human activities, the environment is also contaminated by a mixture of chemical agents, including antibiotics, biocides and heavy metals, which can exert selection pressure, enriching the antibiotic resistance reservoir (Buelow et al., 2021).

Wastewater treatment plants (WWTPs) are one of the main focal point for the emergence of resistance in pathogens because favorable conditions are found, including the presence of commensal, pathogenic, and environmental bacteria at high concentrations and cocktails of chemical agents, including antibiotics (Rizzo et al., 2013). Apart from effluents from pharmaceutical industries producing antibiotics, antibiotic residues are present at sub-inhibitory concentrations in wastewater due to antibiotic use in human and veterinary medicine (Chow et al., 2021). Several studies have shown that these sub-inhibitory concentrations can lead to antibiotic resistance selection (Andersson and Hughes, 2014; Sanchez-Cid et al., 2022). Precursor studies defined the minimal selective concentration (MSC) as the smallest concentration that allows the selection of the resistant mutant at the expense of the susceptible strain (Gullberg et al., 2011; Liu et al., 2011). Competition curves between a sensitive bacterium and its isogenic resistant mutant must be performed to determine MSCs. This approach has several limitations in the context of complex microbial communities, as it has been shown that the MSC assessed in a semi-natural microbial community (pig fecal flora) is higher than that assessed when the competition curves are performed without this microbial community (Klumper et al., 2019). Other authors have developed an approach to determine the lowest observed effect concentration (LOEC) on complex aquatic bacterial communities using phenotypic (culture-based methods) and genotypic (qPCR and metagenomic analyses) criteria (Lundstrom et al., 2016; Murray et al., 2018). Such studies aim to determine the predicted no-effect concentrations for resistance (PNEC-Rs) to assess the risk of antibiotic resistance selection in the environment (Murray et al., 2021).

Fluoroquinolones (FQs) are frequently detected in wastewater and environmental matrices because these molecules are persistent in the environment (Mathur et al., 2021). In France and the United Kingdom, the comparison of measured environmental FQ concentrations to PNEC-Rs highlighted that these antibiotics represented a high risk of selection of resistant bacteria in treated wastewater (Haenni et al., 2022; Hayes et al., 2022). FQ resistance mechanisms are also detected in bacteria from the environment. For example, plasmid-mediated quinolone resistance (PMQR) genes, particularly *qnr* genes, have been detected and quantified in WWTPs (Pazda et al., 2019). These genes reduce susceptibility, but usually not to the point of clinical non-susceptibility (Hooper and Jacoby, 2016). In *Escherichia coli*, most resistance mutations generating clinical resistance to FQs are located in the quinolone resistance-determining region (QRDR) in *gyrA* and *parC* genes. These resistance mutations have been shown common in *E. coli* isolated from the environment, even in the absence of FQs (Johnning et al., 2015).

In the context of research exploring the relationship between antibiotic exposure and the selection of resistance in the environment, it would be interesting to focus on the effects of FQs on a particular compartment: the biofilms from sewers. Indeed, until recently, only a few studies have been conducted on sewer biofilms. However, the internal walls of the sewers are covered with biofilms composed of very dense microbial communities, continually exposed to cocktails of chemical agents, including antibiotic residues, with the highest concentrations detected at the entries of WWTPs (Jelic et al., 2015). Moreover, these bacterial communities have also been shown to contain many ARGs (Balcázar et al., 2015; Auguet et al., 2017; Flores-Vargas et al., 2021). In this way, sewer biofilms are likely to be hotspots for the selection and accumulation of ARB and ARGs. Additionally, biofilms in aquatic environments have been identified as focal points for horizontal gene transfer (HGT) of ARGs (Abe et al., 2020).

This study aimed to develop an *in vitro* biofilm model for studying the impact of FQs on bacterial communities in sewer biofilms. Different culture conditions were tested in bioreactors to determine their effect on *in vitro* biofilm production. Once the culture conditions were set, *in vitro* biofilms were exposed to FQs at high and low concentrations, and changes in taxonomic composition, abundances of PMQR genes and proportions of mutations in *gyrA* and *parC* were tracked.

2 Materials and methods

2.1 Field sample collection and processing

Wastewater and sewer biofilm samples were taken at one of the entries of a WWTP, servicing a population equivalent to 300,000 inhabitants and including hospital wastewater. Wastewater was sampled during four sampling campaigns (A, B, E, and F) in 2021 (Table 1). An average 24-h sample was performed using automatic samplers. Sewer biofilm was only sampled during the first campaign due to technical constraints. The samples were transported at 4°C to the laboratory and placed at 4°C until processing. Wastewater samples were first filtered (170 µm) to remove the larger particles, and used to inoculate bioreactors, as described below. Aliquots of the filtered wastewater were stored in plastic tubes at −80° C until FQ quantification, and others were concentrated 100 times by centrifugation (6,000 ×*g*, 10 min), and were stored at −80°C until DNA extraction. Sewer biofilm was homogenized with 0.9% saline in a homogenizer-mixer (BagMixer 400 W, Intersciences, France), subjected to sonication for 15 min, and filtered using the BagMixer (280 µm). The filtrate was centrifuged for 10 min at 6,000 ×*g*, and the pellets were resuspended in 30 mL of peptone glycerol water (both from Sigma-Aldrich, United States) at 30%. Aliquots of resuspended

TABLE 1 Date of wastewater sampling campaigns, counts of heterotrophic bacteria and *Escherichia coli* and percentage of resistance to ciprofloxacin.

Sampling campaign ID	Sampling date	Heterotrophic bacteria counts (log_{10} CFU/mL)	Heterotrophic bacteria resistant to ciprofloxacin (%)	*E. coli* counts (log_{10} CFU/mL)	*E. coli* resistant to ciprofloxacin (%)
A	03/02/2021	6.5	7.7	4.4	5.7
B	03/23/2021	5.9	6.8	3.0	7.0
E	06/08/2021	7.6	0.2	4.2	2.6
F	07/13/2021	6.9	3.6	3.8	1.8
Mean (SD)		6.7 (0.7)	4.6 (3.4)	3.8 (0.6)	4.3 (2.5)

pellets were stored at −80°C until use for the initial inoculation of bioreactors. Aliquots of sewer biofilm were stored at −80°C for further analysis, as described below.

2.2 Bioreactor experiments

The sewer environment was simulated using CDC Biofilm Reactors® (Biosurface Technologies, United States). Bioreactors were fed continuously (0.1 mL/min) with nutrients (R2A broth without magnesium sulfate) and inoculum (1/100 v/v diluted wastewater in R2A broth without magnesium sulfate). The R2A composition for 1 L is as follows: 0.5 g yeast extract (ThermoFisher scientific, United States), 0.5 g proteose-peptone (Sigma-Aldrich, United States), 0.5 g Hy-Case® SF (Sigma-Aldrich, United States), 0.5 g D-(+)-Glucose (Sigma-Aldrich, United States), 0.5 g starch (Acros Organics, United States), 0.3 g potassium phosphate dibasic (Sigma-Aldrich, United States). Wastewater was diluted in order to prevent the impact of chemical agents. Feeding bottles containing nutrients and inoculum were placed at 4°C. At the start of the experiment, the bioreactors were filled with 350 mL of a mixture containing 1/100 diluted wastewater and R2A broth without magnesium sulfate (1:1 v/v). The bioreactors were kept at 21°C. The cultures were stirred on a magnetic plate at 100 rpm. Biofilms were formed on removable coupons placed on rods supported by the top of the bioreactor. Each bioreactor had eight rods containing three coupons each (Supplementary Figure S1).

Four experiment sets were conducted (Supplementary Table S1). The first two experiments aimed to optimize the culture conditions to obtain an *in vitro* biofilm mimicking the biofilm collected in sewers. Wastewater collected at campaigns A and B was used to inoculate the first and the second experiment, respectively. Different culture conditions were tested: (i) initial inoculum: diluted wastewater with vs. without sewer biofilm, (ii) coupon material: concrete (C) vs. polycarbonate (PC), and (iii) time of culture: 7 days (D07) vs. 14 days (D14). In each experiment, four bioreactors were used in parallel; sewer biofilm and diluted wastewater were used for the initial inoculation of two bioreactors, and diluted wastewater alone for the other two. In addition, each bioreactor was equipped with 12 polycarbonate coupons and 12 concrete coupons (Supplementary Figure S1). Once the growth conditions were set (concrete coupons, sewer biofilm in the initial inoculum, and an incubation time of 14 days), the impact of FQs on *in vitro* biofilm microbiota was evaluated in two other experiments.

The third and fourth experiments aimed to evaluate the impact of FQs on bacterial communities in sewer biofilms. The inoculation was performed with wastewater from campaign E (third experiment) and F (fourth experiment). Biofilms were established under high and low concentrations of FQs. In the 3rd experiment, ciprofloxacin (CIP) (≥98% purity, Sigma-Aldrich, United States) and norfloxacin (NOR) (≥98% purity, Sigma-Aldrich, United States) were added at 5,000 µg/L (high exposure), and at 2.5 µg/L (low exposure) in the 4th. At the start of the experiments, FQs were added to the initial inoculum, and bioreactors were fed with R2A broth containing FQs to obtain target concentrations in bioreactors. For each FQ exposure experiment, three conditions were tested: (i) control (no FQ), (ii) CIP, and (iii) NOR (Supplementary Figure S1).

Biofilms formed on coupons were collected on D07 and D14. During the experiments dedicated to the testing of different growing conditions (first and second experiments), at each sampling time, two rods containing concrete coupons (one replicate) and two rods containing polycarbonate coupons (one replicate) were taken from each bioreactor (Supplementary Figure S1). During the FQ exposure experiments (third and fourth experiments), at each sampling time, two replicates, consisting of two rods, were taken from each bioreactor (Supplementary Figure S1). After rinsing with 0.9% saline, the biofilm was harvested by swabbing the external surface of the three coupons of the same rod. The operation was repeated for the coupons of the other rod of the replicate. The two swabs were placed in 0.9% saline and subjected to sonication for 5 min. The sample was concentrated and stored at −80°C prior to DNA extraction. Additionally, during FQ exposure experiments, wastewater samples from the bioreactors were collected on days 2, 7, 9, and 14 to quantify FQs. A sample description is available in Supplementary datasheet S1.

2.3 Quantification of FQs in bioreactors

CIP and NOR were quantified by ultra-high-performance liquid chromatography with UV and fluorescence detection (Acquity UPLC, Waters, MA United States). One hundred microliters of the sample were added to 100 µL of internal standard (IS, marbofloxacin 0.5 µg/mL for UV detection and danofloxacin 2 ng/mL for fluorescence detection) diluted in 6.6% trichloroacetic acid. The mixture was vortexed for 2 min at 1,400 rpm and 10°C and centrifuged for 10 min at 20,000 ×*g* and 4°C. The supernatant (10 µL) was injected into an Acquity UHPLC® BEH C_{18} column (2.1 × 50 mm, 1.7 µm) (Waters Inc., Milford, MA, United States) and eluted at 40°C and a flow rate of 0.3 mL/min with a gradient consisting of H_2O, 0.1% formic acid (FA) and methanol (MeOH). Detection was carried out at 278 nm for UV detection, and 280 nm excitation wavelength and 425 nm emission wavelength for fluorescence detection. The concentration ranges for calibration were 0.5–10 µg/L for the low-exposure experiment and 250 ng/mL – 10,000 µg/L for the high-exposure experiment.

2.4 Quantification of FQs in field samples

Ten FQs and quinolones (Qs) were assayed in field samples: five FQs for human use (ciprofloxacin, lomefloxacin, moxifloxacin, norfloxacin, and ofloxacin), as well as five FQs/Qs for veterinary use (danofloxacin, enrofloxacin, marbofloxacin, flumequine, and oxolinic acid). All antibiotics were assayed by ultra-high-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Nexera LCMS8045, Shimadzu, Japan). Samples (10 µL) were eluted at 0.3 mL/min with a gradient consisting of H_2O/acetonitrile (ACN) acidified with 0.1% FA and at 40°C onto an Acquity UHPLC® BEH C_{18} column (2.1 × 100 mm, 1.7 µm). FQs and Qs were ionized by electrospray in positive mode (ESI+) and detected using multiple reaction monitoring mode. Multiple reaction monitoring transitions, cone voltage, and collision energies are reported in Supplementary Table S2. Wastewater samples (250 µL) were vortexed for 1 min at 1,000 rpm, centrifuged at 14,000 rpm and 4°C for 20 min, and filtered using 0.45 µm-polytetrafluoroethylene (PTFE) syringe filters. Filtrates (95 µL) were completed by adding 5 µL of ACN acidified with 2% FA and 10 µL of IS (ciprofloxacin-d_8 at 100 ng/mL). The biofilm was previously lyophilized for 48 h and stored at −20°C until extraction. The lyophilized biofilm (200 mg) was mixed with 25 µL of IS (ciprofloxacin-d_8 100 ng/mL) and 1 mL of MeOH acidified with 1% FA. The mixture was ground for 3 min at a frequency of 1/30 Hz with two glass beads (Retsch MM400, Verder Scientific GmbH & Co. KG) and then centrifuged at 20,000 ×*g* at 4°C for 10 min. This process was repeated, and the two supernatants were added and evaporated to dryness under nitrogen. The extract was suspended in 250 µL of H_2O/ACN (95:5 v/v) acidified with 0.1% FA. The mixture was vortexed for 5 min and filtered using a 0.45 µm-PTFE syringe filter. FQs and Qs concentrations were estimated from a calibration curve at concentrations ranging from 0.05 to 10 ng/mL for both matrices. Each sample was directly assayed in a first injection and was enriched with a mixture containing the 10 FQs and Qs at 5 ng/mL for the biofilm and 1 ng/mL for the wastewater to check for matrix effects. The LOQ was set at 0.05 ng/mL for all FQs and LOD was 0.005 ng/mL.

2.5 Bacterial counts

R2A agar (as described in 2.2 with 15 g agar (Sigma-Aldrich, United States)) and *E. coli* ChromoSelect Agar B (Sigma-Aldrich, United States) plates were inoculated to count heterotrophic bacteria and *E. coli*, respectively. CIP-resistant bacteria and CIP-resistant *E. coli* were counted on R2A agar supplemented with 2 mg/L of CIP and on ChromoSelect Agar B plates supplemented with 0.25 mg/L of CIP, respectively. R2A agar plates were incubated for 48 h at 31°C, and ChromoSelect Agar B plates overnight at 45°C. Percentage of resistant heterotrophic bacteria and *E. coli* were then calculated, and statistical significance assessed using ANOVA and Dunnett's test from DescTools package at a 95% family-wise confidence level in RStudio (Version 2023.6.0).

2.6 DNA extraction

DNA was extracted from field samples (wastewater and sewer biofilm) using the FastDNA™ SPIN for Soil Kit (MP Biomedicals, United States), as described by the manufacturer, with the PPS and SEWS-M wash steps repeated twice. DNA was extracted from *in vitro* biofilms using the DNeasy Blood and Tissue kit (QIAGEN, The Netherlands), as described by the manufacturer. The purity and quantity of DNA were analyzed by spectrometry with NanoDrop™ One (ThermoFisher Scientific, United States).

2.7 Relative abundance of plasmid-mediated quinolone resistance (PMQR) genes

Six plasmid-mediated quinolone resistance (PMQR) genes (*qnrA, qnrB, qnrC, qnrD, qnrS* and *qepA*) and the 16S rRNA gene were quantified. DNA fragments of each target DNA were synthesized and subsequently inserted into pEX-A258 to construct recombinant plasmids (Eurofins Genomics, Luxembourg) and generate standard curves (Supplementary Table S3). DNA extracts were submitted to 35 PCR cycles using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Canada) and a mix of primers (300 nM final concentration each). The cycling conditions were as follows: initial denaturation at 98°C for 3 min, followed by 35 cycles of 98°C for 15 s, 60°C for 15 to 60 s (depending on the targeted gene). Primers for each targeted DNA are listed in Supplementary Table S3. The quantity of each gene was calculated using a standard curve from 10 to 10^8 copies/µL with Bio-Rad CFX Manager 3.1 analysis software. The number of copies of PMQR genes per µL was normalized by the number of copies of the 16S rRNA gene per µL to assess the relative abundance of PMQR genes in samples. Comparisons between samples were done in RStudio (Version 2023.6.0) and statistical analysis was performed using Kruskal–Wallis test followed by Dunn's test from FSA package, with Holm's correction for multiple comparisons, for sample comparison of 1st and 2nd experiments, and Dunnett's test from DescTools package at the 95% family-wise confidence level for 3rd and 4th experiments.

2.8 *Escherichia coli gyrA* and *parC* genes sequencing and analysis

From DNA extracted from selected samples, *gyrA* and *parC* amplicons were obtained from two independent PCRs using AmpliTaqGold360 Master Mix (ThermoFisher Scientific) and a mix of primers (1 µM final concentration each). The cycling conditions consisted of denaturation at 95°C for 6 min, followed by 40 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min, followed by 72°C for 7 min. Primers for each targeted DNA are listed in Supplementary Table S3. Resulting PCR products were purified and sequenced at the GeT-Biopuces core facility (Toulouse, France) using Illumina MiSeq V3, yielding 2 × 250 reads leading to 504,000–616,000 sequences per sample for *gyrA* amplicon (311 bp) and 476,000–655,000 sequences per sample for *parC* amplicon (287 bp) per sample. Adapters and low-quality bases were trimmed (−q25 length 150) using TrimGalore (Version 0.6.5). The remaining reads were aligned using bwa-mem (Version 2.2) to a database of *gyrA* and *parC* genes of *E. coli* and *Shigella* (NCBI nucleotide "(*e_coli*[orgn] OR *shigella*[orgn]) AND (*gyrA*[gene] OR *parC*[gene]) AND (*gyrA*[title] OR *parC*[title])") to select *E. coli* or *Shigella gyrA/parC* reads for subsequent analysis.

The paired reads were merged using vsearch - fastq_mergepairs (Version 2.6.2) and dereplicated using u-search - fastx_uniques (Version 11.0.667). Chimeric sequences were removed using usearch - uchime_denovo. After these steps, 2,239–29,271 reads were left for the subsequent analysis for *gyrA* and 13,084 to 42,800 for *parC*. To identify mutations and their abundances in *gyrA*/*parC*, sequences were aligned read by read to the reference (*E. coli* K-12 MG1655 RefSeq locus tags *gyrA*: b2231 *parC*: b3019) using MAFFT -keeplenght -addfragments (restrict the length of the alignments to the length of the reference sequence).

2.9 16S rRNA sequencing and analysis

The V3-V4 region of the 16S rRNA gene was amplified with the primers F343 (CTTTCCCTACACGACGCTCTTCCGATCT ACGGRAGGCAGCAG) and R784 (GGAGTTCAGACGTGTG CTCTTCCGATCTTACCAGGGTATCTAATCCT) using 30 amplification cycles with an annealing temperature of 65°C (Drouilhet et al., 2016). Single multiplexing was performed using an in-house 6 bp index, which was added to R784 during a second PCR with 12 cycles using forward primer (AATGATACGGCGA CCACCGAGATCTACACTCTTTCCCTACACGAC) and reverse primer (CAAGCAGAAGACGGCATACGAGAT-index-GTGAC TGGAGTTCAGACGTGT). The resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge (2 × 250 PE) according to the manufacturer's instructions. The sequences were analyzed using the standardized pipeline FROGS version 4.1.0 (Escudie et al., 2018). Paired-end reads were merged using Vsearch software (Version 2.17.0) with a 0.1 mismatch rate. Unmerged reads were discarded, and only the amplicons between 400 and 490 bp were kept. Primers were trimmed from the sequences. Merged reads were clustered using the swarm tool (Mahé et al., 2014) with an aggregation distance of 1, and chimeras were removed. Then, clusters containing <0.005% of the total sequences were further filtered as recommended elsewhere (Bokulich et al., 2013). Taxonomic affiliation was completed by blasting the sequences against the 16S SILVA Pintail100 38.1 database (Quast et al., 2013). These parameters were validated with microbial community standards (ZymoBIOMICS, D6300, and D6310), extracted and sequenced with our samples. The resulting data were then computed in RStudio (Version 2023.6.0). The vegan and phyloseq packages were used to analyze alpha and beta diversity. The differences between Shannon diversity were assessed with ANOVA and Tukey's post-hoc test, with a 95% family-wise confidence level. Constrained analysis of principal components (CAP) and principal coordinate analysis (PCoA) were performed using phyloseq package, and permutational multivariate analysis of variance (PERMANOVA) using the vegan package. PCoA and PERMANOVA were performed using the Bray–Curtis Dissimilarity metric.

2.10 Metagenomic analysis

Selected samples were sequenced at the Get-PlaGe core facility (Toulouse, France) using Illumina Novaseq 6000 (2 × 150 bps). They included the sewer biofilm with ~167 million read pairs, four wastewater samples with ~232 million reads pairs, ~190 million reads pairs, ~228 million reads pairs, and ~249 Million read pairs, and eight *in vitro* biofilm samples (~169, ~278, ~205, ~225, ~240, ~200, ~208, and ~180 million reads pairs). Reads were cleaned using the metagWGS v2.2 workflow cleaning step, meaning adapters were removed and reads trimmed if quality <20 by cutadapt v2.10 and Sickle v1.33. Read pairs mapping the humanGrch38 genome were removed (bwa-mem2 v0.7.17-r1188, samtools v1.10) (Fourquet et al., 2022).[1] Due to lack of enough sequencing efforts and higher diversity, field samples (sewer biofilm and wastewater) assemblies were much more fragmented than the *in vitro* ones. This difference in diversity between field and *in vitro* samples was highlighted by looking at the histograms of the frequency of kmers (with kat 2.4.2, kmer size of 21). Since our goal was to compare the two in terms of ARG content, we decided not to use the assemblies but the reads so as not to bias the results of the comparison between the two types of samples. These cleaned reads were then used to find and quantify ARGs and efflux pump genes (EPGs) by using RGI v5.2.1 (rgi bwt with bwa aligner, 8 threads, default values for all the other parameters) against CARD database v3.2.1(Alcock et al., 2020). We used sortmerna v4.3.2 (Kopylova et al., 2012) against the 16S database provided here:[2] with default parameters to quantify reads corresponding to 16S rRNA gene. Then, ARGs/EPGs were filtered according to reads quality (Average % of reference allele(s) covered by reads ≥50, number of reads completely mapped to these alleles ≥10, average MAPQ (Completely Mapped Reads) ≥ 30 for at least one sample). We excluded ARGs/EPGs for which resistance resulted from point mutational alterations that were species-specific, genes of efflux pumps not known to expel antibiotics, genes conferring resistance by their absence or under-expression, and regulators of efflux pumps (Supplementary datasheet S2). A total of 349 ARGs (Supplementary datasheet S3) and 149 EPGs (Supplementary datasheet S4) were kept. Data were analyzed in R (version 4.2.2) using phyloseq package (version 1.42.0). To account for possible variations in bacterial proportion reads across samples, we normalized by the 16S rRNA reads. We created a matrix for ARG or EPG counts that included the 16S rRNA counts and resampled it with the lowest 16S rRNA count among libraries (126,759 reads in the sewer biofilm). This was achieved using rarefy function from R package "phyloseq". Thus, normalized 16S reads were similar to those of the sewer biofilm sample, and the proportion of ARG/16S rRNA or EPGs/16S rRNA is maintained in each of the samples after normalization. We then calculated the alpha and beta diversity. Wilcoxon rank-sum (Mann–Whitney) test was used to compare Shannon diversity between *in vitro* biofilm and wastewater. PCoA was performed using the Bray–Curtis Dissimilarity metric. The differential abundance of ARGs/EPGs reads between *in vitro* biofilm and another sample type (sewer biofilm or wastewater) was visualized with the Log2 fold change ratio. To limit false positive, only genes showing a Log2FC ≥ 3.32 or ≤ −3.32 (representing a change of at least 10-fold) were taken into account when at least one of the 2 types of samples contained a minimum of 100 reads (after rarefaction). To compare *in vitro* biofilm to wastewater, the p-adjusted value obtained from poisson linear model and a FDR correction for multiple testing were applied.

1 https://forgemia.inra.fr/genotoul-bioinfo/metagwgs

2 https://github.com/biocore/sortmerna/tree/master/data/rRNA_databases/silva-bac-16s-id90.fasta

10.3389/fmicb.2024.1377047

2.11 Procrustes analysis

Procrustes analysis was performed using the R vegan package to determine whether the changes in ARGs revealed by metagenomic were linked to microbial community changes showed by 16S rRNA sequencing. ARGs data from metagenomics were rarefied to the lowest 16S rRNA. Corresponding 16S rRNA sequencing samples were reanalyzed as described previously. PCoA was performed using the Bray-Curtis distance for both datasets, and similarity was assessed using the "protest" function with 99,999 permutations.

3 Results

3.1 Fluoroquinolones and ciprofloxacin resistant bacteria in field samples

Among the ten Qs/FQs analyzed in wastewater and biofilms, only ciprofloxacin and ofloxacin were detected in wastewater at 187.5 ± 63.3 and 363.2 ± 97.6 ng/L, respectively. In sewer biofilm, ciprofloxacin, norfloxacin and ofloxacin concentrations were 3,900, 1,330, and 48,410 ng/kg of dry matter, respectively (Table 2). The concentration of the other antibiotics remained below the LOD.

Bacteria counts in wastewater ranged from 5.9 to 7.6 $\log_{10}$ CFU/mL for heterotrophic bacteria, and from 3.0 to 4.4 $\log_{10}$ CFU/mL for *E. coli* (Table 1). The mean of CIP-resistant heterotrophic bacteria and *E. coli* in the wastewater of the four campaigns was 4.6 ± 3.4% and 4.3 ± 2.5%, respectively. In sewer biofilm, these percentages were 6.0 and 2.4% for CIP-resistant heterotrophic bacteria and *E. coli*, respectively.

TABLE 2 Fluoroquinolone concentration in field samples (wastewater and biofilm) and bioreactors during FQ exposure experiments.

FQ concentration			
	CIP (ng/L)	NOR (ng/L)	OFL (ng/L)
Wastewater (campaign ID)			
A	182.9	<LOD	408.2
B	122.8	<LOD	240.9
E	274.2	<LOD	467.5
F	170.0	<LOD	336.2
Mean (SD)	187.5 (63.3)	<LOD	363.2 (97.6)
	CIP (ng/kg of dry matter)	**NOR (ng/kg of dry matter)**	**OFL (ng/kg of dry matter)**
Sewer biofilm	3,900	1,330	48,410
	CIP (µg/L)	**NOR (µg/L)**	
Bioreactors			
High-exposure			
Mean (SD)	2,125.4 (1,749.3)	2,083.9 (1,464.4)	
Low-exposure			
Mean (SD)	2.7 (0.2)	2.7 (0.1)	

CIP, ciprofloxacin; NOR, norfloxacin; OFL, ofloxacin; LOD, limit of detection; FQ, fluoroquinolone.

3.2 Optimization of *in vitro* biofilm culture conditions

3.2.1 Bacterial biomass

The results of counts of heterotrophic bacteria in *in vitro* biofilms showed that there was no significant difference in bacterial counts between biofilms obtained following the initial inoculation of the bioreactors with wastewater, whether or not supplemented with sewer biofilm (Kruskal–Wallis, $p = 0.598$). Similarly, no difference was observed between biofilms harvested after 7 or 14 days of culture (Kruskal–Wallis, $p = 0.365$; Supplementary Figure S2A). On the other hand, a significant difference was observed between the bacterial counts of biofilms formed on concrete and polycarbonate coupons. Indeed, concrete coupons facilitated a higher level of bacteria forming the biofilm (Kruskal–Wallis, $p = 2.31 \times 10^{-5}$). The quantification of 16S rRNA, as a marker of bacterial biomass, also demonstrated significantly greater quantities of biomass for the biofilms formed on concrete coupons (Supplementary Figure S2C). Regarding *E. coli* counts, the effect of coupon material on biofilm formation was not significant (Supplementary Figure S2B).

3.2.2 Taxonomic composition

The taxonomic composition determined by sequencing 16S rRNA V3-V4 section from amplicon as described in section 2.9 showed that *in vitro* biofilms were predominantly composed of *Enterobacterales* (42.8% on average) (Figure 1). The impact of three parameters on microbial diversity was assessed: (i) the material of coupons (concrete vs. polycarbonate), (ii) the initial inoculation (wastewater supplemented or not with sewer biofilm), and (iii) the duration of culture (7 vs. 14 days). These factors did not exhibit any effect on alpha diversity, as estimated by the Shannon index [ANOVA $p = 0.251$ (i), $p = 0.115$ (ii) and $p = 0.288$ (iii)]. However, differences in alpha diversity (Shannon index) based on the experiment set (first vs. second experiment) were observed (ANOVA $p = 0.008$; Figure 2A). A dissimilarity matrix using the Bray-Curtis distance was calculated to compare the bacterial compositions of *in vitro* biofilms considering the experiment set and the sampling day. The CAP plot revealed that communities were primarily differentiated by the sampling day, with also an influence from the experiment set (Supplementary Figure S3).

Moreover, considering field samples, a PCoA analysis on the Bray-Curtis distance showed a separation of samples according to their type (wastewater, sewer biofilm, and *in vitro* biofilms) (Figure 2B). It also revealed a distinction between *in vitro* biofilms from the first and second experiment sets. The relative abundance of the ten most prevalent orders in field samples (wastewater and sewer biofilm) and *in vitro* biofilms showed that samples share common orders but in varying proportions (Figure 1). Sewer biofilm was primarily characterized by *Burkholderiales* (21.8%), *Pseudomonadales* (12.6%) and *Enterobacterales* (11.2%). Wastewater samples were dominated by *Campylobacterales* (39.9% on average). After rarefaction, the heatmap of the most abundant genera indicated that *Pseudomonas* was the predominant genus in sewer biofilm, while *Arcobacter* dominated wastewater (Supplementary Figure S4). The most abundant genera in *in vitro* biofilms were scarce in sewer biofilm.

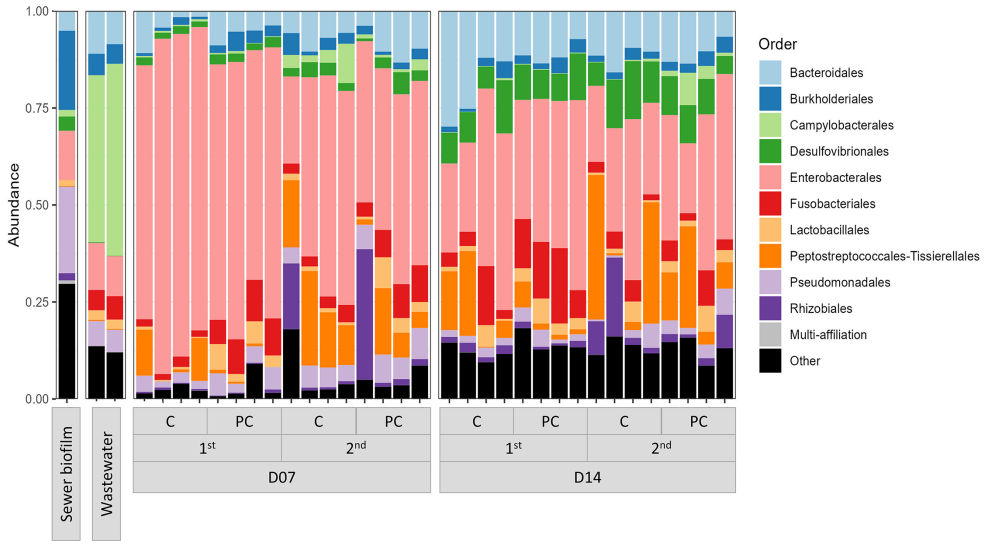

FIGURE 1
Bar chart representing the relative abundance of the ten most abundant orders in field samples (wastewater and sewer biofilm) and *in vitro* biofilms. The samples were categorized based on sampling time (D07 and D14), experiment set (first and second), and coupon material (C, concrete; PC, polycarbonate).

3.2.3 Resistance to FQs

The percentages of CIP-resistant heterotrophic bacteria were consistently low in *in vitro* biofilms across the tested conditions (Figure 3A). This pattern was similarly observed for the percentages of CIP-resistant *E. coli* (Figure 3B). Concerning the quantification of PMQR genes, *qnrC* and *qepA* genes were not detected in any sample. A significantly higher relative abundance for biofilms obtained on polycarbonate coupons than concrete coupons was observed for *qnrB* and *qnrS* genes. The relative abundances of *qnrA, qnrB, qnrD* and *qnrS* tended to be higher in *in vitro* biofilms than in sewer biofilms but were similar to those in wastewater (Figure 4A).

Additionally, sequencing of *gyrA* and *parC* amplicons from DNA extracted from both field samples and *in vitro* biofilms from the first experiment at D14 was conducted. The most common non-synonymous mutations in *gyrA* were observed in codon S83 (11.0–31.6%) and D87 (10.1–23.7%) and in *parC* in codon S80 (17.1–28.0%) and E84 (7.9–10.5%) (Table 3). Conditions tested in the formation of *in vitro* biofilms did not impact the relative abundance mutations, and they closely resembled those observed for field samples. The most common substitutions in *gyrA* were S83L and D87N in *in vitro* biofilms (16.0 ± 4.5% and 12.0 ± 5.4%) and in wastewater (14.3 and 7.3%), whereas they were S83G (6.7%) and D87L (6.2%) in sewer biofilm (Supplementary Table S4). The relative abundance of co-occurrence of mutations in *gyrA* at codons S83L and D87N ranged from 0.3% (sewer biofilm) to 16.5% (*in vitro* biofilm) (Table 4). The most common substitution in *parC* was S80I for all samples (Supplementary Table S5).

3.2.4 Resistome

Thirteen samples, meeting the required quantity and quality parameters, were subjected to metagenomic analysis, including *in vitro* biofilms sampled at D14 on concrete coupons (6 samples) and polycarbonate coupons (2 samples), sewer biofilm (1 sample) and wastewater (4 samples). The sewer biofilm's resistome (ARGs and EPGs) exhibited the highest Shannon diversity index compared to wastewater and *in vitro* biofilms (Figure 5A). The resistome of *in vitro* biofilms showed a significantly higher Shannon diversity index than that of wastewater (Mann–Whitney, $p < 0.05$). A PCoA analysis on the Bray-Curtis distance illustrated a differentiation of samples based on the nature of the sample (sewer biofilm, wastewater and *in vitro* biofilms) (Figure 5B).

For ARG data after rarefaction, 18,139 reads were counted in sewer biofilm, 27,560 ± 528 in wastewater and 20,978 ± 2,008 in *in vitro* biofilms. A total of 349 ARGs were detected and grouped according to the antibiotic classes for which they confer resistance. The results indicated that ARGs conferring resistance to tetracycline and peptides were more abundant in *in vitro* biofilms than in sewer biofilm. Conversely, ARGs conferring resistance to MLS, macrolide or aminoglycoside were less abundant in *in vitro* biofilm than in sewer biofilm (Figure 6A; Supplementary Table S6). Among the 61 ARGs with read counts ≥100 in *in vitro* biofilms or sewer biofilm, 32 were differentially abundant according to the Log2FC estimate: 22 ARGs were under-represented and 10 ARGs were over-represented in *in vitro* biofilm (Supplementary Figure S5). Concerning ARGs conferring resistance to FQs, only three genes (*qnrA1, qnrB35*, and *qnrVC6*) were detected in sewer biofilm, and only the *qnrVC6* contained more than ten reads in one sample (sewer biofilm). Finally, to assess whether the differences in ARGs observed between samples were linked to changes in the microbial community, a Procrustes analysis was performed on the PCoA based on the Bray–Curtis dissimilarity index from metagenomic results and the corresponding 16S rRNA community results. The Procrustes analysis was significant ($M^2 = 0.66$, $p = 0.021$).

For EPG data after rarefaction, 92,348 reads were counted in sewer biofilm, 22,162 ± 2,843 in wastewater and 44,081 ± 15,685 in *in vitro* biofilm. Among the 149 EPGs detected, 64 were RND-EP (resistance-nodulation-division efflux pump), primarily involved in multidrug efflux, and 62 were MFS-EP (major facilitator superfamily efflux pump). EPGs were grouped according to the antibiotic classes

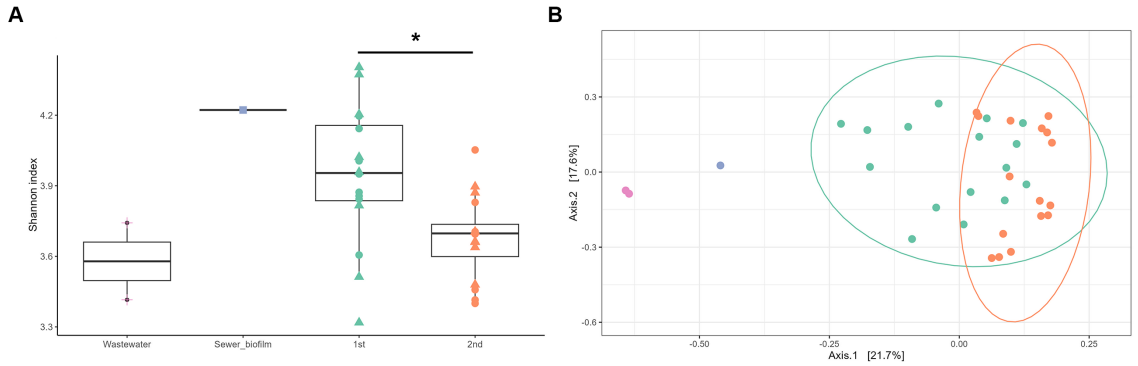

FIGURE 2
(A) Alpha diversity (Shannon index) of field samples (wastewater, purple and sewer biofilm, blue) and *in vitro* biofilms as a function of the experiment set (first, green and second, orange) and the day of sampling (D_7, circle, and D_{14}, triangle). Statistical differences were assessed with ANOVA ($p < 0.01$) and Tukey *post-hoc* test (*$p < 0.05$). **(B)** Visualization by principal coordinate analysis (PCoA) of the beta-diversity analysis carried out using the Bray-Curtis distance, constrained by the type of samples (wastewater, purple; sewer biofilm, blue; first set of experiment, green and second set of experiment, orange).

for which they confer resistance (Figure 6B). The comparison of *in vitro* biofilm to sewer biofilm showed that multidrug, macrolide- and phenicol-specific EPGs were under-represented in *in vitro* biofilm (Figure 6B; Supplementary Table S7). Among the 92 EPGs with read counts ≥100 in *in vitro* biofilms or sewer biofilm, 43 were differentially abundant, 33 being under-represented in *in vitro* biofilm, and ten over-represented in *in vitro* biofilm (Supplementary Figure S6). Regarding resistance to FQs, among the 39 multidrug-EP excluding FQ, 17 were under- and 3 were over-represented in *in vitro* biofilms compared to sewer biofilm. Five FQ-specific EP were detected, including two EPG over-represented in sewer biofilm (*MdtK*, *amrA*) and one under-represented (*ifrA*) compared to sewer biofilm.

3.3 FQ exposure experiments

Biofilms were established under two concentrations of FQs: high (5,000 µg/L) and low (2.5 µg/L), with testing of two specific FQs, namely CIP and NOR. The measured FQ exposure concentrations in bioreactors were closely approximated by the nominal target concentrations (Table 2). High exposure to FQs decreased alpha diversity (Shannon index) in the *in vitro* biofilms (Supplementary Figure S7A). Conversely, this reduction in diversity was not observed when biofilms were exposed to low concentrations of FQs. Notably, biofilms formed during the high FQ exposure experiment exhibited a distinct microbial composition compared to other *in vitro* biofilms (Supplementary Figure S7B). The *Peptostreptococcales-Tissierellales* order was predominant in all conditions tested during this experiment, including the control ones. It is worth noting that the wastewater used for the inoculation of this experiment set (E) had a microbial composition similar to that of wastewater from the other campaigns (A, B, and F) (Figure 1, Supplementary Figure S7B). The β-diversity between wastewater samples was measured, and campaign E was not specifically more distant (Supplementary Figure S8). High-concentration exposure significantly increased the percentage of CIP-resistant heterotrophic bacteria and *E. coli*, whereas these percentages remained low during low-concentration exposure (Figures 3C–F). Among *qnr* genes, a significantly higher relative abundance was only observed for *qnrS* when exposed to CIP (Figure 4B).

Finally, when *in vitro* biofilms were exposed to low concentrations of FQs, the relative abundance of mutations in *gyrA* S83 ranged from 25.8 to 67.5%, increasing to 95.3–96.8% after high exposure (Table 3). The relative abundance of mutations in *gyrA* D87 remained around 12.9–18.4% in non-exposed or exposed to low concentrations of FQs samples but increased to 97.0–97.8% after high FQ exposure. Reads evidenced S83L and D87N substitutions (Supplementary Table S4). In the absence of FQs and after exposure to low concentrations of FQs, only a few reads containing the S83L substitution showed concomitant D87N substitution (2.4–17.5%), but after high FQ exposition, almost all reads showed both mutations (80.4–83.0%; Table 4). Regarding *parC*, the relative abundance of S80 increased after high FQ exposure (86.0–36.1%), whereas it was not the case for E84 (Table 3). The S80I substitution was predominant (Supplementary Table S5).

4 Discussion

This study aimed to establish an *in vitro* biofilm model designed to investigate the influence of FQs on bacterial communities within sewer biofilms. The assessment involved examining the effects of various culture conditions on both the microbial community composition and the characteristics of FQ resistance. Subsequently, upon determining the optimal culture conditions, *in vitro* biofilms were exposed to high and low concentrations of FQs.

4.1 Optimization of culture conditions

In the optimization of culture conditions for *in vitro* biofilms cultivation, evaluating three culture parameters was imperative. These parameters involved the coupon material acting as the foundation for subsequent biofilm development, the biofilm development duration, and the composition of the initial inoculum serving as a subtract for biofilm growth. The ensuing discussion analyzes the outcomes of assessing these three parameters, elucidating their impact on the development and characteristics of *in vitro* biofilms.

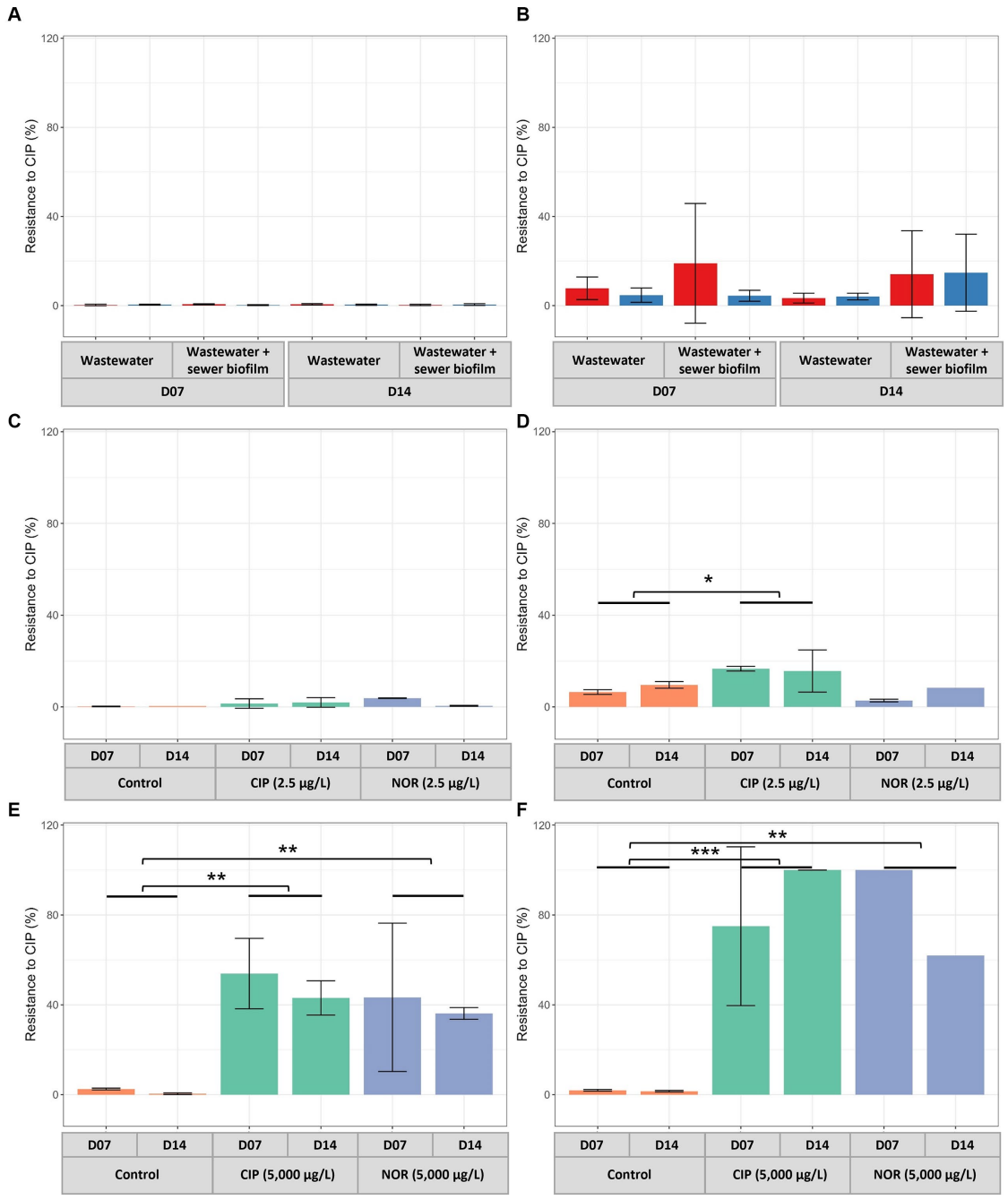

FIGURE 3
Percentages of resistance to ciprofloxacin of heterotrophic bacteria **(A,C,E)** and *E. coli* **(B,D,F)** in *in vitro* biofilms at D07 and D14. Experiments were conducted without exposure to FQs **(A,B)** with two coupon materials (concrete, red and polycarbonate, blue) and with exposure to FQs at low concentration (2.5 µg/L) **(C,D)** and high concentration (5,000 µg/L) **(E,F)**. CIP, ciprofloxacin; NOR, norfloxacin. *p*-values for **C-F** were calculated with Dunnett's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

To explore the potential impact of sewer material on biofilm composition, we conducted tests using two types of coupons designed to support biofilm growth. For instance, recent studies have revealed that the material of pipes can exert a significant influence on both biofilm structure and microbial community within water distribution systems (Lee et al., 2021; Zhang et al., 2022). In our study, concrete coupons were tested because they matched the material used in the pipes at the entrance of the WWTP at which the sewer biofilm had been sampled. Polycarbonate was also tested as it is a material commonly used for the culture of *in vitro* biofilms. Our results showed that using concrete coupons led to biofilms with higher heterotrophic bacteria counts and greater quantities of 16S rRNA compared to polycarbonate coupons. Previous experiments simulating a sewer pipe environment reported similar concentrations of 16S rRNA genes per area for polyvinylchloride (PVC) and concrete (Medina et al., 2020). However, in drinking water distribution systems, biofilms formed on

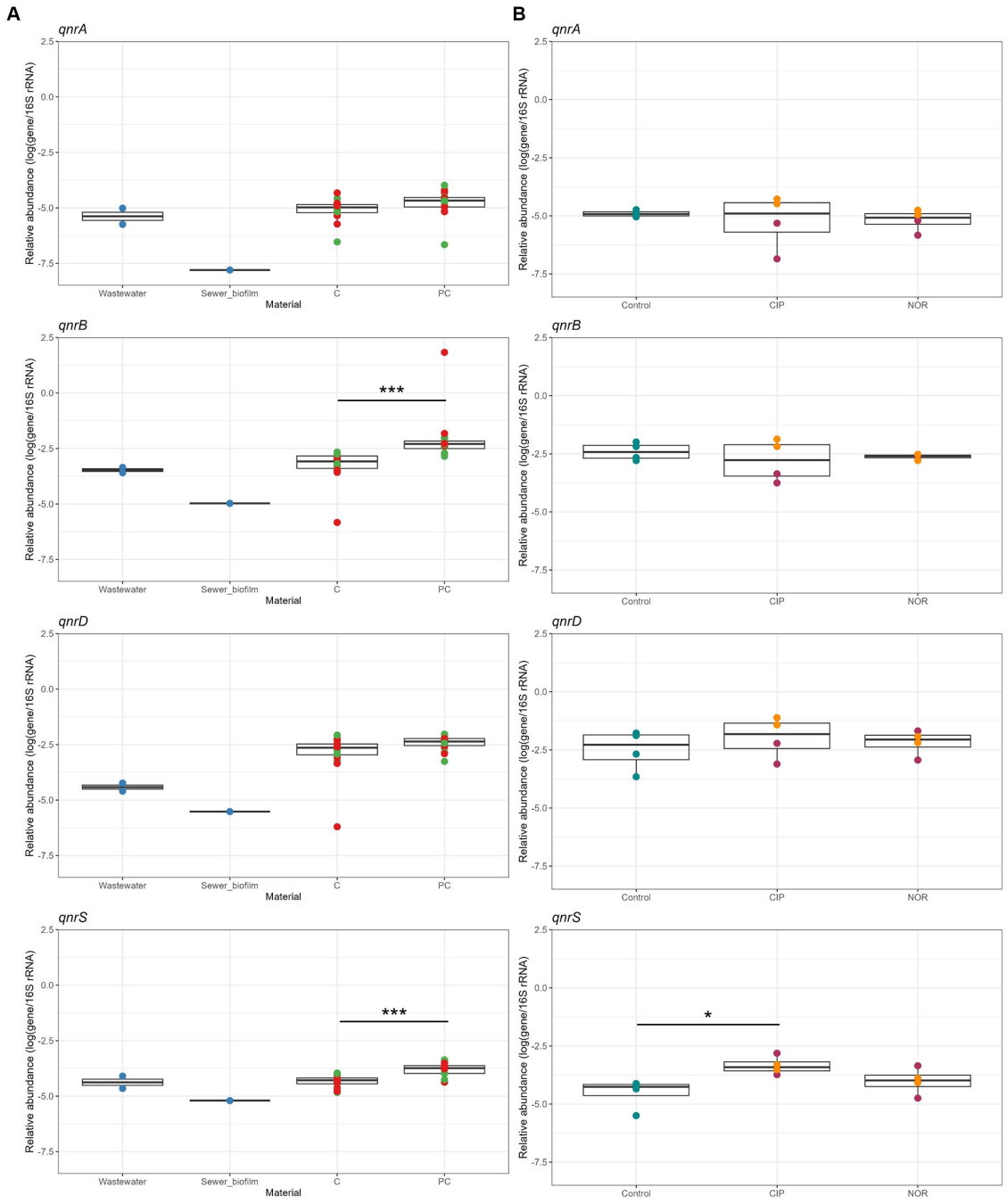

FIGURE 4
(A) Relative abundance of *qnr* genes in field samples (wastewater and sewer biofilm, blue) and *in vitro* biofilms from the 1st and 2nd experiment at D07 and D14, depending on the addition (red) or not (green) of sewer biofilm at the initial inoculation of bioreactors, and the material of the coupons (C, concrete; PC, polycarbonate). **(B)** Relative abundance of *qnr* genes in *in vitro* biofilms not exposed (Control) and exposed to ciprofloxacin (CIP) and norfloxacin (NOR) at high (5,000 µg/L, purple) and low (2.5 µg/L, orange) concentration. Statistical differences were assessed with Kruskal–Wallis test and Dunn *post-hoc* test for **(A)**, and Dunnett's multiple comparison test for **(B)** (*$p < 0.05$; ***$p < 0.001$).

concrete exhibited higher total cell counts and heterotrophic plate counts within 12 days compared to other various materials (copper, stainless steel, cast iron and polyethylene) (Zhu et al., 2014). Finally, the material of the coupons did not impact the taxonomic composition of *in vitro* biofilms, consistent with previous findings that PVC and concrete material did not lead to different prokaryotic communities (Medina et al., 2020). As such, the material of the coupons used in our experiments appears to influence biofilm growth in terms of bacterial biomass but not taxonomic diversity.

In this study, two culture duration were examined to understand how the taxonomic composition of sewer biofilms evolves over time, as highlighted by Zheng et al. (2021), who observed time-dependent bacterial succession in rural sewer biofilms. CAP analysis revealed that microbial community composition varied significantly with incubation

TABLE 3 Relative abundance of mutations in *Escherichia coli gyrA* (S83 and D87) and *parC* (S80 and E84) in field samples, *in vitro* biofilms at D_{14} not exposed to fluoroquinolones considering the coupon material (polycarbonate and concrete) and the supplementation or not of the initial inoculum of bioreactors with sewer biofilm at the initial inoculation of bioreactors, and *in vitro* biofilms at D_{14} exposed to FQs (CIP, ciprofloxacin and NOR, norfloxacin) at low and high concentrations.

Relative abundance of mutations in *E. coli* (%)							
	Field samples		*In vitro* biofilms without FQs				
	Wastewater	Sewer biofilm	Polycarbonate Without sewer biofilm	Polycarbonate With sewer biofilm	Concrete Without sewer biofilm	Concrete With sewer biofilm	Mean (SD)
gyrA S83	24.7	11.0	28.1	19.9	31.6	21.0	25.2 (5.6)
gyrA D87	15.4	10.1	20.8	13.3	23.7	14.4	18.1 (5.0)
parC S80	17.6	17.1	27.3	17.7	28.0	18.2	22.8 (5.6)
parC E84	10.5	9.2	9.7	8.7	9.3	7.9	8.9 (0.8)

	In vitro biofilms with FQs					
	Control		CIP		NOR	
			Low-exposure	High-exposure	Low-exposure	High-exposure
gyrA S83	16.4	19.3	67.5	95.3	25.8	96.8
gyrA D87	15.7	12.9	19.3	97.0	18.4	97.8
parC S80	15.7	13.7	27.9	86.0	10.8	86.1
parC E84	8.2	8.0	11.3	16.7	10.3	15.5

TABLE 4 Relative abundance of mutations in *Escherichia coli gyrA* (S83L and D87N and co-occurrence of these mutations) and *parC* (S80I) in field samples, *in vitro* biofilms at D14 not exposed to fluoroquinolones considering the coupon material (polycarbonate and concrete) and the supplementation or not of the initial inoculum of bioreactors with sewer biofilm at the initial inoculation of bioreactors, and *in vitro* biofilms at D_{14} exposed to FQs (CIP, ciprofloxacin and NOR, norfloxacin) at low and high concentrations.

Relative abundance of mutations in *E. coli* (%)							
	Field samples		*In vitro* biofilms without FQs				
	Wastewater	Sewer biofilm	Polycarbonate Without sewer biofilm	Polycarbonate With sewer biofilm	Concrete Without sewer biofilm	Concrete With sewer biofilm	Mean (SD)
S83L without D87N	8.8	0.6	6.3	6.7	4.8	6.3	6.0 (0.8)
D87N without S83L	1.8	1.2	1.8	1.8	2.3	2.1	2.0 (0.2)
S83L and D87N	5.5	0.3	11.8	5.2	16.5	6.4	10.0 (5.2)
S80I	8.7	8.3	18.5	9.1	18.9	10.2	14.2 (5.2)

	In vitro biofilms with FQs					
	Control		CIP		NOR	
			Low-Exposure	High-exposure	Low-exposure	High-exposure
S83L without D87N	2.9	5.9	51.8	2.2	12.8	2.6
D87N without S83L	1.4	1.7	1.1	7.6	1.6	7.5
S83L and D87N	5.8	3.7	5.5	80.4	5.4	83.0
S80I	9.0	6.0	17.5	75.5	2.4	75.8

time, corroborating Zheng et al.'s findings. This variation likely accounts for the observed differences between the sewer biofilm samples and our *in vitro* biofilms, considering that the latter are relatively younger. A culture duration of 14 days was selected for this reason, as it gave the biofilm more time to establish and get closer in composition to sewer biofilm. Additionally, alpha diversity measures and PCoA analysis

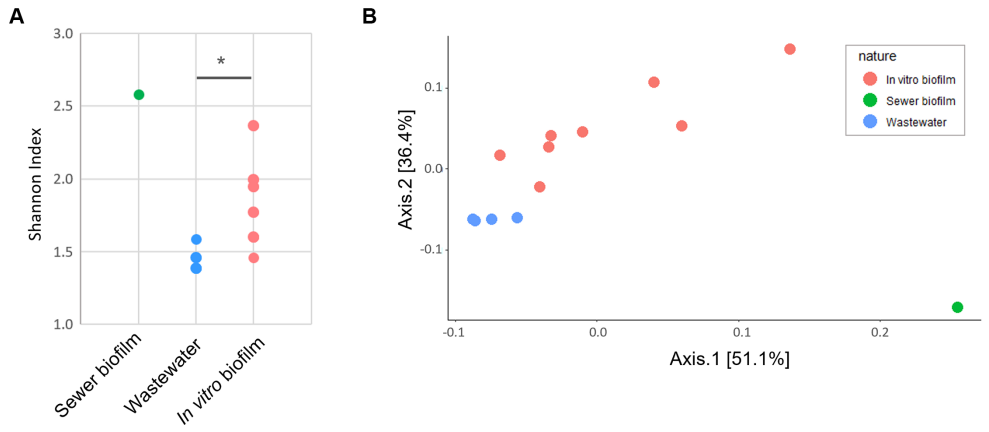

FIGURE 5
(A) Shannon diversity index based on antibiotic resistance genes and efflux pump genes counts derived from metagenomic data (*$p < 0.05$ Mann–Whitney U test). **(B)** PCoA based on Bray-Curtis of ARG and EPG counts for metagenomic data. The ARG and EPG counts were rarified with the lowest 16S rRNA counts.

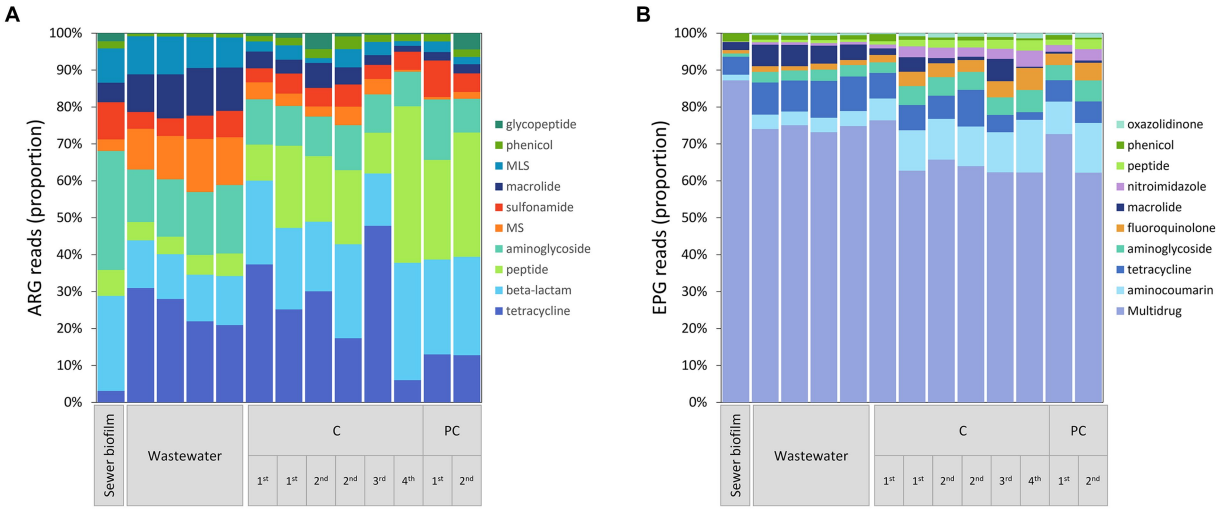

FIGURE 6
(A) Relative abundance of ARGs grouped according to drug family resistance. For MS, and MLS, M, macrolide; L, lincosamide; S, streptogramin. **(B)** Relative-abundance of EPGs grouped according to resistance profile. ARG and EPG that showed an average reads ≥10 in at least one of the sample categories (sewer biofilm, wastewater or *in vitro* biofilm) were included. The samples were grouped by the type of sample, the experiment (1st to 4th) and coupon material (C, concrete; PC, polycarbonate).

indicated distinct microbial communities across different experimental setups. Notably, in one experiment, *in vitro* biofilms formed under high FQ exposure, including control groups, exhibited a different taxonomic composition compared to *in vitro* biofilms from other experiments. While the taxonomic composition of the wastewater used for inoculating the bioreactors appeared similar, subtle variances in these samples could be accentuated under the specific conditions of the bioreactors, leading to variable taxonomic composition, as observed. Even if a single wastewater had been used for the inoculation of all bioreactors, a recent article reported the dominant role of stochastic assembly in creating variations of microbial diversity using a well-controlled laboratory system (Zhou et al., 2013).

Finally, testing the addition of sewer biofilm in the initial inoculation was based on previous findings that sewer biofilms and wastewater share microorganisms, albeit in varying proportions (Auguet et al., 2017; McLellan and Roguet, 2019). The 16S rRNA analysis showed that the dominant microorganisms in the sewer biofilm were from the *Burkholderiales* and *Pseudomonadales* orders, consistent with the review by Li et al. (2019). In contrast, wastewater bacterial communities were predominantly composed of bacteria from the *Campylobacterota* phylum, especially *Arcobacter*, aligning with studies indicating that wastewater microbiota are distinct from human fecal microbiota (Guo et al., 2019; LaMartina et al., 2021). Interestingly, the taxonomic composition of the *in vitro* biofilms differed significantly from both the sewer biofilm and wastewater, being largely dominated by *Enterobacterales*. Moreover, the initial addition of a sewer biofilm at the onset did not alter the taxonomic composition of the biofilms formed. This observation aligns with Medina et al., who reported distinct dominant family taxa in biofilms from simulated sewer experiments compared to actual sewer biofilms (Medina et al., 2020).

Regarding FQ resistance indicators, the percentage of heterotrophic bacteria and *E. coli* resistant to CIP was low both in sewer and *in vitro* biofilms, indicating limited FQ resistance. Kraupner et al. (2018) also assessed *E. coli* resistance to CIP in biofilms developed from treated sewage effluent. Contrary to our results, they reported a high percentage of heterotrophic bacteria resistant to CIP, attributed to the prevalence of intrinsically resistant bacterial species. Further, we quantified quinolone resistance genes *qnrA*, *qnrB*, *qnrD* and *qnrS* in both field samples and in *in vitro* samples. This approach aligns with Pazda et al., who documented these genes in WWTPs (Pazda et al., 2019). Coupons of polycarbonate and concrete material did not lead to a significant difference in accumulation of ARGs, corroborating the findings of Medina et al. (2020) who showed that pipe material did not affect the abundance of ARGs in *in vitro* biofilms. The most common mutations detected were S83L and D87N in the *gyrA* gene and S80I in the *parC* gene. These mutations, also predominantly found in sediment samples (Johnning et al., 2015), are known to confer FQ resistance in *E. coli* (Hooper and Jacoby, 2016). Overall, the various conditions tested in our study did not significantly impact the levels of FQ resistance.

Metagenomic analysis revealed distinct resistomes in each type of sample: sewer biofilm, wastewater, and *in vitro* biofilms. Given the observed differences in taxonomic composition among these sample types, we performed a Procrustes analysis based on the Bray-Curtis distance to investigate the correlation between observed changes in ARG and the microbial community. The significant results from this analysis confirmed that the observed ARG variations were indeed associated with changes in microbial community composition, as it has been described by others (Bengtsson-Palme et al., 2016; Auguet et al., 2017). For instance, genes *mphG* and *aac(3)-IIIc*, which confer resistance to macrolides and aminoglycosides respectively, were under-represented in *in vitro* biofilms compared to sewer biofilm. According to CARD 3.2.4 (Alcock et al., 2023), these genes are more prevalent in bacteria like *Alcaligenes faecalis* of the *Burkholderiales* order or *Pseudomonas aeruginosa* of the *Pseudomonadales* order, both of which are dominant in sewer biofilm. Conversely, several genes related to resistance against antibiotic peptides (*eptA, eptB, MCR.7.1, bacA,* and *pmrF*) associated with *Enterobacterales* (CARD 3.2.4) were over-represented in *in vitro* biofilms. These genes were also more abundant in *in vitro* biofilms than in sewer biofilm.

4.2 Impact of FQ exposure

In vitro biofilms were exposed to two concentrations of FQs to assess their effects on microbiota composition and indicators quantifying FQ resistance. A high concentration of 5,000 µg/L was chosen to assess the potential of such level in selecting resistance. This concentration is notably higher than the EUCAST epidemiological "cut-off" of CIP for *E. coli* (60 µg/L) and the clinical breakpoint for *Enterobacterales* (500 µg/L) (EUCAST, 2023). Additionally, a low concentration of 2.5 µg/L was tested to simulate environmental exposure levels. This concentration falls in the range of FQ concentrations typically found in wastewater in various high-income countries, reported to be between 0.1 and 9.9 µg/L (Jelic et al., 2015; Auguet et al., 2017; Hayes et al., 2022). In our samples, among the 10 FQs/Qs screened in wastewater, only CIP and OFL were detected, with average concentrations of 170.0 (±56.1) and 347.1 (±80.4) ng/L respectively, aligning with levels found in treated urban wastewater in France (Haenni et al., 2022). Importantly, the 2.5 µg/L concentration was likely to have an impact on biofilm microbiota, as the PNEC-R of CIP is estimated to be between 0.004 and 10.8 µg/L, depending on the method used for determining the selective concentration of this antibiotic (Hayes et al., 2022).

High FQ concentration exposure resulted in a reduction in biofilm diversity, a phenomenon paralleling the decreased diversity in the digestive microbiota of patients undergoing antibiotic treatment, as reported by Schwartz et al. (2020). Specifically, high FQ concentrations significantly increased the proportion of CIP-resistant heterotrophic bacteria and *E. coli*. Notably, this condition also dramatically increased the relative abundance of chromosomal mutations in key *E. coli* genes: *gyrA* S83 and D87, and *parC* S80. The most frequent substitutions were a co-occurrence of S83L, D87N and S80I, with S83L and D87 almost systematically being detected on the same read. In contrast, at the lower FQ concentration, only the *gyrA* gene showed a significant increase in its relative abundance, and the S83L substitution without the D87N substitution was the most frequent. Furthermore, the relative abundance of *qnrB*, *qnrS*, *qnrD*, and *qnrA* genes remained largely unchanged after FQ exposure, excepted for an increase of *qnrA* gene with the addition of CIP. These observations are consistent with the results of Kraupner et al. (2018), who determined the selective concentration for CIP in complex aquatic biofilms. They reported that a concentration of 10 µg/L drastically increased the percentage of CIP-resistant *E. coli* and selected chromosomal resistance mutations, predominantly the triple mutation in *gyrA* S83L/D87N and *parC* S80I, similar to our findings using amplicon sequencing from community DNA. However, in contrast to the findings of Kraupner et al., who observed an increase in *qnrB*, *qnrD*, and *qnrS* genes at 10 µg/L, our study did not result in the selection of PMQR genes under high exposure conditions. This disparity could be attributed to the fact that PMQR genes typically confer low-level resistance (Hooper and Jacoby, 2016).

5 Conclusion

In this study, we optimized an *in vitro* model to investigate the impact of FQs on the microbiota of sewer biofilms. Our experimental design included: (i) the use of concrete coupons, chosen for their capacity to yield higher bacterial biomass; (ii) a 14-day incubation time, facilitating the development of more mature biofilms; and (iii) the addition of sewer biofilm to the initial inoculum. Although the inclusion of sewer biofilm did not significantly influence the experimental outcomes, we maintained this approach to better mimic real-world conditions, with biofilm that can break away and migrate in the sewers, and to potentially incorporate population unique to sewer biofilms. Our results indicated differences between *in vitro* and sewer biofilm in terms of microbial composition, abundance of *qnr* genes, and resistome. This finding aligns with recent research by Buelow et al. (2023), which also reported challenges in reproducing field biofilms *in vitro*, noting particularly that the microbiota and extracellular polymeric substance composition of *in vitro* biofilms were more akin to each other than to their natural counterparts (Buelow et al., 2023). We acknowledge that, like all experimental models, ours may have limitations in accurately representing the resistome and microbiota of actual sewer biofilms. However, we demonstrated that our model was effective in tracking multiple changes in biofilms upon FQ exposure. It had no significant impact on the abundance of *qnr* genes whatever the

concentration, but high-concentration exposure increased the proportion of mutations in *gyr*A (codons S83L and D87N) and *par*C (codon S80I). These results underscore the utility of our experimental approach to clarify the role of bacterial communities of sewer biofilms in the dissemination of resistance to FQs in the environment.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, PRJEB69678.

Author contributions

SN: Data curation, Formal analysis, Validation, Writing-original draft, Writing-review & editing. AF: Conceptualization, Investigation, Methodology, Writing-review & editing. PI: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing-review & editing. NA: Investigation, Writing – review & editing. CM: Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. MV: Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. SD: Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. AB-M: Conceptualization, Methodology, Writing-review & editing. FR-P: Conceptualization, Investigation, Methodology, Writing-review & editing. ML: Conceptualization, Investigation, Methodology, Writing-review & editing. CH: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing-review & editing. MB: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing-review & editing. VD: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Validation, Writing-review & editing. DB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Investigation, Validation, Writing-original draft, Writing-review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

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Bacterial enrichment prior to third-generation metagenomic sequencing improves detection of BRD pathogens and genetic determinants of antimicrobial resistance in feedlot cattle

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Introduction: Bovine respiratory disease (BRD) is one of the most important animal health problems in the beef industry. While bacterial culture and antimicrobial susceptibility testing have been used for diagnostic testing, the common practice of examining one isolate per species does not fully reflect the bacterial population in the sample. In contrast, a recent study with metagenomic sequencing of nasal swabs from feedlot cattle is promising in terms of bacterial pathogen identification and detection of antimicrobial resistance genes (ARGs). However, the sensitivity of metagenomic sequencing was impeded by the high proportion of host biomass in the nasal swab samples.

Methods: This pilot study employed a non-selective bacterial enrichment step before nucleic acid extraction to increase the relative proportion of bacterial DNA for sequencing.

Results: Non-selective bacterial enrichment increased the proportion of bacteria relative to host sequence data, allowing increased detection of BRD pathogens compared with unenriched samples. This process also allowed for enhanced detection of ARGs with species-level resolution, including detection of ARGs for bacterial species of interest that were not targeted for culture and susceptibility testing. The long-read sequencing approach enabled ARG detection on individual bacterial reads without the need for assembly. Metagenomics following non-selective bacterial enrichment resulted in substantial agreement for four of six comparisons with culture for respiratory bacteria and substantial or better correlation with qPCR. Comparison between isolate susceptibility results and detection of ARGs was best for macrolide ARGs in *Mannheimia haemolytica* reads but was also substantial for sulfonamide ARGs within *M. haemolytica* and *Pasteurella multocida* reads and tetracycline ARGs in *Histophilus somni* reads.

Discussion: By increasing the proportion of bacterial DNA relative to host DNA through non-selective enrichment, we demonstrated a corresponding increase

in the proportion of sequencing data identifying BRD-associated pathogens and ARGs in deep nasopharyngeal swabs from feedlot cattle using long-read metagenomic sequencing. This method shows promise as a detection strategy for BRD pathogens and ARGs and strikes a balance between processing time, input costs, and generation of on-target data. This approach could serve as a valuable tool to inform antimicrobial management for BRD and support antimicrobial stewardship.

KEYWORDS

long-read metagenomic sequencing, bovine respiratory disease, antimicrobial resistance, feedlot cattle, antimicrobial resistance genes

1 Introduction

Bovine respiratory disease (BRD) is an important cause of morbidity and mortality and is responsible for most of the injectable antimicrobial use in feedlot cattle in western Canada (Brault et al., 2019). Respiratory disease is complex and multifactorial, often involving a combination of bacterial and viral pathogens exacerbated in animals stressed by weaning, transportation, and comingling (Griffin et al., 2010). Antimicrobial use (AMU) is necessary for managing the impacts of BRD on animal health and welfare. Recent WHO guidelines recommended antimicrobial prescriptions for livestock on diagnostic test data (Aidara-Kane et al., 2018).

Laboratory diagnostics to inform AMU for BRD have traditionally relied on bacterial culture, with or without antimicrobial susceptibility testing (AST). Culture-based diagnostic strategies can take up to a week to provide actionable information and, therefore, have limited utility to guide rapid therapeutic decisions that are critical for ensuring BRD treatment success (Wolfger et al., 2015). Typically, culture-based methods test susceptibility of a single isolate per sample and might not represent the susceptibility status of the population of all bacteria of interest within the sample. Additionally, multi-drug-resistant strains of BRD pathogens have emerged (Michael et al., 2012; Lubbers and Hanzlicek, 2013; Klima et al., 2016, 2020; Rainbolt et al., 2016; Snyder et al., 2017), highlighting the importance of identifying bacterial BRD pathogens and characterizing antimicrobial resistance (AMR) to inform prudent AMU.

Molecular methods have been used in veterinary diagnostic testing for decades and provide comparatively faster and potentially more sensitive results than traditional culture (Loy, 2020). Quantitative polymerase chain reaction (qPCR) is widely used in BRD diagnostic testing in the form of commercially available kits (Pneumo4, DNA Diagnostic A/S, Risskov, Denmark) and has been used for detection and quantitation of antimicrobial resistance genes (ARG) in the nasopharyngeal microbiota of Canadian feedlot cattle (Holman et al., 2018; Guo et al., 2020). While qPCR can be superior to classical culture-based methods in terms of turnaround time, it is inherently limited in scope to the known assay targets for which primers have been developed and tested. Typically, multiple reactions are required to test for the presence of multiple pathogens and ARGs.

In contrast, whole genome sequencing (WGS) produces high resolution genomic information for outbreak and AMR surveillance and management (Harrison et al., 2013; Besser et al., 2019; Delgado-Suárez et al., 2021). However, WGS requires culture, isolation, and nucleic acid extraction prior to sequencing.

A shotgun metagenomic sequencing approach combines the rapidness of using DNA extracted directly from clinical samples with a broad, untargeted view of all genetic information in a sample. Metagenomics has the potential to find multiple pathogens and ARGs in a single sequencing run without the need for pathogen isolation or specifying a known genetic target (Adewusi et al., 2024).

Previous proof-of-concept work using third generation Oxford Nanopore Technology (ONT) for long-read metagenomic sequencing of nasal swabs collected from feedlot cattle has shown multiple advantages compared with traditional laboratory methods (Freeman et al., 2022). Bacterial BRD pathogens, including the difficult-to-culture *Mycoplasma bovis*, were reliably identified. Not only did long-read metagenomic sequencing detect BRD pathogens more frequently than bacterial culture but it was also faster and produced some information about the presence of ARGs in the sample. However, concordance between ARGs detected by long-read metagenomic sequencing and phenotypic resistance detected by AST was limited likely due to relatively low sequence coverage of target BRD organisms and excess of bovine-derived host sequence, even with extensive host-depletion. Among 25 samples, the average proportion of non-host-derived sequence was 6%. An excessive host to non-host ratio in sequencing output reduced the ability to characterize the sample for the presence of pathogen and ARG. Analyses with short-read sequencing have shown that high ratios of host to non-host data reduced the sensitivity of microbiome and resistome profiling (Zaheer et al., 2018; Pereira-Marques et al., 2019).

Developing a reliable and cost-effective detection strategy for BRD pathogens and ARGs based on long-read metagenomic sequencing of samples requires more bacterial sequencing coverage than was previously achieved. This additional coverage could be facilitated by increasing the relative amount of non-host to host in the sequenced DNA. Our objective was to evaluate the use of a low-cost non-selective bacterial enrichment of bovine nasopharyngeal swabs as a means of increasing the proportion of target species of interest relative to host biomass. In this study we demonstrate that bacterial enrichment enhances the detection of BRD pathogens (*Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni*) and species-associated ARGs, in addition to allowing us to detect *M. bovis* and *Bibersteinia trehalosi*. Our approach of using non-selective enrichment increased the proportion of sequenced on-target DNA, resulting in the reliability and utility of long-read metagenomic sequencing of nasal swab samples for diagnostics and surveillance. Additionally, using previously collected frozen swabs provided an opportunity to evaluate the potential of this method to assess archived samples.

2 Methods

2.1 Sample selection

We tested frozen swab heads from deep nasopharyngeal (DNP) swabs collected from 20 beef calves stored at −80°C as part of a larger sample collection. The samples used in this experiment were selected for a range of culture and phenotypic AST outcomes. Overall, 10 of the 20 samples were collected in 2020 from a single pen of calves sampled at 6 days on feed (DOF). Six samples were collected from calves sampled at 13 DOF from other pens from the same study, where calves arrived from October to early December 2020. Four samples were collected in 2021 from different early-, mid-, and late-filled feedlot pens (Supplementary Table S1A). The research protocols and procedures for this study were approved by the University of Saskatchewan Animal Care Committee (AUP 20190069).

2.2 Animals and sample collection

Samples were collected at a research feedlot operated by the University of Saskatchewan from 1,600 recently weaned mixed-breed steers purchased from a western Canadian auction market in the fall of 2020 and 2021. Calves were restrained in a hydraulic chute with a neck-extender, and three DNP swabs were collected from each calf from alternating nostrils (collection protocol details in Supplementary material). All three DNP swabs per calf were pooled in the same vial.

2.3 Initial sample processing

Samples were transported to the University of Saskatchewan for processing. The pooled samples (three swabs from each calf) were vortexed for 1 min to release biomass from the swab to the transport medium, and an aliquot was submitted to Prairie Diagnostic Services, Inc. (PDS; Saskatoon, Saskatchewan, Canada) for culture and AST.

2.4 Culture and antimicrobial susceptibility testing

In total, 10 µL inoculation loop of sample was cultured on 5% Columbia sheep blood (BA) and chocolate agar (CHOC) plates and incubated at 35°C for 18 h in an environment of 5% CO_2 to isolate *M. haemolytica*, *P. multocida*, and *H. somni*. Bacterial colonies were incubated for 18 h and 42 h. Isolates of interest were identified using the MALDI-TOF MS Microflex LT instrument (Bruker Daltonik, Bremen, Germany) and the MALDI-TOF MS Biotyper Microflex LT Compass version 1.4 software with MSP library, according to the manufacturer's guidelines. Isolate abundance was scored using a semi-quantitative scale (trace, 1+ to 4+) based on counts of visible colonies following streaking plates by quadrants (identification and quality control details in Supplementary material).

Susceptibility was measured by a commercially available serial broth microdilution panel using the Sensititre™ platform and the commercially available bovine BOPO7F Plate (ThermoFisher Scientific™, Thermo Fisher Scientific, Waltham, Massachusetts, United States), following the manufacturer's instructions and recommendations for testing and quality control (antimicrobials and concentration ranges in Supplementary material). *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *H. somni* ATCC 700025 were used to evaluate the performance. The minimum inhibitory concentration (MIC) plate was placed and read on the BIOMIC® V3 microplate reader. The MIC value was considered equal to the lowest concentration of antimicrobial that inhibited visible growth.

The MICs were compared against breakpoints designated by the Clinical and Laboratory Standards Institute (CLSI) for the pathogens of interest. Isolates with MIC values considered intermediate were categorized as "susceptible" for all analyses (CLSI, 2023).

2.5 Sample processing for molecular and genomic testing

For samples collected in 2020, 2 mL of the remaining transport medium was centrifuged at 4,000 × g for 10 min to pellet biomass. Then, 900 µL of supernatant was decanted, and the pellet was resuspended in the remaining transport medium and host-depleted using the HostZERO™ Microbial DNA Kit (Zymo Research, Irvine, California, United States), according to the manufacturer's recommended protocol and extracted using the QIAGEN DNEasy Blood and Tissue Vacuum Kit (QIAGEN, Hilden, Germany). The dry, swab heads were then stored in cryovials at −80°C without media or cryoprotectant.

Dry swab heads for all samples/years were removed from −80°C for enrichment. The swab head was thawed briefly at room temperature and placed aseptically into sterile glass vials with 14 mL of BHI medium containing 1% glucose and a stir bar. Glass vials were sealed with air permeable Air 'o Top membranes (Thomson Instrument Company, Oceanside, California, United States). Cultures were grown with vigorous aeration at 35°C for 22 h. During this period, 1 mL of each sample was retrieved at 0 h and 8 h and, subsequently, every 2 h until the sample had reached the stationary phase of growth. Stationary phase was determined by optical density (OD) that indicated culture saturation at three consecutive timepoints. Each 1 mL sample was pelleted at 4,000 × g for 10 min, and the medium was discarded. Bacterial pellets were stored at −20°C overnight. Nucleic acid extraction of the resulting enriched bacterial pellets was performed using the Gentra Puregene Buccal Cell Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

DNA concentration was determined using Qubit™ 1x dsDNA Broad Range (BR) or High Sensitivity (HS) Assay Kits (Invitrogen, Carlsbad, California, United States), according to the manufacturer's specifications. The extracted DNA was stored at 4°C for <1 month in TE-buffer until library preparation and qPCR.

2.6 qPCR

To identify *M. haemolytica*, *P. multocida*, and *H. somni*, qPCR was performed in triplicate on the Aria MX (Agilent Technologies, Santa Clara, California, United States) using Taqman™ Fast Advanced MasterMix (Invitrogen, Carlsbad, California, United States). All samples were normalized to 10 ng/µl for

amplification and compared against a standard curve. Total microbial load was quantified by targeting the bacterial 16S rRNA gene (Nadkarni et al., 2002), and the abundance of organisms of interest was quantified by targeting species-specific marker genes (Kishimoto et al., 2017). The results from qPCR were examined to identify time point(s) with the highest concentrations among all BRD organisms of interest for comparative testing after the stationary phase of growth was attained.

2.7 Library preparation and sequencing

Two candidate optimal enrichment timepoints were identified based on qPCR results for *M. haemolytica*, *P. multocida*, and *H. somni*. DNA from the optimal timepoints and an aliquot of DNA that was extracted without enrichment were selected for further testing. Sequencing libraries were prepared using the ONT ligation kit SQK-LSK109 and native barcoding kit (EXP-NBD104 and EXP-NBD114), according to the manufacturer's instructions, with the following reductions to reaction volumes: repair and end prep reactions were scaled to 15 μL, and barcode ligation was scaled to 20 μL. Eight barcoded samples were normalized and pooled into each library, resulting in seven sequencing runs, each run containing eight samples. Sequencing libraries were quantified with the Qubit™ 1x High Sensitivity (HS) Assay Kit (Invitrogen, Carlsbad, California, United States). Overall, 200 ng of each prepared library was loaded onto an FLO-MIN106 flow cell and sequenced on an ONT GridION device for 72 h.

2.8 Bioinformatic analysis

ONT GridION default run parameters and high-accuracy Guppy (v4.0) real-time base calling were used to process raw signal data and remove reads with an average quality < Q7, after which terminal and internal adapters in split reads were removed with Porechop v0.2.4 (Wick et al., 2017). Reads shorter than 100 bp were removed using NanoFilt v2.6.0, and sequence statistics were calculated using NanoStat v1.5.0 (De Coster et al., 2018).

Kraken2 v2.0.8-beta (Wood et al., 2019) was used to classify host and non-host reads with a custom database. The database included all complete genomes in NCBI RefSeq for the bacterial, viral, and archaeal domains on 17 October 2020, as well as the *Bos taurus* reference genome assembly ARS-UCD1.2_Btau5.0.1Y, which consists of the ARS-UCD1.2 genome assembly (Elsik et al., 2009; Rosen et al., 2020). Following classification, reads were divided into two groups using the KrakenTools v1.0 utility extract_kraken_reads.py: those assigned to the *B. taurus* genome were placed into the "host" dataset and those were classified as any other taxa or were unclassified and placed into the "non-host" dataset. Chimeric reads were retrieved from the host dataset as described by Freeman et al. (2022). Taxonomic abundance estimates for organisms of interest were computed by Bracken v2.5 (Lu et al., 2017) from the "non-host" dataset after removing host-like sequences using the KrakenTools script filter_bracken_out.py. The number of reads and total base pairs were reported for BRD bacteria, such as *M. haemolytica*, *P. multocida*, *H. somni*, *M. bovis*, and *B. trehalosi*.

Antimicrobial resistance genes were identified in non-host reads using ABRicate v.1.0.1 (Seemann, n.d.) and AMRFinderPlus v3.9.8 (Feldgarden et al., 2019), both with the NCBI Bacterial Antimicrobial Resistance Reference Gene Database (PRJNA313047, version 2020-12-17). ABRicate was also run using the Comprehensive Antimicrobial Resistance Database (CARD) (Alcock et al., 2019) and MEGARes 2.0 database (Doster et al., 2019). For AMRFinderPlus, the minimum percent identity and percent coverage thresholds were set to 60%, as it is more stringent in reporting ARGs, and the *-plus* option was used to direct the program to search for genes involved in virulence, biocide, heat, metal, and acid resistance. Default parameters were used for ABRicate (80% minimum percent identity and percent coverage).

Theoretical genome coverage was calculated as the sum of the lengths of reads classified as a particular BRD pathogen divided by the size of its reference genome (*M. haemolytica*: 2.8 Mb [NCBI GCF_002285575.1], *P. multocida*: 2.3 Mb [NCBI GCF_002073255.2], *H. somni*: 2.2 Mb [NCBI GCF_000019405.1], *M. bovis*: 0.9 Mb [NCBI GCF_001930225.1], and *B. trehalosi*: 2.3 Mb [NCBI GCF_000521725.1]).

2.9 Statistical analysis

Sequence statistics (number of reads, total base pairs, and theoretical genome coverage) and qPCR results for organism detection were summarized as medians. qPCR copy numbers and sequence statistics were compared between culture-positive and culture-negative samples for no enrichment and 10h and 14h enrichment using the Wilcoxon rank sum test for each organism (StataSE ver 18.0, StataCorp, College Station, TX). The Wilcoxon signed-rank test was used to compare the total base pairs detected for each organism among matched pairs of samples that were unenriched to samples enriched for 10h and 14h. The number of total base pairs was also compared with qPCR concentration for the 10h and 14h enrichment scenarios using Spearman's correlation coefficient.

Concordance was assessed between genomic detection, defined by surpassing a threshold of sequencing reads per organism, and the traditional culture results (either positive or negative) using the kappa statistic for each organism and enrichment duration. In the present study, the cutoff representing species detection with metagenomics was estimated based on the distribution of read counts to optimize the distinction between culture positive and negative samples, leveraging previously reported metagenomic sequencing examples from the literature that used cutoffs of 100 and 1,000 reads (Zhang et al., 2022; Liu et al., 2023). Kappa was interpreted as 0.81–1.0 almost perfect agreement; 0.61–0.80 substantial agreement, 0.40–0.60 moderate agreement, 0.21–0.40 fair agreement, and 0.01–0.20 none to slight agreement (Dohoo et al., 2009).

For AMR and ARG detection for antimicrobials of interest in managing BRD, statistical analyses included kappa to assess agreement between MIC-based AST results and detection of ARGs, Wilcoxon rank sum test to assess differences in ARG numbers between isolates with and without phenotypic AMR, and the Wilcoxon singed-rank test to assess differences in ARGs among unenriched samples and samples enriched for 10h and 14 h. p-values $\leq$ 0.05 were considered significant.

3 Results

3.1 Trajectory of bacterial growth from frozen swabs

Sequential qPCR testing was used initially to screen samples from all time periods and select candidate enrichment time points for metagenomic sequencing. Total bacterial abundance continued to increase up to 10 h of incubation, as determined by qPCR targeting the 16S rRNA gene (Figures 1A,B). *M. haemolytica* plateaued after 8 h. *P. multocida* and *H. somni* were highest at 14 h. Samples from 10 and 14 h were selected for metagenomic sequencing; 10 h was the first time point after total bacterial abundance peaked and 14 h represented as the highest joint concentrations of *M. haemolytica, P. multocida*, and *H. somni.*

3.2 Sequence statistics

After removal of short (<100 bp) and low-quality (<Q7) sequences, the median total base pairs of data available for analyses, number of total reads, read lengths, and read quality were summarized for unenriched, 10- and 14-h enriched samples (Supplementary Tables S1A,B). The median read lengths were longer for enriched than unenriched samples. Non-selective enrichment increased the percentage of non-host DNA in most samples (Table 1). The percentage of non-host sequence measured by total base pairs and total non-host reads was less than 7% for all non-enriched samples. The median percentage of non-host sequence was higher for enriched samples; 54% (range 7–93%) for 10 h and 61% (range 38–94%) for 14 h (Table 1).

Bacterial enrichment increased the percentage of sequence data from BRD pathogens relative to unenriched samples. The increase was

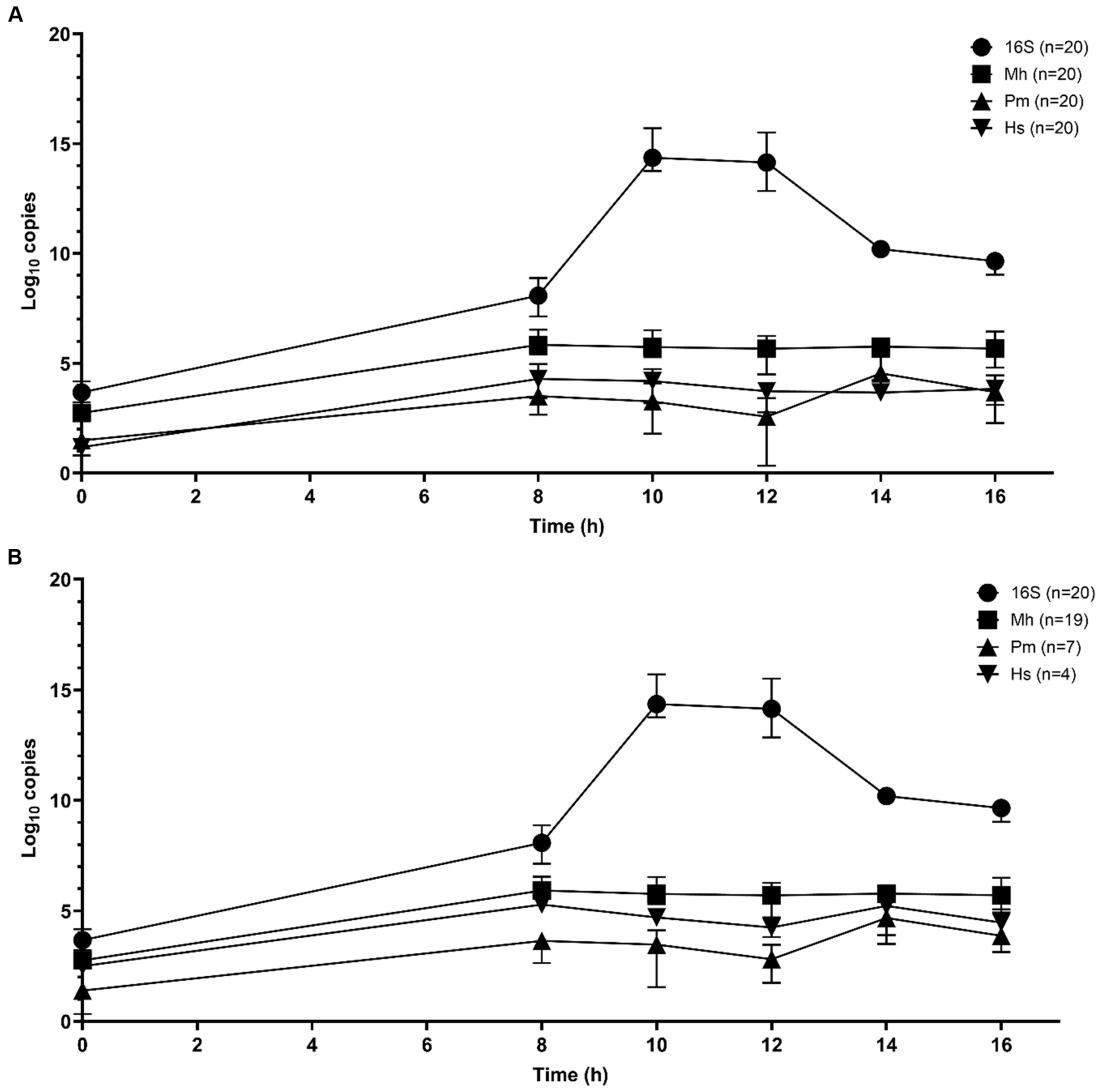

FIGURE 1
Mean qPCR copy numbers (three repeats per n samples—median value and interquartile range) of bacterial Bovine Respiratory Disease pathogens for DNA samples extracted from frozen swabs from 0 h to 16 h of O_2 incubation in BHI broth with 1% glucose (n = 20 samples) for total 16S rRNA gene copies, *M. haemolytica* (Mh), *P. multocida* (Pm), and *H. somni* (Hs) (Nadkarni et al., 2002; Kishimoto et al., 2017). **(A)** All samples and **(B)** the results restricted to samples that were culture-positive for *M. haemolytica* (19 samples), *P. multocida* (7 samples), and *H. somni* (4 samples).

TABLE 1 Percentage non-host sequence based on total base pairs and reads for samples undergoing bacterial enrichment for 10h and 14h compared with DNA from samples that were not enriched.

		Subset matching no enrichment DNA ($n = 16$)			Full data set ($n = 20$)		
		Median	Minimum	Maximum	Median	Minimum	Maximum
Percent non-host sequence (based on total base pairs)	No enrichment	0.3	0.1	6	–	–	–
	10 h	54	7	93	56	7	93
	14 h	61	38	94	61	38	94
Percent non-host reads	No enrichment	0.9	0.3	7	–	–	–
	10 h	38	6	76	34	6	76
	14 h	37	17	84	36	17	84

two to almost four orders of magnitude higher for read numbers and total sequence length (bp) for *M. haemolytica* and two orders of magnitude for *P. multocida* (Table 2, Figure 2, Supplementary Tables S2A,B). There were no comparable culture-positive unenriched samples for *H. somni* (Supplementary Table S2C).

In the absence of enrichment, only one sample produced >1x theoretical genome coverage for any organism (Supplementary Tables S2A–C). This sample had 2.8x coverage for *M. haemolytica* (Supplementary Table S2A) and was the only sample where a culture was semi-quantitatively scored as 3+ out of a possible 4+ abundance of colony growth on the culture plate (Table 2). In contrast, at 14 h, *M. haemolytica* was detected at >30x theoretical coverage in 11 of 19 culture-positive samples (all samples >1.8x coverage) (Supplementary Table S2A). At 14 h, *P. multocida* was detected at >1.6x theoretical coverage in five of seven culture-positive samples (Supplementary Table S2B), and *H. somni* was detected at >8x theoretical coverage in all four culture-positive samples (Supplementary Table S2C). The median theoretical coverage was ≤0.1 for culture negative samples of *P. multocida* and *H. somni* at 10h and 14 h (Supplementary Tables S2B,C). There was only one sample culture negative for *M. haemolytica* with a theoretical coverage of 1.3 for 10 h and 1.9 for 14 h (Supplementary Table S2A). Notably, this sample was also qPCR positive.

M. bovis was only detected in enriched samples (6/20 at 10 h and 2/20 at 14 h) and *B. trehalosi* was detected in more enriched (12/20 at 10 h and 18/20 at 14 h) than unenriched samples (3/16) ($p < 0.05$) (additional details are included in Supplementary material).

3.3 Concordance for species detection

M. haemolytica had been cultured from 19 of 20 samples, followed by *P. multocida* ($n = 7$) and *H. somni* ($n = 4$). Two BRD pathogens were co-isolated from 10 of 20 samples (Supplementary Tables S2A–C).

Culture detection for each of the three organisms of interest was compared with taxonomic classification of sequencing data (Table 2). All 16 unenriched samples (Table 2, Supplementary Table S2A) were *M. haemolytica* positive, and kappa could not be calculated. One unenriched sample with a 3+ abundance culture score had >1,000 *M. haemolytica* reads and the rest had <250 reads. In total, 6 of the 16 culture-positive unenriched samples from 2020 had <10 reads for *M. haemolytica*.

As there was only one culture-negative sample, the estimation of kappa for *M. haemolytica* was limited for the 20 enriched samples. However, there was substantial agreement ($\kappa = 0.64$) between culture positivity and samples with >1,000 reads for 14h. There was very good correlation between the qPCR results and total base pairs sequenced for *M. haemolytica* at 10 h ($\rho = 0.86$) and 14h ($\rho = 0.87$) (Table 2, Supplementary Table S2A).

There were no samples with >100 *P. multocida* reads in the unenriched group (Table 2, Supplementary Table S2B). If samples were considered positive at ≥1 *P. multocida* reads, agreement of unenriched sequencing with culture results was poor ($\kappa = 0.11$). Agreement between detection of >100 reads and culture positivity was substantial ($\kappa = 0.68$) at 14 h and moderate ($\kappa = 0.56$) at 10 h (Table 2). At 14 h, two *P. multocida* culture-negative samples had >100 reads; one was strong positive for *P. multocida* on qPCR suggesting a false-negative culture (Supplementary Table S2B). At 10h and 14h, culture-positive-enriched samples had higher ($p < 0.01$) numbers of *P. multocida* reads, total base pairs, and theoretical genome coverage than culture-negative samples; whereas, there were no significant differences without enrichment (Table 2). There was also good correlation between qPCR results and total base pairs for *P. multocida* at 10h ($\rho = 0.77$) and 14 h ($\rho = 0.79$) (Table 2).

For *H. somni* (Table 2, Supplementary Table S2C), all 2020 samples were culture-negative. In the 10h- and 14h-enriched samples, the agreement between total *H. somni* reads >100 and culture-positive samples was substantial ($\kappa = 0.63$, $\kappa = 0.74$). Correlation was satisfactory to good between qPCR results and total base pairs of *H. somni* at 10 ($\rho = 0.49$) and 14 h ($\rho = 0.68$) (Table 2). The 10h- and 14h-enriched samples had higher ($p < 0.001$) numbers of *H. somni* reads, total base pairs, and theoretical genome coverage in culture-positive versus culture-negative samples (Table 2).

3.4 ARG detection and concordance for enriched samples

Concordance was summarized between culture of target BRD bacteria with phenotypic AST based on MICs (Supplementary Table S3) and identification of known ARGs (Supplementary Tables S4, S5) for unenriched samples, where ARGs were detected and for all samples enriched for 10 h and 14 h (Tables 3–5). The most common types of phenotypic resistance based on CLSI breakpoints were for the macrolides gamithromycin and tulathromycin, with only one tested sample with a tetracycline resistance isolate; all three antimicrobials are used in BRD management (Brault et al., 2019) (Tables 3–5, Supplementary Table S3). Samples with sulfadimethoxine MICs

>256 μg/mL were also considered in the analysis based on the prevalence (Supplementary Table S3) and the use of trimethoprim-sulfamethoxazole in the treatment of BRD (Brault et al., 2019).

3.5 Antimicrobial resistance genes in unenriched samples

Detection of ARGs in unenriched samples was limited to ≤3 reads in two samples (Tables 3–5). In one sample, where the unenriched theoretical coverage of *M. haemolytica* was 2.8 (Supplementary Table S2A, sample 2045Bi2-023), and AST phenotypes included macrolide resistance and sulfadimethoxine MICs >256 μg/mL, 2 *sul2*, 2 *mphE*, 1 *msrE*, and 1 *tet(34)* genes were detected in the unenriched sequence data (Table 3, Supplementary Tables S4, S5A). In the second sample (2045Bi2-003), which was culture-positive for *H. somni* (Supplementary Table S2C) with no detected phenotypic resistance (Table 5), a single *tet(H)* gene was identified in the unenriched sequence data (Table 5, Supplementary Tables S4, S5C).

Resistance genes were detected more frequently in enriched samples than in unenriched samples, particularly where phenotypic resistance was most prevalent (Tables 3–5). The differences between unenriched and enriched samples were significant for macrolide and sulfonamide resistance genes within *M. haemolytica* reads (Table 3) and for tetracycline resistance genes within *P. multocida* reads (Table 4). Furthermore, ARGs were more likely to be detected (> 1 ARG) regardless of enrichment status with increased total number of base pairs for *M. haemolytica* ($p < 0.001$), *P. multocida* ($p < 0.001$), or *H. somni* ($p = 0.03$).

3.6 Detection of ARGs coding for macrolide resistance from enriched samples

Macrolide ARGs were identified within at least two *M. haemolytica* reads (Table 3, Supplementary Tables S4, S5A) at 10h (11/20 samples) and 14 h (8/20 samples). Agreement between detection of at least two *M. haemolytica* reads with macrolide ARGs with phenotypic AMR for gamithromycin and tulathromycin was substantial ($\kappa = 0.68$) for 10 h and almost perfect ($\kappa = 0.83$) for 14 h (Table 3). The number of *M. haemolytica* reads with macrolide ARGs was also higher at 10h ($p = 0.02$) and 14 h ($p = 0.01$) in samples where *M. haemolytica* isolates displayed phenotypic macrolide resistance compared with samples susceptible *M. haemolytica* isolates.

Genes coding for macrolide resistance were identified in 463 unique *M. haemolytica* reads sequenced in the enriched samples by at least one of the NCBI, CARD, or MEGARes databases (Supplementary Tables S4, S5A). The 859 macrolide genes on *M. haemolytica* reads had a median identity of 96% (IQR, 93–97%) and a median coverage of 98% (IQR, 97–99%) (Supplemental Table S5A). The macrolide resistance-associated genes identified on *M. haemolytica* reads in these samples were *mphE* (53%, 457/859) and *msrE* (47%, 402/859). Both *mphE* and *msrE* were identified on 81% of the 463 reads that contained at least one of these genes. Both genes were also identified on *M. haemolytica* reads in all eight samples with macrolide-resistant isolates and five of the remaining eight samples with susceptible isolates.

Macrolide ARGs were also identified within at least two *P. multocida* reads (Table 4, Supplementary Tables S4, S5B) from samples at 10h (3/20 samples) and 14 h (5/20 samples). However, there were no samples with cultured macrolide-resistant *P. multocida* (Table 4, Supplementary Table S3). Macrolide ARGs (53% *msrE* (25/47) and 47% *mphE* (22/47)) were identified on 24 *P. multocida* reads in enriched samples by at least one database. The median gene identity was 96% (IQR, 89–99%) and the median coverage was 98% (IQR, 95–100%) (Supplementary Table S5B).

No macrolide resistance-associated genes were identified within the *H. somni* reads from the 20 enriched samples (Table 5, Supplementary Tables S4, S5C). None of the samples had *H. somni* isolates that displayed phenotypic resistance to macrolides (Table 5, Supplementary Table S3).

Macrolide ARGs (*msrE*, *mphE*) were identified in 46 *B. trehalosi* reads from four samples enriched for 10 h and the same four samples plus two more enriched for 14 h. Both genes had been identified in *M. haemolytica* reads from the same six samples.

3.7 Detection of ARGs coding for sulfonamide resistance for enriched samples

M. haemolytica reads containing sulfonamide ARGs (Table 3, Supplementary Tables S4, S5A) were identified at least twice in samples for 10 h (12/20 samples) and 14 h (13/20 samples). Agreement for detection of at least two *M. haemolytica* reads containing sulfonamide ARGs (*sul2* gene) and samples culture-positive for *M. haemolytica* with MIC >256 μg/mL for sulfadimethoxine were substantial ($\kappa = 0.65$) for 10 and 14 h (Table 3, Supplementary Table S3). The number of *sul2* genes detected in *M. haemolytica* reads was higher for samples with *M. haemolytica* isolates with sulfadimethoxine for MICs >256 μg/mL than those without sulfadimethoxine in 10 h-enriched samples ($p = 0.03$) but not in 14-h-enriched samples ($p = 0.11$). The *sul2* gene ($n = 761$) was identified in 735 *M. haemolytica* reads from enriched samples. The median *sul2* gene identity was 95% (IQR, 92–97%) and the median coverage was 98% (IQR, 97–99%).

Sulfonamide ARGs within *P. multocida* reads (Table 4, Supplementary Tables S4, S5B) were identified in at least two reads for samples for 10h (3/20) and 14 h (4/20). Agreement between detection of *sul2* genes and phenotypic MICs >256 μg/mL was moderate for 10h- ($\kappa = 0.60$) and 14h-enriched samples ($\kappa = 0.48$) (Table 4). There was no significant difference in the number of *sul2* genes detected for samples with *P. multocida* isolates with MICs for sulfonamides >256 μg/mL and those without MICs (Table 4). Forty *sul2* genes were identified in 28 *P. multocida* reads from enriched samples; median identity was 95% (IQR, 93–97%) and median coverage was 98% (IQR, 97–99%).

No sulfonamide ARGs were detected within *H. somni* sequence data from samples enriched for either 10h or 14 h (Table 5, Supplementary Tables S4, S5C). However, the sulfadimethoxine MIC for all four *H. somni* isolates was >256 μg/mL.

A sulfonamide ARG (*sul2*) was identified within *B. trehalosi* in 56 reads from four samples enriched for 10 h and the same four samples plus five additional samples enriched for 14 h.

TABLE 2 Taxonomic classification statistics for Bovine Respiratory Disease pathogens: culture results, number of reads, amount of sequence (total base pairs—bp), and the theoretical genomic coverage of that sequence for different sample enrichment treatments.

Sample type and ID	Culture	No enrichment			10h enrichment				14h enrichment			
		Number of reads	Total base pairs	Theoretical coverage	qPCR (copies)	Number of reads	Total base pairs	Theoretical coverage	qPCR (copies)	Number of reads	Total base pairs	Theoretical coverage
Mannheimia haemolytica												
Median all samples	$n = 20$	16	5.6×10^4	0.02	1.94×10^6	2.30×10^3	2.42×10^7	8.6	2.93×10^6	1.19×10^4	1.06×10^8	37.9
Median culture positives	$n = 19$	16	5.6×10^4	0.02	2.38×10^6	2.52×10^3	2.56×10^7	9.2	3.31×10^6	1.26×10^4	1.11×10^8	39.7
Median all 2020 samples	$n = 16$	16	5.6×10^4	0.02	1.94×10^6	2.30×10^3	2.42×10^7	8.6	2.93×10^6	1.19×10^4	1.06×10^8	37.9
Median 2020 culture positives	$n = 16$	16	5.6×10^4	0.02	1.94×10^6	2.30×10^3	2.42×10^7	8.6	2.93×10^6	1.19×10^4	1.06×10^8	37.9
Median culture negatives	$n = 1$	Not sequenced			782	430	3.62×10^6	1.3	71.4	697	5.24×10^6	1.9
Kappa (reads > 1,000 vs. culture)	$n = 20$	NA			$\kappa = 0.35$ Fair agreement				$\kappa = 0.64$ Substantial agreement			
Spearman's ρ/*p*-value (PCR ng vs. total bp)	$n = 20$				0.86/$p < 0.001$				0.87/$p < 0.001$			
WSR Test *p*-value (exact): comparison of total base pairs	$n = 16$	NE vs. 10 h $p < 0.001$	NE vs. 14 h $p < 0.001$	10h vs. 14 h $p = 0.07$								
Pasteurella multocida												
Median all samples	$n = 20$	3	4.22×10^3	0.002	2.04×10^3	72	4.89×10^5	0.2	4.79×10^4	68	6.05×10^5	0.3
Median culture positives	$n = 7$	7	1.39×10^4	0.01	1.66×10^3	420	2.24×10^6	1	3.04×10^4	504	4.15×10^6	1.8
Median all 2020 samples	$n = 16$	3	4.22×10^3	0.002	1.10×10^3	72	5.85×10^5	0.3	3.64×10^4	68	7.80×10^5	0.3
Median 2020 culture positives	$n = 6$	7	1.39×10^4	0.01	1.66×10^3	420	2.24×10^6	1	3.04×10^4	504	4.15×10^6	1.8
Median culture negatives	$n = 13$	1	5.62×10^2	0.0002	1.10×10^3	22	1.87×10^5	0.1	2.25×10^5	43	3.11×10^5	0.1
WRS test: *p*-value (exact)	$n = 20$	$p = 0.11$	$p = 0.23$	p = 0.23	$p < 0.001$	$p < 0.001$	$p = 0.001$	p = 0.001	$p = 0.001$	$p = 0.006$	$p = 0.005$	$p = 0.005$
Kappa (reads > 100 vs. culture)	$n = 20$	NA	($\kappa = 0.11$ - reads >1 vs. culture)		$\kappa = 0.68$ Substantial agreement				$\kappa = 0.56$ Moderate agreement			
Spearman's ρ/*p*-value (PCR ng vs. total bp)	$n = 20$				0.77/$p < 0.001$				0.79/$p < 0.001$			
WSR Test *p*-value (exact): comparison of total base pairs	$n = 16$	NE vs. 10 h $p < 0.001$	NE vs. 14 h $p < 0.001$	10 vs. 14 h $p = 0.03$								
Histophilus somni												
Median all samples	$n = 20$	0	0	0	1.22×10^4	42	3.50×10^5	0.2	4.60×10^3	17	5.83×10^4	0.03
Median culture positives	$n = 4$	Not sequenced			6.81×10^4	482	6.81×10^4	482	5.56×10^6	2.5	1.85×10^5	4,298
Median all 2020 samples	$n = 16$	0	0	0	3.90×10^3	15	6.37×10^4	0.03	4.02×10^3	14	4.33×10^4	0.02

(Continued)

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TABLE 2 (Continued)

Sample type and ID	Culture	No enrichment			10h enrichment				14h enrichment			
		Number of reads	Total base pairs	Theoretical coverage	qPCR (copies)	Number of reads	Total base pairs	Theoretical coverage	qPCR (copies)	Number of reads	Total base pairs	Theoretical coverage
Median 2020 culture positives	$n = 0$	No culture positives: 2020 samples			No culture positives: 2020 samples				No culture positives: 2020 samples			
Median culture negatives	$n = 16$	0	0	0	3.90×10^3	24	2.39×10^5	0.1	3.67×10^3	11	2.35×10^4	0.01
WRS test p-value (exact)	$n = 20$	NA	NA	NA	$p = 0.02$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.03$	$p < 0.001$	$p < 0.001$	$p < 0.001$
Kappa (reads > 100 vs. culture)	$n = 20$	NA			κ = 0.63 Substantial agreement				κ = 0.74 Substantial agreement			
Spearman's ρ/p-value (PCR ng vs. total bp)	$n = 20$				$0.49/p = 0.03$				$0.68/p = 0.001$			
WSR Test p-value (exact): comparison of total base pairs	$n = 16$	NE vs. 10h $p < 0.001$	NE vs. 14 h $p = 0.005$	10h vs. 14h $p = 0.19$								

WRS test: Wilcoxon Rank-Sum test of value (qPCR concentration, number of reads, total bp, or coverage) of culture-positive compared with culture-negative samples for 10h and 14h enrichment. NA: Kappa not calculated no culture positives; NE: no enrichment samples.

WSR test: Wilcoxon Signed-Rank test of read counts of comparisons between unenriched and enriched for 10h and enriched for 14h.

3.8 Detection of ARGs coding for tetracycline resistance for enriched samples

Tetracycline ARGs were detected within at least two *M. haemolytica* reads (Table 3, Supplementary Tables S4, S5A) in 10h (11/20) and 14h-enriched (14/20) samples; however, there was no detected phenotypic resistance in culture-positive isolates (Table 3, Supplementary Table S3). In all enriched samples, 277 tetracycline ARGs were detected in 275 *M. haemolytica* reads. The primary tetracycline ARGs included *tet(H)* (60%, 167/277) and *tet(34)* (39%, 109/277). The median identity for the *tet(H)* genes was 95% (IQR, 93–97%) and median coverage was 98% (IQR, 97–99%) but median identity for the *tet(34)* genes was only 62% (IQR, 61–62%) and median coverage was 77% (IQR, 67–84%). The *tet(34)* genes were not detected by the Abricate option and MEGARes databases likely due to the higher cutoff of 80% identity. The *tet(H)* genes were detected in the same six samples for 10h and 14h.

Tetracycline ARGs were detected within at least two *P. multocida* reads (Supplementary Tables S4, S5B) for 10h (4/20) and 14h (6/20) (Table 4). The *tet(H)* gene was detected in at least two *P. multocida* reads in four samples for 10h and five samples for 14h. One sample contained two *tet(34)* genes with low identity scores (62%) as reported for the same gene from *M. haemolytica*. No *P. multocida* isolates were phenotypically resistant to tetracycline (Table 4, Supplementary Table S3).

The *tet(H)* genes were detected in at least two *H. somni* reads for two samples for 10h and 14h. The *tet(H)* gene was identified in one of four *H. somni* culture-positive samples with tetracycline-resistant isolates (Table 5, Supplementary Tables S3, S4, S5C).

The tetracycline resistance gene (*tet(H)*) was identified within *B. trehalosi* in eight reads from one sample for 10h and the same sample enriched for 14h.

3.9 Other resistance genes

The only other ARGs reported at least twice in enriched samples conferred aminoglycoside resistance. The most common was *APH(3′)*. There were 282 *APH(3′)-Ia* genes identified on 278 *M. haemolytica* reads from 14 samples and 13 genes on 12 *P. multocida* reads from 4 samples (Supplementary Tables S4A,B, S5A,B). There were also 232 *APH(3′)-Ib* genes identified on 230 *M. haemolytica* reads from 14 samples, and 20 genes were identified on 18 *P. multocida* reads from eight samples.

Another identified aminoglycoside resistance gene was *APH(6)-Id*, with 245 *APH(6)-Id* genes identified on 240 *M. haemolytica* reads from 14 samples and 13 genes on 12 *P. multocida* reads from 4 samples (Supplementary Tables S4A,B, S5A,B).

No aminoglycoside ARGs were identified in *H. somni* reads (Supplementary Tables S4A,B, S5C) or *B. trehalosi* reads.

3.10 Detection of multiple ARGs on single reads

Multiple ARGs were detected on 739 long individual reads for 31 metagenomic analyses of 19 unique samples (Supplementary Table S6).

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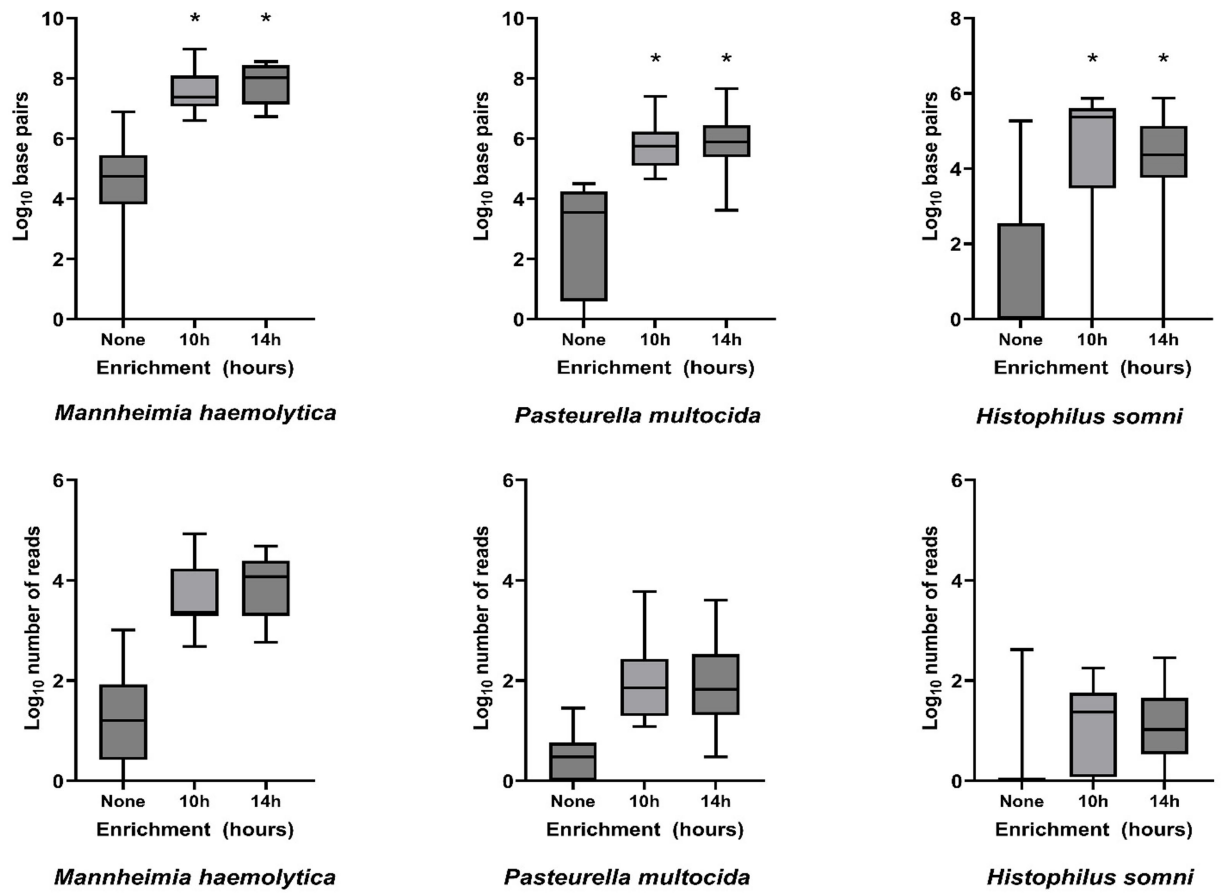

FIGURE 2
Log_{10}-transformed total DNA base pairs and total number of DNA read counts for each enrichment treatment (none, 10 h, and 14 h, $n = 16$ for each) of frozen swabs to detect bacterial Bovine Respiratory Disease pathogens by metagenomic sequencing. Box and whisker plots: boxes include the median and upper and lower quartiles; whiskers include the minimum and maximum values. *$p < 0.01$ on Wilcoxon signed-rank test of the total base pairs for enrichment treatment compared with no enrichment.

In total, 2 of the 739 reads with multiple ARGs were from unenriched samples.

The median number of unique ARGs on each read was three (5th percentile = two, 95th percentile = four). The median length of individual reads on which multiple ARGs were found was 21,941 bp (5th percentile 7,971, 95th percentile 53,678 bp). Most reads with multiple ARGs were *M. haemolytica* ($n = 694$), with smaller numbers of *P. multocida* (n = 42) and only one *H. somni* read. The most common pattern of multiple ARGs per read was *msrE*, *mphE*, and *sul2* in 349 reads followed by *APH(3″)-Ib*, *APH(6)-Id*, *APH(3′)-Ia*, and *sul2* in 188 reads. Both macrolide ARGs and sulfonamide ARGs were detected in 45% of the 774 *M. haemolytica* reads with at least one of these genes and in at least one *M. haemolytica* read from 78% (14/18) of samples with at least one of these genes.

4 Discussion

Non-selective bacterial enrichment increased the amount of on-target data available from metagenomic sequencing of nasal samples from feedlot cattle enabling previously unreported robust detection of ARGs on species-specific respiratory bacterial reads in complex respiratory samples from healthy animals. Incubating the sample in growth medium for 10h and 14 h increased bacterial numbers for three important BRD pathogens of interest relative to unenriched samples and for *B. trehalosi*. In the case of *M. haemolytica* and *B. trehalosi*, enrichment also increased the read detections of ARGs for macrolide and sulfonamide resistance, and tetracycline ARGs for *P. multocida*.

Host DNA is a major impediment in metagenome analysis, particularly in nasal swab samples, where >90% of sequencing reads can be host-derived (Marotz et al., 2018; Chen and Xu, 2023; Ring et al., 2023). High proportions of host DNA reduce the sensitivity of metagenomic sequencing, especially for detecting low-abundance bacteria (Pereira-Marques et al., 2019). In this study, all 20 enriched samples resulted in >93% non-host DNA for both the 10h and 14h protocols. For the three BRD bacterial pathogens of most interest, *M. haemolytica*, *P. multocida,* and *H. somni*, non-selective enrichment increased the detection of targeted sequence by several orders of magnitude, particularly in culture-positive samples. Increased coverage of the pathogen genomes was directly associated with the detections of ARGs within identified bacterial reads for antimicrobials of interest such as macrolides and tetracyclines. Another recent study of metagenomic identification of pathogens in blood cultures also

described increased ARG detection with increased total base pairs reported for target organisms of interest (Liu et al., 2023), confirming the clinical relevance of additional on-target bacterial sequence data.

At least some reads for all three BRD pathogens were detected in sequence data from samples with negative culture results, particularly for enriched samples. This finding could partially be explained by the ability of sequence-based approaches to detect DNA from growth-inhibited or dead pathogens (Shao et al., 2022). This discrepancy could also be due to the inherent sampling bias of streak plate-based identification of pathogens, wherein only 10 µL loop of the original sample is used for analysis, which could miss low-abundance organisms. In previous studies comparing detection of BRD pathogens, sequencing demonstrated positive results more frequently than culture, and the concordance between these techniques varied by organism (Bell et al., 2014; Freeman et al., 2022). However, in the present study, the sequence and qPCR data generated for enriched culture-positive samples were significantly higher than culture-negative samples.

In the present study, one advantage of metagenomic sequencing for BRD pathogen detection is that it can provide a snapshot of the nasal bacteria beyond the detection of specific organisms targeted by routine culture, such as *M. bovis* and *B. trehalosi*. However, non-selective bacterial enrichment can modify the relative quantities of identified organisms, as the community structure can be altered by variation in replication rate and overgrowth of some organisms (Jarvis et al., 2015; Leonard et al., 2015). In the present study, differences in the absolute read numbers and relative depth of sequence data were recognized for target organisms of interest. There was a higher chance of detecting ARGs of interest in samples that had higher reads and more base pairs for BRD organisms. However, the protocol was optimized to include 14h enrichment based on qPCR detection of the three primary organisms of interest. The long-read metagenomic sequencing of our samples had good agreement with culture and reflected what we expected to observe in calves when they arrive at the feedlot; initial recovery of *M. haemolytica* and *P. multocida* with increasing frequency of *H. somni* and *M. bovis* in the feeding period (Alhamami et al., 2021; Andrés-Lasheras et al., 2021; Younes et al., 2022).

While the impact of non-selective enrichment on description of the microbiome requires further research, this study demonstrates that this approach was successful in identifying primary bacteria of interest for managing BRD and enhancing detection of ARGs in these organisms. While not all organisms were expected to benefit equally from the enrichment protocol, culture times were relatively short; DNA from unculturable (i.e., dead) or difficult to culture bacteria was unlikely to be lost with enrichment, and therefore, the risk of losing taxonomic breadth was minimal (Lennon et al., 2018; Shao et al., 2022). Even *M. bovis*, which replicates slowly and requires additional nutrients not provided by the enrichment medium used in this study (McVey et al., 2013), was detected following bacterial enrichment. Furthermore, *M. bovis* was identified in calves sampled early in the feeding period when we did not expect a high prevalence (Freeman et al., 2022). However, the resulting sequencing data were limited with 0.23 as the highest observed theoretical coverage of *M. bovis* in a single sample.

In a proof-of-concept study (Freeman et al., 2022), metagenomic sequencing without bacterial enrichment detected BRD pathogens of interest more frequently than did culture, but ARGs from relevant taxa were not reliably detected due to high levels of host DNA. Our findings show that with enrichment and the resulting increased sequencing coverage of target organisms, hundreds of ARGs were detected with taxonomic resolution to the species level, with moderate and better concordance with phenotypic resistance.

Samples with *M. haemolytica* isolates displaying phenotypic resistance to the 15-member macrolides, tulathromycin and gamithromycin, were selected for this pilot study. Agreement between AST results for macrolides in *M. haemolytica* and the detection of ARGs on *M. haemolytica* reads was in agreement with 14h enrichment. The genes *mphE* and *msrE* encode a macrolide phosphotransferase protein and an ABC transporter protein, respectively (Kadlec et al., 2011), and have been consistently associated with macrolide resistance in *Pasteurellaceae* isolates derived from feedlot cattle (Alhamami et al., 2021; Andrés-Lasheras et al., 2021; Younes et al., 2022). These two genes were recovered together on 81% of individual *M. haemolytica* reads with any macrolide resistance and all of the samples with phenotypic macrolide-resistant *M. haemolytica*. The colocation of these two genes was expected as was found in previous reports using PCR and assemblies based on whole genome sequencing (Clawson et al., 2016; Snyder et al., 2019; Klima et al., 2020; Stanford et al., 2020) but has not been previously described on individual raw reads from metagenomic data.

The *erm42* gene is often present in isolates exhibiting resistance to gamithromycin, tulathromycin, and tilmicosin (Desmolaize et al., 2011; Rose et al., 2012; Snyder et al., 2019) but was not detected in these samples. Although macrolide resistance can emerge from point mutations in the 23S rRNA gene (Olsen et al., 2015); the phenotypic resistance in this study was explained by the presence of previously characterized macrolide ARGs. Macrolide ARGs were also detected on reads of BRD organisms where the specific organisms were absent on culture or AST reported for the tested isolate from the sample was susceptible to macrolides. However, smaller number of macrolide ARGs were present in the absence of phenotypic macrolide-resistant *M. haemolytica*. Metagenomics could have detected ARG reads in samples with isolates that were either not detected or not selected for MIC testing.

In this study, high MICs for sulfadimethoxine (MIC >256 µg/mL) were observed for isolates of all three BRD pathogens. Agreement between the *sul2* gene and high sulfadimethoxine MICs was at least moderate for *M. haemolytica* and *P. multocida*. However, in the four samples where sulfadimethoxine-resistant *H. somni* isolates were recovered, no *sul2* genes were detected suggesting another potential, undetected resistance mechanism. Furthermore, almost half of all *M. haemolytica* reads and four out of five samples with either macrolide or sulfonamide ARGs had both macrolide and sulfonamide ARGs. This suggests that these genes are frequently collocated potentially on integrative and conjugative elements (ICE) (Clawson et al., 2016; Beker et al., 2018). Colocation of macrolide and sulfonamide ARGs was also observed in *B. trehalosi* reads.

In this study, tetracycline phenotypic resistance was only reported in one *H. somni* isolate. The tetracycline resistance gene *tet(H)* was identified in this sample and encoded a tetracycline efflux protein. Other studies have found variable concordance with phenotypic resistance to tetracycline and *tet* genes (Owen et al., 2017; Snyder et al., 2020). The *tet(34)* gene was also identified in several samples; however, given the lack of concordance with phenotypic resistance

TABLE 3 Concordance between phenotypic antimicrobial resistance and number of reads with antimicrobial resistance genes (ARGs) within *Mannheimia haemolytica* reads.

	Antimicrobial resistance and counts of resistance genes: *Mannheimia haemolytica*											
	Macrolides				Sulfonamides				Tetracyclines (*tet(H)*)			
Sample ID	AST	Unenriched	10 h	14 h	AST	Unenriched	10 h	14 h	AST	Unenriched	10 h	14 h
2045Bi2-003	S	0	2	0	SUL	0	3	0	TET-I	0	1 (1)	1 (1)
2045Bi2-004	No MIC	0	3	0	No MIC	0	40	48	No MIC	0	41 (36)	52 (46)
2045Bi2-018	S	0	0	0	SUL	0	12	37	TET-I	0	6 (6)	38 (33)
2045Bi2-023	GAM, TUL	3	304	50	SUL	2	183	31	S	1 (0)	20 (0)	5 (0)
2045Bi2-046	No MIC	0	2	0	No MIC	0	9	4	No MIC	0	7 (7)	2 (2)
2045Bi2-053	No MIC	0	4	0	No MIC	0	4	4	No MIC	0	1 (1)	0 (0)
2045Bi2-055	No MIC	0	0	4	No MIC	0	0	2	No MIC	0	0 (0)	0 (0)
2045Bi2-063	No MIC	0	1	0	No MIC	0	1	0	No MIC	0	0 (0)	0 (0)
2045Bi2-067	GAM, TUL	0	2	59	SUL	0	3	39	S	0	0 (0)	7 (0)
2045Bi2-070	No MIC	0	0	0	No MIC	0	16	39	No MIC	0	11 (9)	18 (16)
2045Ai2-012	GAM, TUL	0	6	33	SUL	0	4	23	S	0	1 (0)	2 (0)
2046bi2-013	GAM, TUL	0	34	127	SUL	0	26	80	S	0	0 (0)	9 (0)
2045Bix2-015	GAM, TUL	0	33	23	SUL	0	20	19	S	0	4 (0)	3 (0)
2048Ai2-036	GAM, TUL	0	60	107	SUL	0	32	72	S	0	2 (0)	4 (0)
2048Ai2-083	GAM, TUL	0	0	2	SUL	0	0	7	S	0	2 (2)	4 (3)
2046Ai2-095	GAM, TUL	0	2	0	SUL	0	1	0	S	0	0 (0)	0 (0)
2148Bdev3009	No MIC	No data	0	0	No MIC	No data	0	0	No MIC	No data	3 (0)	13 (0)
2145Ax8-011	S	No data	0	0	S	No data	0	0	S	No data	0 (0)	0 (0)
2146Bdev014	S	No data	0	1	S	No data	0	1	S	No data	3 (0)	13 (0)
2146Bdev13	ND	No data	0	1	ND	No data	0	1	ND	No data	2 (2)	2 (2)
Median (R)	8/12 = 67%	0	6	42	10/12 = 83%	0	8	27	0/12 = 0%		N/T	N/T
Median (S)	4/12 = 33%	0	0	0	2/12 = 17%	N/T	0	0.5	12/12 = 100%		2 (0)	4 (0)
Median (ND)	$n = 1$	No data	0	1	$n = 1$	N/T	0	1	$n = 1$		2 (2)	2 (2)
Median (No MIC)	$n = 7$	0	1	0	$n = 7$	0	4	4	$n = 7$		3 (1)	2 (0)
Kappa (>1 ARG)	$n = 13$	N/T	κ = 0.68 substantial	κ = 0.84 almost perfect	$n = 13$	N/T	κ = 0.65 substantial	κ = 0.65 substantial	$n = 13$	N/T	N/T	N/T
WRS Test *p*-value (exact)		$p = 0.99$	$p = 0.02$	$p = 0.01$		$p = 0.99$	$p = 0.03$	$p = 0.11$		$p = 0.99$	N/T	N/T
WSR Test *p*-value (exact)	$n = 16$	NE vs 10 h $p < 0.001$	NE vs 14 h $p = 0.008$	10 vs 14 h $p = 0.91$	$n = 16$	NE vs 10 h $p < 0.001$	NE vs 14 h $p = 0.0002$	10 vs 14 h $p = 0.14$	$n = 16$	NE vs 10 h $p = 0.50$	NE vs 14 h $p = 0.25$	10 vs 14 h $p = 0.99$

AST: antimicrobial susceptibility testing result (CLSI breakpoints specific to *M. haemolytica*). GAM: gamithromycin (MIC ≥ 16 ug/ml), TUL: tulathromycin (MIC ≥ 64 ug/ml), SUL: sulfamethoxazole (MIC > 256 ug/ml) (no CLSI breakpoint), TET: tetracycline (MIC ≥ 8ug/ml), TET-I: tetracycline intermediate susceptibility (MIC = 4 ug/ml). ND: not detected (not cultured), No MIC (minimum inhibitory concentration and phenotypic susceptibility not available—excluded from concordance), S: susceptible. R: resistant. N/T: not tested (did not have ≥1 isolates with phenotypic resistance or ≥1 isolates with ARG reads). Kappa based on comparison of >1 ARG to phenotypic AMR where negative = S or ND (isolates with No MIC excluded). WRS Test: Wilcoxon Rank Sum test of read counts compared between phenotypic AMR detection where negative = S or ND. WSR Test: Wilcoxon Signed Rank test comparison of read counts between unenriched and enriched for 10h and enriched for 14h. Tetracycline resistant reads, first value is total tetracycline ARGs and value in () is specifically for *tet(H)*.

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TABLE 4 Concordance between phenotypic antimicrobial resistance and number of reads with antimicrobial resistance genes (ARGs) within *Pasteurella multocida* reads.

	Antimicrobial Resistance and Counts of Resistance Genes: *Pasteurella multocida*											
	Macrolides				Sulfonamides				Tetracyclines (*tet(H)*)			
Sample ID	AST	Unenriched	10 h	14 h	AST	Unenriched	10 h	14 h	AST	Unenriched	10 h	14 h
2045Bi2-003	ND	0	0	0	ND	0	0	0	ND	0	0 (0)	0 (0)
2045Bi2-004	ND	0	0	0	ND	0	1	3	ND	0	16 (16)	16 (16)
2045Bi2-018	ND	0	0	0	ND	0	0	1	ND	0	4 (4)	10 (10)
2045Bi2-023	ND	0	20	2	ND	0	6	0	ND	0	0 (0)	0 (0)
2045Bi2-046	ND	0	0	0	ND	0	0	0	ND	0	0 (0)	3 (3)
2045Bi2-053	S	0	0	0	S	0	1	0	S	0	0 (0)	3 (1)
2045Bi2-055	ND	0	0	0	ND	0	0	0	ND	0	0 (0)	0 (0)
2045Bi2-063	ND	0	0	0	ND	0	0	0	ND	0	0 (0)	0 (0)
2045Bi2-067	ND	0	0	0	ND	0	0	0	ND	0	0 (0)	0 (0)
2045Bi2-070	ND	0	0	0	ND	0	0	0	ND	0	6 (6)	21 (21)
2045Ai2-012	ND	0	0	0	ND	0	0	0	ND	0	0 (0)	0 (0)
2046bi2-013	S	0	0	6	S	0	0	2	S	0	0 (0)	0 (0)
2045Bix2-015	S	0	2	8	SUL	0	4	3	S	0	0 (0)	1 (1)
2048Ai2-036	S	0	2	5	SUL	0	0	0	S	0	0 (0)	0 (0)
2048Ai2-083	S	0	0	0	SUL	0	8	11	S	0	2 (2)	14 (12)
2046Ai2-095	S	0	0	0	S	0	0	0	S	0	0 (0)	0 (0)
2148Bdev3009	ND	No data	0	2	ND	No data	0	0	ND	No data	0 (0)	0 (0)
2145Ax8-011	ND	No data	0	0	ND	No data	0	0	ND	No data	0 (0)	0 (0)
2146Bdev014	ND	No data	0	0	ND	No data	0	0	ND	No data	0 (0)	0 (0)
2146Bdev13	No MIC	No data	0	0	No MIC	No data	0	1	No MIC	No data	0 (0)	1 (1)
Median (R)	0/6 = 0%	N/T	N/T	N/T	3/6 = 50%	N/T	4	3	0/6 = 0%	N/T	N/T	N/T
Median (S)	6/6 = 100%	0	0	3	3/6 = 50%	N/T	0	0	6/6 = 100%	0	0 (0)	0.5 (0.5)
Median (ND)	$n = 13$	0	0	0	$n = 13$	0	0	0	$n = 16$	0	0 (0)	0 (0)
Median (No MIC)	$n = 1$	N/T	0	0	$n = 1$	N/T	0	1	$n = 1$	N/T	N/T	N/T
Kappa (>1 ARG)	$n = 19$	N/T	N/T	N/T	$n = 19$	N/T	$\kappa = 0.60$ Substantial	$\kappa = 0.48$ Moderate	$n = 19$	N/T	N/T	N/T
WRS Test *p*-value (exact)		N/T	N/T	N/T		N/T	$p = 0.08$	$p = 0.08$		N/T	N/T	N/T
WSR Test *p*-value (exact)	$n = 16$	NE vs 10 h $p = 0.25$	NE vs 14 h $p = 0.13$	10 vs 14 h $p = 0.63$	$n = 16$	NE vs 10 h $p = 0.06$	NE vs 14 h $p = 0.06$	10 vs 14 h $p = 0.70$	$n = 16$	NE vs 10 h $p = 0.13$	NE vs 14 h $p = 0.02$	10 vs 14 h $p = 0.03$

AST: antimicrobial susceptibility testing result (CLSI breakpoints specific to *P. multocida*). GAM: gamithromycin (MIC ≥ 16 ug/ml), TUL: tulathromycin (MIC ≥ 64 ug/ml), SUL: sulfamethoxazole (MIC > 256 ug/ml) (no CLSI breakpoint), TET: tetracycline (MIC ≥ 8 ug/ml), TET-I: tetracycline intermediate susceptibility (MIC = 4 ug/ml). ND: not detected (not cultured), No MIC (minimum inhibitory concentration and phenotypic susceptibility not available—excluded from concordance), S: susceptible. R: resistant. N/T: not tested (did not have ≥1 isolates with phenotypic resistance or ≥1 isolates with ARG reads). Kappa based on comparison of >1 ARG to phenotypic AMR where negative = S or ND (isolates with No MIC excluded). WRS Test: Wilcoxon Rank Sum test of read counts compared between phenotypic AMR detection where negative = S or ND. WSR Test: Wilcoxon Signed Rank test comparison of read counts between unenriched and enriched for 10 h and enriched for 14 h. Tetracycline resistant reads, first value is total tetracycline ARGs and value in () is specifically for *tet(H)*.

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TABLE 5 Concordance between phenotypic antimicrobial resistance and number of reads with antimicrobial resistance genes (ARGs) within *Histophilus somni* reads.

	Antimicrobial resistance and counts of resistance genes: *Histophilus somni*											
	Macrolides				Sulfonamides				Tetracyclines (*tet(H)*)			
Sample ID	AST	Unenriched	10 h	14 h	AST	Unenriched	10 h	14 h	AST	Unenriched	10 h	14 h
2045Bi2-003	ND	0	0	0	ND	0	0	0	ND	1 (1)	0 (0)	0 (0)
2045Bi2-004	ND	0	0	0	ND	0	0	0	ND	0 (0)	4 (4)	1 (1)
2045Bi2-018	ND	0	0	0	ND	0	0	0	ND	0 (0)	1 (1)	1 (1)
2045Bi2-023	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2045Bi2-046	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2045Bi2-053	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2045Bi2-055	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2045Bi2-063	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2045Bi2-067	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2045Bi2-070	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	2 (2)
2045Ai2-012	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2046bi2-013	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2045Bix2-015	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2048Ai2-036	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2048Ai2-083	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2046Ai2-095	ND	0	0	0	ND	0	0	0	ND	0 (0)	0 (0)	0 (0)
2148Bdev3009	S	No data	0	0	SUL	No data	0	0	S	No data	0 (0)	0 (0)
2145Ax8-011	S	No data	0	0	SUL	No data	0	0	S	No data	0 (0)	0 (0)
2146Bdev014	S	No data	0	0	SUL	No data	0	0	S	No data	0 (0)	0 (0)
2146Bdev13	S	No data	0	0	SUL	No data	0	0	TET	No data	4 (4)	5 (5)
Median (R)	0/4 = 0%	N/T	N/T	N/T	4/4 = 100%	N/T	0	0	1/4 = 25%	N/T	4 (4)	5 (5)
Median (S)	4/4 = 100%	N/T	0	0	0/4 = 0%	N/T	N/T	N/T	3/4 = 75%	N/T	0 (0)	0 (0)
Median (ND)	$n = 16$	0	0	0	$n = 16$	0	0	0	$n = 16$	0 (0)	0 (0)	0 (0)
Median (No MIC)	$n = 0$	N/T	N/T	N/T	$n = 0$	N/T	N/T	N/T	$n = 0$	N/T	N/T	N/T
Kappa (>1ARG)	$n = 20$	N/T	N/T	N/T	$n = 20$	N/T	N/T	N/T	$n = 20$	N/T	$\kappa = 0.65$ Substantial	$\kappa = 0.65$ Substantial
WRS Test *p*-value (exact)		N/T	N/T	N/T		N/T	$p = 0.99$	$p = 0.99$		N/T	0.20	0.10
WSR Test *p*-value (exact)	$n = 16$	NE vs 10 h $p = 0.99$	NE vs 14 h $p = 0.99$	10 vs 14 h $p = 0.99$	$n = 16$	NE vs 10 h $p = 0.99$	NE vs 14 h $p = 0.99$	10 vs 14 h $p = 0.99$	$n = 16$	NE vs 10 h $p = 0.75$	NE vs 14 h $p = 0.50$	10 vs 14 h $p = 0.99$

AST: antimicrobial susceptibility testing result (CLSI breakpoints specific to *H. somni*). GAM: gamithromycin (MIC ≥ 16 ug/ml), TUL: tulathromycin (MIC ≥ 64 ug/ml), SUL: sulfamethoxazole (MIC > 256 ug/ml) (no CLSI breakpoint), TET: tetracycline (MIC ≥ 8 ug/ml), TET-I: tetracycline intermediate susceptibility (MIC = 4 ug/ml). ND: not detected (not cultured), No MIC (minimum inhibitory concentration and phenotypic susceptibility not available—excluded from concordance), S: susceptible. R: resistant. N/T: not tested (did not have ≥1 isolates with phenotypic resistance or ≥1 isolates with ARG reads). Kappa based on comparison of >1 ARG to phenotypic AMR where negative = S or ND (isolates with No MIC excluded). WRS Test: Wilcoxon Rank Sum test of read counts compared between phenotypic AMR detection where negative = S or ND. WSR Test: Wilcoxon Signed Rank test comparison of read counts between unenriched and enriched for 10 h and enriched for 14 h. Tetracycline resistant reads, first value is total tetracycline ARGs and value in () is specifically for *tet(H)*.

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and the low percentage identity of the *tet(34)* genes, these results were unlikely to be clinically relevant.

Concordance between genotypic ARG detection and phenotypic AST varied, and there were sufficient samples with phenotype positive and negative isolates to generate reliable metrics. However, most calculations suggested substantial agreement and significant differences in read numbers between samples with AST-positive and AST-negative isolates. Concordance can vary depending on the organism and antimicrobial, level of transcriptional expression, and sequence quality. In some previous reports, phenotypic resistance was highly correlated with known resistance determinants. Concordance between AST and WGS for *M. haemolytica* from 20 stocker calves calculated from raw data was very good for tilmicosin ($\kappa = 0.96$), tulathromycin ($\kappa = 0.96$), and tetracycline ($\kappa = 1.0$) (Snyder et al., 2020). Agreement was lower between oxytetracycline resistance and detection of *tet(H)* ($\kappa = 0.66$) and sulfonamide resistance and detection of *sul2* ($\kappa = 0.38$) for 64 WGSs for *M. haemolytica, P. multocida,* and *H. somni* isolates from beef and dairy calves (Owen et al., 2017).

This study demonstrated the use of long-read metagenomic sequencing for the detection of ARGs linked directly to bacterial species without the need for genome assembly. The detection of ARGs has been reported from long-read metagenomic sequencing for one study of aspirates from human ventilator-associated pneumonia (Chen et al., 2023) but more typically from samples with substantially less host DNA, such as those recovered from positive blood cultures (Taxt et al., 2020; Liu et al., 2023), bile cultures (Whittle et al., 2022), milk cultures (Ahmadi et al., 2023), and urine cultures (Zhang et al., 2022; Ring et al., 2023).

There were no previous reports of ARG detection within identified pathogen reads using long-read metagenomic sequencing in human or veterinary upper respiratory tract samples (Chen and Xu, 2023; Adewusi et al., 2024) with the exception of the limited success in earlier study published by our group (Freeman et al., 2022). Resistance genes were not recovered in a veterinary study of skin infections in dogs due to the high percentage of host DNA (Ring et al., 2023). None of the metagenomic studies reporting ARGs explicitly reported either individual or multiple ARGs on single long reads of the organisms of interest at the species level as described in the present study, potentially increasing the clinical relevance of the ARG detection.

Short-read data can be used for read-based detection of ARGs; however, it typically requires higher genome coverage and more computing resources compared with long-read detection (Gupta et al., 2020), making long-read metagenomic options such as our method attractive as genomics moves toward rapid diagnostic solutions to inform antimicrobial stewardship. Long-read methods such as ONT also offer access to taxonomic real-time data during the sequencing run, which may also speed up the time for results and is not currently available for short-read sequencing methods (Votintseva et al., 2017; Charalampous et al., 2019; Chan et al., 2020; Ring et al., 2023).

Although bacterial enrichment of samples has improved the sensitivity of both taxonomic and ARG detection, this method is not without drawbacks. Indeed, it adds time and complexity to sample preparation for sequencing, and it does not offer the same unbiased insight into the microbial community structure for those whose objective might be traditional microbiome research as does metagenomic sequencing of unenriched samples. However, in our case, the goal was to detect reads of specific BRD pathogens with ARGs to inform clinical decisions and not to produce a general description of the nasal microbiome. Larger studies including more animals will further validate this tool against alternative testing methods and assess whether this method might be a cost-effective option to help inform antimicrobial stewardship.

5 Conclusion

Long-read metagenomic sequencing of enriched DNP samples from feedlot cattle to detect BRD pathogens and ARGs shows promise as a diagnostic testing strategy for feedlot cattle production. Agreement between pathogen detection and traditional culture-based methods was improved by the enrichment step. While this step adds time and makes the process less portable, the relative benefits of improving sequence quantity for non-host reads and ARG detection outweighed these costs. This method provides additional promise for the characterization of species not specifically targeted by routine culture and susceptibility protocols with no additional diagnostic costs.

Data availability statement

The data presented in this study were deposited to the Sequence Read Archive as submission SUB14263813 and as BioProject PRJNA1096931.

Ethics statement

The animal study was approved by University of Saskatchewan Animal Care Committee (AUP 20190069). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

EH: Methodology, Writing – original draft, Software. SL: Methodology, Conceptualization, Project administration, Writing – review & editing. CF: Methodology, Writing – original draft. SO: Funding acquisition, Writing – review & editing. EM: Writing – review & editing, Data curation, Software. ML: Data curation, Software, Formal analysis, Writing – review & editing. PS: Software, Writing – review & editing. CW: Writing – review & editing, Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1386319/full#supplementary-material

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Genomic characterisation of *Escherichia coli* isolated from poultry at retail through Sink Surveillance in Dhaka, Bangladesh reveals high levels of multi-drug resistance

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The surveillance of antimicrobial resistance (AMR) in commensal *Escherichia coli* from livestock at slaughter is widely employed to assess the potential for risk to humans. There is currently a limited understanding of AMR in Bangladesh poultry at retail in live bird markets, with studies focussing solely on phenotypic characterisation of resistance. To address this evidence gap we performed antimicrobial susceptibility testing and whole genome sequencing on *E. coli* obtained from chickens from live bird markets in Dhaka in 2018 ($n = 38$) and 2020 ($n = 45$). *E. coli* were isolated from caeca samples following ISO guidelines and sequenced using short and long read methods. Multidrug resistance was extremely common ($n = 77$) and there was excellent concordance between AMR phenotype and the presence of corresponding AMR genes or mutations. There was considerable genomic diversity, with 43 different sequence types detected. Public health considerations included the high occurrence of resistance to ciprofloxacin ($n = 75$) associated with plasmid-residing *qnrS* or mutations in the *gyrA* and *parC* chromosomal genes; and the detection of a tigecycline resistant isolate harbouring *tet*(X4) on an IncHI1A/B-IncFIA mosaic plasmid. Thirty-nine isolates were resistant to azithromycin and harboured *mphA*, with a significant increase in the incidence of resistance between 2018 and 2020. Although azithromycin is banned for veterinary use in Bangladesh it remains an important treatment option for humans. Interestingly, *mphA* confers high-level resistance to azithromycin and erythromycin, and the latter is commonly used on poultry farms in Bangladesh. Seven isolates were colistin resistant and carried *mcr1*. For two isolates hybrid assemblies revealed that *mcr1* resided on a highly conserved IncHI2 plasmid that had 93% nucleotide identity to a plasmid from the published genome of an *E. coli* isolate of Bangladeshi human origin. Six isolates had resistance to third generation cephalosporins, associated with plasmid-residing $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-65}}$, or $bla_{\text{DHA-1}}$. By employing phenotypic and genomic approaches for AMR surveillance we have provided new insights

into the potential for One Health AMR linkages in Bangladesh. Employing similar approaches in human and environmental sectors will help inform the One Health approach to addressing AMR, and generate evidence to support mitigation measures such as improved antimicrobial stewardship.

KEYWORDS

antimicrobial resistance, *E. coli*, Bangladesh, poultry, plasmid, prevalence

Introduction

Antimicrobial resistance (AMR) is one of the most pressing issues of the 21st century. AMR leads to increased morbidity, disease burden, healthcare costs and mortality. It is currently estimated that AMR will lead to 300 million global deaths, an 11% loss of livestock production and ~$100 trillion in financial loss by 2050 (Murray et al., 2022). Such is the concern, the World Health Organisation (WHO) implemented a Global Action Plan (GAP) in 2015, based on a 'One Health' approach, with a plan to reduce the developing threat of AMR at the sources (WHO, 2015). AMR does not just affect humans, as the 'One Health' approach recognises the health of humans is interconnected with the health of animals and the environment (CDC, 2022; FAO, WHO and WOAH, 2022).

Bangladesh is tackling the threat of AMR through the implementation of a National Action Plan (NAP) incorporating a 'One Health' approach (Alam et al., 2017), with substantial coordination between the human health and food producing agriculture sectors (including livestock, aquaculture and crops) (Ahmed et al., 2022). Bangladesh has a large livestock sector, with 403 million terrestrial animals, accounting for 1.5% of the GDP for the national economy. Farming in Bangladesh is comprised of a mixture of intensive and extensive farms. Poultry is the most important and advanced segment of the livestock sector in Bangladesh mostly used for domestic production. The majority of poultry farms are small-medium scale with some larger commercial farms, where farms are categorised as follows: large commercial (>10,001 birds), medium (1,001–10,000 birds), small (101–1,000 birds). Antimicrobial usage on the farms is largely unregulated and antibiotics can be bought off the shelf without the need of veterinary prescriptions, a review paper found 86% of antimicrobials used in livestock from Bangladesh were non-prescription (Morgan et al., 2011; Netherlands Enterprise Agency, 2020).

A 2016 study by Islam et al., observing 73 broiler farms, found all farms were using antibiotics, 31% for prophylaxis and 8% were using antimicrobials for growth promotion. Over 60% of the farms in that study were using antimicrobials without prescription. Nearly 70% of the antimicrobials identified in that study were fluroquinolones, with enrofloxacin and/or ciprofloxacin being used on 19% of farms (Islam et al., 2016). Both enrofloxacin and ciprofloxacin are classified by the WHO as a high priority critically important antimicrobials (HP-CIA) (WHO, 2019). In 2020 a study by Ahmed et al. looked at *E. coli* from 20 broiler farms. The study used whole genome sequencing to characterise isolates resistant to colistin; a final resort antimicrobial reserved for human medicine. The study found that 25% of 1,200 isolates carried *mcr1* genes, aligning to the widespread usage of colistin as a prophylactic for broiler production (Ahmed et al., 2020). In 2022 colistin was banned for use in broiler production by the Bangladeshi government (Directorate General of Drug Administration, 2022).

E. coli is regarded as an important indicator species to characterise the transmission and dissemination of AMR, due to its ability to receive and transfer antimicrobial resistance genes (Anjum et al., 2021). This transfer can be to other *E. coli* strains and other bacterial species. *E. coli* is found in the intestinal tracts of mammalian and poultry livestock as well as on skin, fur, and feathers. There are many different strains of *E. coli* that make up normal gut flora and do not cause illness. There are also several pathogenic strains in poultry which can cause illness, such as avian pathogenic *E. coli*, and other strains that can cause infection in humans, these are commonly caused by enteropathogenic, enterotoxiogenic, enteroinvasive, and enterohemorrhagic *E. coli* (Ievy et al., 2020). Contamination of foodstuffs by *E. coli* is often caused by poor food hygiene practises or poorly prepared/cooked meat.

Between 2016 and 2017, a sink surveillance study was carried out in Bangladesh for Highly Pathogenic Avian Influenza (HPAI) and other emerging zoonotic pathogens at Live Bird Markets (LBMs) (Osmani et al., 2018). The sink surveillance study gave an overall picture of the HPAI prevalence across Dhaka, and its effective sampling strategy was readily replicated and adapted for the purpose of bacterial AMR characterisation. Studies by Sarker et al. (2019) and Mandal et al. (2022) indicate a high level of AMR in *E. coli* from poultry, however, few papers have used whole genome sequencing to characterise the resistance genes present in *E. coli* isolated from LBMs in Bangladesh. In a 2023 systematic review (Islam et al., 2023a) 17 articles were identified looking at AMR in *E. coli* from poultry, some articles used PCR to identify antimicrobial resistance genes. No articles used whole genome sequencing, limiting the number of genes that can be identified.

The purpose of this study was to characterise the antimicrobial resistances and genomic diversity of *E. coli* obtained from poultry at retail in Dhaka, Bangladesh. This study at a point close to consumption aimed to address evidence gaps, including the genetic basis of resistance, and allow assessment of the risk that AMR from food producing animals presents to people.

Materials and methods

Study design and isolation of *Escherichia coli*

The sample collection followed was based on the sink surveillance protocol for the identification of highly pathogenic avian influenza, which had started in 2016 and collected samples from 106 of the largest selling chicken LBMs in Dhaka (Osmani et al., 2018). In our study, sampling commenced in March 2018, with 24 of the 106 LBMs

being randomly selected for sampling. Two whole caeca were collected from each market, one from a Sonali chicken and the other from a broiler chicken, both of which had been freshly slaughtered for retail (no animals were euthanised specifically for this publication). This was to ensure a representation of the two main types of birds consumed in Bangladesh. Sonali is a crossbreed of Rhode Island Red cocks and Fayoumi hens which is well adapted to Bangladesh's environmental conditions and can command higher prices at retail. Sample collection was not undertaken in April and May 2020 due the COVID-19 pandemic.

For the isolation of *E. coli*, 1 g of caecal contents from chickens was inoculated in 9 mL Buffered Peptone Water (Thermo Scientific) and then incubated at 37°C overnight. The mixture was plated onto MacConkey agar (Thermo Scientific) and incubated for 18–22 h at 37°C. Lactose-fermenting colonies were sub-cultured onto nutrient agar (Thermo Scientific) for biochemical testing. Oxidase and indole testing was carried out and oxidase negative/indole positive isolates were confirmed as *E. coli*. A single colony was selected and stored on beads.

A representative sub-set of 83 *E. coli* isolates was selected for detailed characterisation by antimicrobial susceptibility testing and whole genome sequencing. The selection was undertaken to ensure that both breed and all sampling months were represented, to support the statistical analysis (see below). These 83 isolates were shipped to the UK on charcoal swabs following IATA guidelines and cultured onto CHROMagar ECC (CHROMagar). Isolates were sub-cultured onto MacConkey No.3 (Thermo Scientific) for confirmation of lactose-fermentation. Putative *E. coli* isolates were biochemically tested using oxidase and indole reagents. MALDI-ToF MS (Bruker, Library version 4.7.373.7) was performed for isolates where oxidase/indole testing was inconclusive.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on the 83 isolates by broth microdilution for Minimum Inhibitory Concentration (MIC) determination using commercial plates (Sensititre™ EU Surveillance *Salmonella/E. coli* EUVSEC3 plate, Thermo Fisher Scientific, 2021), according to manufacturer's instructions. Briefly, a suspension of each isolate was adjusted to a density of 0.5 McFarland in 5 mL demineralised water, then 10 µL of the suspension was transferred to 11 mL of Mueller Hinton broth to obtain a target inoculum density of between 1×10^5 and 1×10^6 CFU/mL. Fifty microlitres was dispensed into each well of the microtitre plate using a Sensititre AIM and incubated aerobically at 35–37°C for 18 to 22 h. Fifteen antimicrobials were tested in this manner (amikacin, ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, tigecycline and trimethoprim), and MICs were recorded as the lowest concentration preventing visible growth. *E. coli* NCTC 12241 (ATCC 25922) was used as control strain. Susceptibility was assessed using EUCAST ECOFF values (accessed 21/11/2022) (EUCAST, 2022), except for sulfamethoxazole for which the interpretative criteria proposed by the European Food Safety Authority (EFSA) (Amore et al., 2023) were employed as an ECOFF value was not available, as wild type or non-wild type (Schwarz et al., 2010). ECOFFs distinguish microorganisms without (wild type) and with phenotypically detectable acquired resistance mechanisms (non-wild type) to the antimicrobial in question.[1] In this paper use of the term 'resistance', such as multidrug resistance and phenotypic resistance refers to the non-wild type phenotype, which is not necessarily synonymous with clinical resistance (Schwarz et al., 2010; EUCAST, 2022). Isolates resistant to third generation cephalosporins (cefotaxime MIC ≥0.5 mg/L and/or ceftazidime MIC ≥1 mg/L) were additionally tested on the EUVSEC2 microplate (Sensititre®, Trek Diagnostic Systems, East Grinstead, UK) to determine the presumptive phenotype of Extended-Spectrum Beta-Lactamase (ESBL), AmpC and carbapenemase producers (EFSA and ECDC, 2023). The following antibiotics are included in the EUVSEC2 plate: cefepime, cefotaxime, cefotaxime and clavulanic acid, cefoxitin, ceftazidime, ceftazidime and clavulanic acid, ertapenem, imipenem, meropenem, and temocillin. Where isolates presented with resistance to three or more antimicrobial classes they were classified as multidrug resistant (MDR) (Schwarz et al., 2010).

Whole genome sequencing and analysis

DNA extracts were prepared from overnight Luria-Bertani broth cultures of the 83 isolates with the MagMAX™ CORE extraction kit (Thermo Fisher Scientific, Basingstoke, UK) using the semi-automated KingFisher Flex system (Thermo Fisher Scientific, Basingstoke, UK) according to the manufacturer's instructions. Extracted DNA was processed for whole genome sequencing using the NextSeq® 500/550 Mid Output Kit v2.5, using NextSeq sequencing reagents. The resulting raw sequences were analysed with the Nullabor 2 pipeline (Seemann et al., 2020), using as reference the published genome *E. coli* K12 (Accession number U00096.2), Spades for genome assembly [version 3.14.1; (Prjibelski et al., 2020)] and Prokka for annotation [version 1.14.6; (Seemann, 2014)]. The presence of genes and point mutations conferring AMR, heavy metal stress genes, and virulence genes were assessed using AMRFinderPlus (Feldgarden et al., 2021). AMR genes and plasmid incompatibility types were identified using APHASeqfinder.[2] The Sequence Type (ST) was determined with MLST [version 2.19.0; (Seemann, 2022)] using the pubMLST database (Jolley et al., 2018). Core genome SNPs were generated using SNIPPY (Seemann, 2020). *E. coli* serotypes were determined using ECTyper (Petkau et al., 2017). Phylogenetic trees with 200 bootstraps were built using RAX-ML (Stamatakis, 2014) from the core genome SNPs, and annotated using iTOLv5 (Letunic and Bork, 2021).

Three isolates were selected for long read sequencing using Oxford Nanopore Technologies (ONT), based on the presence of AMR genes and plasmids identified through the short read sequencing (see Results). DNA was extracted using the GenFindV3 extraction kit (Beckman Coulter) according to the manufacturer's instructions. Sample preparation was carried out using the SQK-RBK004 Rapid barcoding kit (ONT) according to manufacturer instructions. Samples were then run-on MinION and MinION flow cell for 72 h. Hybrid assemblies were created using long and short read sequences by Unicycler v2.0 (Wick et al., 2017) to generate closed (fully circularised)

1 https://mic.eucast.org/

2 https://github.com/APHA-AMR-VIR/APHASeqFinder

plasmids. StarAMR was used to map AMR resistance genes to plasmids (Bharat et al., 2022). Blastn and MOB-suite (Robertson and Nash, 2018; NCBI, 2023) was used to identify similar published plasmids from public database (NCBI, 2023) and BRIG (Alikhan et al., 2011) was used to generate an image that compared published plasmids with those identified in this study. Bakta and the CARD AMR database were used for image annotation (Schwengers et al., 2021; Alcock et al., 2023). Easyfig was also used for comparative analysis using published genomes from NCBI Blast (Sullivan et al., 2011).

The whole genome sequences were deposited in the National Center for Biotechnology Information (NCBI) National Library of Medicine under BioProject accession number PRJNA1100899.

Statistical analysis

Logistic regression was used to assess for significant (p-value <0.05) associations between individual AMR and MDR outcomes and the three available explanatory variables (year, month, and breed). We also analysed whether *E. coli* which were ESBL-producers, AmpC-producers or colistin resistant have associations with ST and breed.

Results

A total of 315 caeca samples were collected from apparently healthy poultry from live bird markets in the years 2018 ($n = 180$) and 2020 ($n = 135$). *E. coli* was isolated from 217 samples, with 108 isolated in 2018 and 109 in 2019 (Supplementary File S1). The number of samples positive for *E. coli* each month sampled ranged from 11 to 40. A representative collection of 38 isolates from 2018 and 45 isolates from 2020 was selected for detailed characterisation by antimicrobial susceptibility testing and whole genome sequencing (File S1).

Antimicrobial resistance and carriage of AMR genes

The 83 isolates were assessed for their susceptibilities towards 15 antimicrobials and results are presented in Table 1 and Supplementary File S2. The most common resistances were towards tetracycline ($n = 78$; 94%), ciprofloxacin ($n = 75$; 90%), trimethoprim ($n = 74$, 89%), sulfamethoxazole ($n = 74$; 89%), and ampicillin ($n = 69$; 83%). A noteworthy number of isolates were resistant to critically important antimicrobials (CIAs) as defined by the WHO (2019): azithromycin ($n = 39$; 47%), gentamicin ($n = 29$; 35%), colistin ($n = 7$; 8%) and/or tigecycline ($n = 2$; 2%). Multidrug resistance was common in the panel of isolates (77/83; 93%). All isolates were susceptible to the critically important antimicrobials amikacin and meropenem. Six isolates had resistance to cefotaxime and/or ceftazidime and further susceptibility testing determined that four were Extended-Spectrum Beta-Lactamase (ESBL)-producing *E. coli* and two were AmpC-producers (Supplementary File S2).

There was very high concordance (>99%) between phenotypic data from MIC and the presence of AMR determinants as determined using genotypic data generated from WGS (Figure 1; Supplementary File S2). All isolates with azithromycin resistance harboured *mph(A)* and all gentamicin resistant isolates contained a variant of *aac3*, with the most predominant being *aac3-lld* ($n = 25/28$). Sixty-nine isolates were resistant to ampicillin, and all carried a variant of the bla_{TEM} gene (Supplementary File S2). The two AmpC producers harboured bla_{DHA-1} and the ESBL producers harboured $bla_{CTX-M-55}$ ($n = 2$) or $bla_{CTX-M-65}$ ($n = 2$). Colistin resistance was associated with *mcr1.1* ($n = 7$). Tetracycline resistance was associated with *tet*(A), *tet*(B) and/or *tet*(M) genes; isolates which carried *tet*(M) ($n = 14$) also carried *tet*(A). Two isolates had a non-wild type tigecycline resistance phenotype; both isolates carried *tet*(A) [but not the variant associated with tigecycline resistance (Radisic et al., 2023)] and one isolate additionally harboured *tet*(X4). Sulfamethoxazole resistance was associated with *sul1*, *sul2* and/or *sul3* genes and trimethoprim resistant isolates harboured *dfrA*. The genes *catA1*, *cmlA1* and *floR* were associated with chloramphenicol resistance; 15 isolates harboured both *cmlA1* and *floR*. Isolates with point mutations in DNA gyrase (*gyrA*) and/or DNA topoisomerase (*parC*), most commonly *gyrA* (D87N), *gyrA* (S83L) and *parC* (S80I) respectively, had high ciprofloxacin MIC values, which exceeded the ECOFF value and also the EUCAST clinical breakpoint of >0.5 mg/L (EUCAST, 2023). Several of these isolates also carried a plasmid mediated quinolone resistance (PMQR) gene: *qnrS* ($n = 33$) or *qepA4* ($n = 1$) (Supplementary File S2). Seventeen isolates had ciprofloxacin MIC values which exceed the ECOFF value but not the clinical breakpoint, and these harboured *qnrS* only. Additional AMR genes conferring resistance to antimicrobials not tested by MIC in this study were present in many isolates (Supplementary File S2).

Genomic diversity

Considerable genomic diversity was evident in the isolate collection, summarised in the phylogenetic tree (Figure 1). The isolates comprised 44 different sequence types (ST), of which two were newly identified in this study (ST15234 and ST15267). 29 STs were detected only once (Supplementary File S3). The most frequently identified ST was ST155 (highlighted red in Figure 1), detected in both breeds of bird and in each year. AMR gene content and the presence of plasmid incompatibility types differed between ST155 isolates, with only *tet*(A) present in all ten isolates. Five isolates were ST12066, part of clonal complex 206 (highlighted yellow in Figure 1), which were all detected in 2020, in both broiler and Sonali birds. Each harboured the same 19 AMR genes and four plasmid incompatibility types. These isolates were multidrug resistant and possessed AMR genes conferring resistance to eight antimicrobial classes. There was high sequence identity between the ST12066 isolates, with <10 single nucleotide polymorphisms (SNPs) difference between them, therefore meeting proposed relatedness threshold criteria to be considered clones (Schurch et al., 2018).

Four isolates were ST117 (highlighted in green in Figure 1) and these could be grouped into two clones based on sequence identity. One clone comprised the 2018 isolates CDE012 (Sonali) and CDE019 (broiler) which harboured four plasmid incompatibility types and 13 AMR genes, including $bla_{CTX-M-65}$ and *mcr1.1*. The two 2020 isolates CDE060 and CDE084 (both from Sonali birds) comprised a different clone carrying 12 AMR genes and four plasmid incompatibility types.

TABLE 1 Distribution of minimum inhibitory concentrations from 83 *E. coli* isolates obtained from live bird markets in Dhaka in 2018 and 2020.

Antimicrobial	Antimicrobial concentration test range	ECOFF Values		MIC Range (mg/L)																
		Wild type	Non-Wild type	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	% Resistant
Amikacin	4–128	≤ 8	≥ 16									83								**0%**
Ampicillin	1–32	≤ 8	≥ 16								3	8	3	1	68					**83%**
Azithromycin	2–64	≤ 16	≥ 32								4	12	26	2	6	33				**47%**
Cefotaxime	0.25–4	≤ 0.25	≥ 0.5					77			2	4								**7%**
Ceftazidime	0.25–8	≤ 0.5	≥ 1					76	1	1	2		3							**7%**
Chloramphenicol	9–64	≤ 16	≥ 32										37		1	45				**55%**
Ciprofloxacin	0.015–8	≤ 0.06	≥ 0.12	8			1	8	8	1		3	54							**90%**
Colistin	1–16	≤ 2	≥ 4							76		4	3							**8%**
Gentamicin	0.5–16	≤ 2	≥ 4						42	12	1			28						**34%**
Meropenem	0.03–16	≤ 0.06	≥ 0.12		83															**0%**
Nalidixic Acid	4–64	≤ 8	≥ 16									12	6	6		59				**78%**
Sulfamethoxazole	8–512	≤ 64	≥ 128										6	3					74	**89%**
Tetracycline	2–32	≤ 8	≥ 16								4	1			78					**94%**
Tigecycline	0.25–8	≤ 0.5	≥ 1					75	6	2										**2%**
Trimethoprim	0.25–16	≤ 2	≥ 4					2	6			1		74						**90%**

Susceptibilities have been interpreted using EUCAST ECOFF values (EFSA interpretive criteria for Sulfamethoxazole), which are displayed as vertical black lines. Red fill and blue fill indicate MICs above and below the ECOFF value, respectively.

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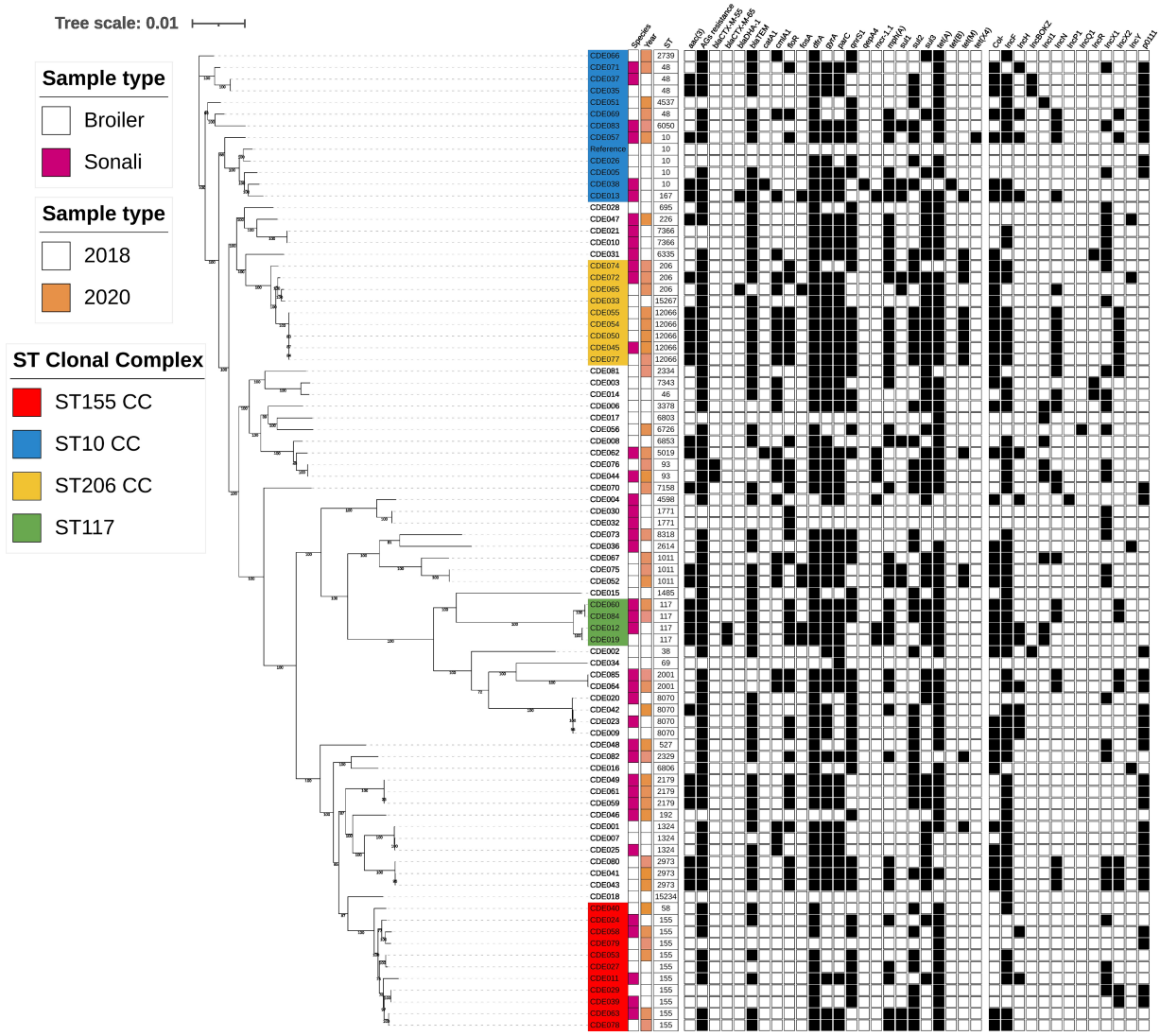

FIGURE 1
Phylogenetic tree generated from core genome single-nucleotide polymorphisms of *E. coli* isolates obtained from poultry at slaughter in 2018 and 2020. Sequence type, year, species, AMR genes, and plasmid incompatibility types are indicated. Isolate names with colour highlights are discussed in the text and correspond to green (ST117), red (ST155), blue (Clonal Complex 10), and yellow (Clonal Complex 206).

Several sequence types from the ST10 clonal complex were detected (highlighted in blue in Figure 1). ST10 isolates were identified in 2018 and 2020, in both broiler and Sonali chickens, but sequence identity exceeded the proposed threshold to indicate a clonal relationship. The ST10 isolate CDE057 from a Sonali bird carried *tet*(X4), which confers resistance to tetracycline and tigecycline, as well as 10 other AMR genes and six plasmid incompatibility types. Considered as group these four ST10 isolates harboured from 5 to 12 AMR genes, and all possessed the *gyrA* (S83L) mutation, *dfrA* and either *tet*(A) or *tet*(B). Isolate CDE013 resided in the same sub-clade as the ST10 isolates (Figure 1) and was ST167, a member of the ST10 clonal complex. CDE013 had an AmpC resistance phenotype and harboured $bla_{\text{DHA-1}}$, the colistin resistance gene *mcr1.1* and 13 other AMR genes. Four ST48 isolates (ST10 clonal complex) were also detected. The *E. coli* serotypes were determined using an *in silico* detection method (ECTyper), a range of serovars were identified but sequence type was used to analyse genomic diversity, and no further analysis of serovar was conducted.

Presence of plasmids harbouring multiple antimicrobial resistance genes

To further explore the diversity of AMR genes and assess whether they resided on plasmids we selected three isolates for long read sequencing as exemplars harbouring resistance to critically important antimicrobials: CDE012 (*mcr1.1*; $bla_{\text{CTX-M-65}}$), CDE013 (*mcr1.1*; $bla_{\text{DHA-1}}$), and CDE057 [*tet*(X4)]. Plasmids were present in all three isolates and every plasmid was fully circularised using the hybrid assembly approach (Table 2). Nine of the ten plasmids identified harboured AMR genes, and of these seven had an AMR gene content which would confer resistance to three or more antimicrobials, and hence an MDR phenotype.

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TABLE 2 Plasmids identified in the MDR isolates CDE012, CDE013 and CDE057.

Isolate	Plasmid ID	Plasmid size (bp)	Plasmid Incompatibility type	AMR genes located on plasmid
CDE012	pCDE012-1	241,725	IncHI2, IncHI2A	*aadA1, aadA2, aph(3")-Ib, aph(3')-Ia, aph(6)-Id, bla*$_{TEM-1B}$, *cmlA1, dfrA14, floR,* ***mcr-1.1****, mph(A), sul3, tet* (A)
	pCDE012-2	117,899	IncI	*aac(3)-IV,* ***bla*** $_{CTX-M-65}$, *bla*$_{TEM-1B}$, *fosA3*
	pCDE012-3	106,361	IncFIB	*aadA5, dfrA17*
CDE013	pCDE013-1	234,667	IncHI2, IncHI2A	*aadA1, aadA2, cmlA1, dfrA14,* ***mcr-1.1****, mph(A), sul3, tet*(A), *tet*(M)
	pCDE013-2	129,417	IncFIB	*aac(3)-Iid, bla*$_{TEM-1B}$, *mph(A)*
	pCDE013-3	63,856	IncN, IncX1	*aph(3')-Iia,* ***bla*** $_{DHA-1}$, *fosA4, sul1*
CDE057	pCDE057-1	217,549	IncFIA, IncHI1A, IncHI1B	*dfrA14, floR,* ***tet*****(X4)**
	pCDE057-2	153,061	p0111	*aac(3)-Iid, aph(3')-Ia, bla*$_{TEM-1B}$, *dfrA14, qnrS1, sul2, tet*(A)
	pCDE057-3	111,908	IncFIB	-
	pCDE057-4	32,570	IncN, IncX2	*mph(A), qnrS1, tet*(A)

Resistance genes in bold are highlighting genes of particular interest which are discussed in the text.

For isolate CDE012, the *mcr1.1* gene resided on a 234,667 bp IncHI2 plasmid (pCDE012-1) together with 12 additional AMR genes (Table 2). Plasmid pCDE012-1 had high sequence identity (>99%) with several published plasmids (Supplementary File S4), including pRS571-MCR-1.1 which was obtained from an *E. coli* reported as isolated from 'human normal flora' in Bangladesh in 2018 (accession number CP034390). These two plasmids of Bangladesh origin shared a substantial degree of sequence identity (>99%) and a largely identical gene synteny, however, the *mcr1.1* gene was present in a different location in each plasmid (Figure 2). In both plasmids the *mcr1.1* region was flanked by IS30 family transposons, and it is probable that *mcr1.1* has inserted into these two very similar plasmids at different locations, following separate recombination events. Isolate CDE012 also harboured a 117,899 bp IncI plasmid (pCDE012-2) on which resided *bla*$_{CTX-M-65}$, *fosA3* (conferring fosfomycin resistance), *aac(3)-IV*, and *bla*$_{TEM-1B}$ (Table 2). This plasmid had high sequence identity (>99%) with many published plasmids harbouring the same AMR genes, which were present in *E. coli* from poultry and human sources in various regions such as China, South Korea, and Bolivia (Figure 3). Similarly, plasmid pCDE012-3 had high sequence identity (>99%) to published plasmids from *E. coli* (Supplementary File S4).

Isolate CDE013 harboured three plasmids. The *bla*$_{DHA-1}$ gene (conferring the AmpC phenotype) was present on plasmid pCDE013-3, a 63,856 bp IncN/IncX1 hybrid plasmid that also carried *aph(3')-IIa*, *fosA4*, and *sul1* (Table 2). pCDE013-3 had a backbone region of approximately 40Kb with high sequence identify to published plasmids from *E. coli*, *Salmonella*, and *Klebsiella*, however, the region containing *bla*$_{DHA-1}$, *aph (3')-Iia*, and *sul1* was not in the backbone region (Figure 4). The most similar plasmids to pCDE013-3 identified by Blast were from *Salmonella* serovar London, however, these plasmids did not contain *bla*$_{DHA-1}$ or *fosA4*. Plasmid pCDE013-2 (harbouring three AMR genes) was similar to previously described plasmids, with high sequence identity and conserved gene synteny (File S4). Plasmid pCDE013-1 was IncHI2 with *mcr1*.1 and eight other AMR genes, and shared extensive gene synteny and nucleotide identity with pCDE012-1 (96% identity) and pRS571-MCR-1.1 (93% identity) (Figure 2), suggesting a highly conserved *mcr1*-containing plasmid is circulating in Bangladesh.

For isolate CDE057, the tigecycline resistance gene *tet*(X4) was located on a 217,549 bp plasmid (pCDE057-1) that also carried *drfA14* and *floR* (Table 2). Comparative analysis with published plasmids revealed a ~ 190Kb region with >98% identity to published plasmid from a *Salmonella* monophasic Typhimurium found in Canada, which did not contain any of the resistance genes identified on this plasmid including *tet*(X4) (CP044958). The remaining ~20Kb region containing the AMR genes had the highest homology (>80%) to an IncHI1 plasmid identified in a *Klebsiella pneumoniae* from China (CP072461) (Figure 5). Plasmids pCDE057-2 and pCDE057-4 had <70% sequence identity to published genomes from NCBI and may represent newly described and/or mosaic plasmids; whereas pCDE057-3 was highly similar to previously published genomes but carried no AMR genes (File S4).

Virulence, disinfectant and heavy metal tolerance genes

The whole genome sequences were also examined for virulence, disinfectant resistance, and heavy metal tolerance genes (Supplementary File S2). All isolates were negative for the *stx* gene, present in Shiga toxin producing *E. coli* (STEC) which can cause serious life-threatening conditions in humans (Melton-Celsa, 2014). One isolate harboured the intimin gene *eae*, a virulence factor

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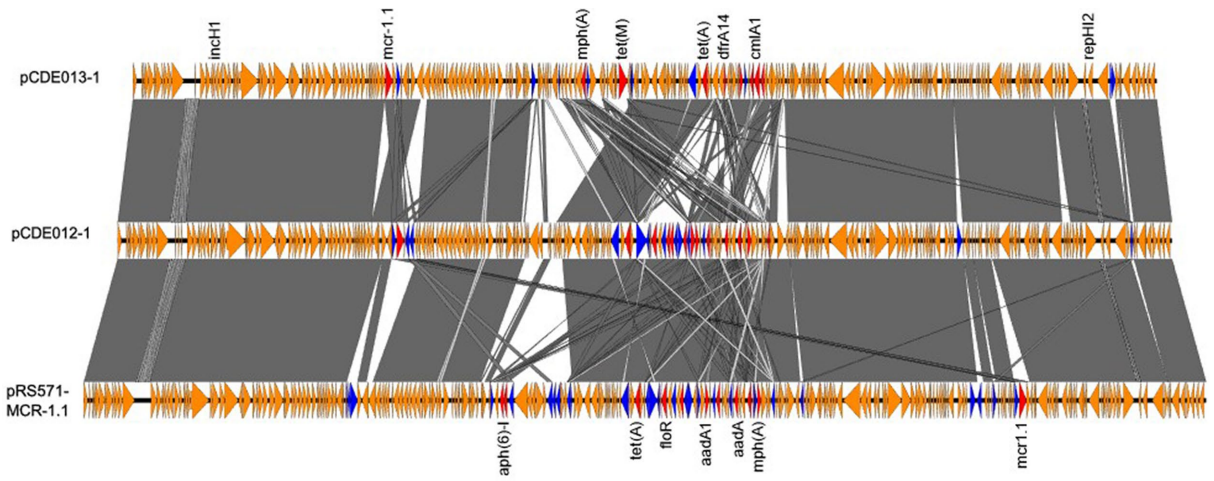

E. coli Isolate	*mcr1* plasmid	Origin	Sequence Type	Cephalosporinase		
				Phenotype	Gene	Location
CDE013	pCDE013-1	Sonali poultry	167	AmpC-Producer	*bla*DHA-1	IncN/IncX plasmid
CDE012	pCDE012-1	Sonali poultry	117	ESBL-Producer	*bla*CTX-M-65	IncI plasmid
RS571	pRS571-MCR1.1	Human	648	ESBL-Producer	*bla*CTX-M-15	Chromosome

FIGURE 2
Linear comparison of *mcr1.1* plasmids pCDE013-1 (top), pCDE012-1 (middle) and pRS571-MCR-1.1 (lower). AMR genes are denoted in red, IS family transposons in blue, and other genes in orange. The grey area indicates the blast identities, and the percentage of identity is indicated in the legend. The arrow size is proportional to the gene length. The image was generated using EasyFig with default parameters.

associated with the locus of enterocyte effacement (Stevens and Frankel, 2014).

Statistical analysis

The results of the statistical analyses indicated there was a significantly greater risk of azithromycin (odds ratio (OR) = 3.25, *p*-value 0.011), chloramphenicol (OR 2.75, *p*-value 0.027) and gentamicin (OR 3.88, *p*-value 0.008) resistance in year 2020 when compared to 2018. The month analysis was difficult as many months either had all positives or all negatives and so the analyses predicted success or failure perfectly. However, there was a significantly greater risk of ampicillin resistance in October (OR 9.33 *p*-value 0.038) when compared against the baseline of September, and a significantly lower risk of gentamicin resistance in June (OR 0.07 *p*-value 0.028) when compared against the baseline of January. Due to the study design, however, these associations may be biassed by which year or markets were sampled in those months. The analysis detected no significant associations with bird type.

Similarly, when considering resistances, no significant associations with bird type were detected for the colistin resistance, ESBL, or AmpC outcomes. There were seven *mcr-1.1* isolates, and these had a significantly greater number of resistance genes detected than the other isolates (mean 17.9 compared to 10.4, OR 1.51, *p*-value 0.005). There were four ESBL isolates, and these had a significantly greater number of resistance genes detected than the other isolates (mean 19.5 compared to 10.6, OR 1.96, *p*-value 0.019). The two AmpC isolates had a greater number of resistance genes detected than the other isolates (mean 18.5 compared to 10.9) but this was only approaching significance (OR 1.51, *p*-value 0.094). These three outcomes were not significantly associated with ST or breed.

Discussion

In this study we assessed the burden of AMR in indicator *E. coli* in poultry at retail in live bird markets in Dhaka, Bangladesh in 2018 and 2020. A representative panel of 83 isolates, from the total of 217 obtained, was selected for detailed characterisation by antimicrobial susceptibility testing using the gold standard broth microdilution and WGS. The use of WGS provided detailed insights into the genetic determinants responsible for the resistance phenotypes observed and showed that resistance is present in a wide diversity of bacterial phylogenies in both broiler and Sonali breeds. Importantly the bacterial isolates did not harbour an *stx* virulence gene, associated with Shiga-toxin and serious life-threatening conditions in humans. Through the use of a hybrid assembly approach, we have demonstrated that many resistances resided on plasmids, which would facilitate the potential for dissemination of resistance. An example from this study is the detection of a highly conserved IncHI2 plasmid carrying *mcr1* and additional AMR genes that was present in *E. coli* isolates CDE012 and CDE013, which are of different STs (i.e., separate phylogenetic lineages). Isolate CDE012 was ST117 and carried an IncI plasmid with $bla_{\text{CTX-M-65}}$, and was hence an ESBL-producer. Isolate CDE013 was ST167 and carried an IncN/IncX1 plasmid with $bla_{\text{DHA-1}}$, and hence an AmpC-producer. These data suggest that both isolates have acquired the IncHI2 plasmid independently, demonstrating the potential for AMR dissemination via plasmids, and highlights how accumulation of AMR plasmids can reduce treatment options. Furthermore, database screening showed that the IncHI2 plasmid was also present in an *E. coli* of human origin in Bangladesh from the same year as the poultry isolates. These findings provide an exemplar for the challenge of AMR, where mobile genetic elements are able to move within a bacterial population, and

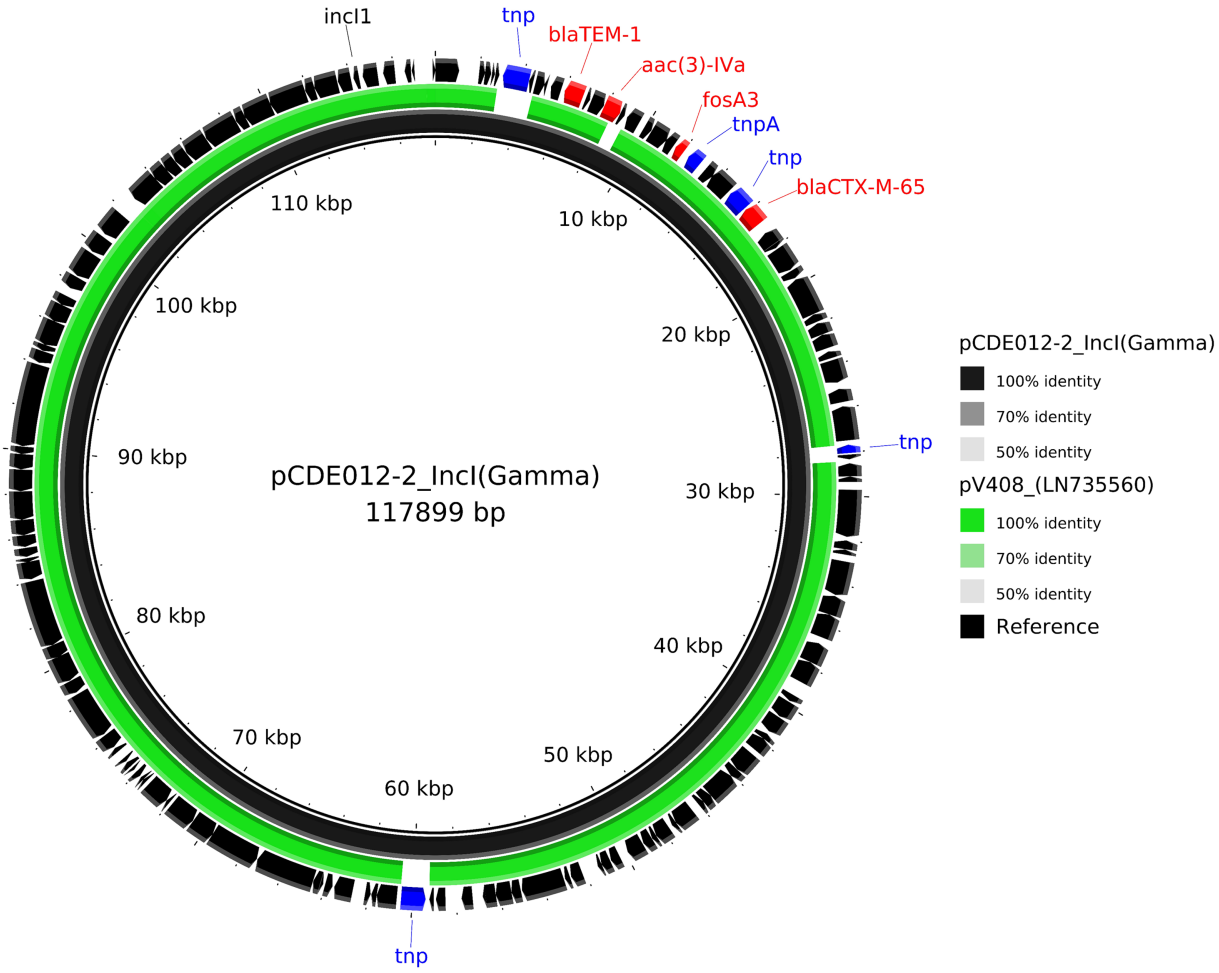

FIGURE 3
Comparative analysis of the IncI harbouring $bla_{CTX\text{-}M\text{-}65}$ plasmid pCDE012-2 (black inner ring) with the most similar mash neighbour identified using MOB-suite looking at other published plasmids. Each concentric ring displays the nucleotide similarity between pCDE012-2 and the other sequence in the outer ring; LN735560. The shade of colour indicates the BLAST percentage identity. The arrows around the map indicate deduced Coding Sequences and their orientation. AMR genes are highlighted in red, and transposons highlighted in blue. The image was generated using BRIG.

highlight the importance of a One Health approach to AMR surveillance and potential for risk to people via the food chain.

The occurrence of MDR was high at 93%, although similar to recent studies from Bangladesh which have reported MDR at 93 and 100% at LBMs (Azad et al., 2019; Parvin et al., 2022) and 88 and 100% at poultry farms (Rafiq et al., 2022; Ibrahim et al., 2023). Indeed, a recent review paper showed an MDR occurrence of 10–100% in poultry and poultry environment samples in Bangladesh, and of the 14 articles examined nine recorded that 100% of isolates tested had MDR (Islam et al., 2023a). Inappropriate antimicrobial use and inadequate farm biosecurity (which can contribute to disease occurrence and hence antimicrobial use) have been reported for poultry farms in Bangladesh (Imam et al., 2021; Siddiky et al., 2022) and antimicrobial use at poultry farms has been correlated with AMR (Ibrahim et al., 2023). The occurrence of MDR *E. coli* in Bangladesh poultry contrasts with that observed in broilers from other countries, such as the United Kingdom at 27.2% and European Union nations which have a median of 38.3%, although the range is broad at 0.4 to 86.0% (EFSA and ECDC, 2022). To consider the one health context we note that a study of 100 human clinical *E. coli* isolates from Dhaka, Bangladesh, reported 98% MDR (Jain et al., 2021), reflecting the prevalence seen in poultry-derived isolates.

Resistance towards antimicrobials from the WHO AWaRe groups Access or Watch (Zanichelli et al., 2023) was common. Resistance to the Access antibiotics tetracycline, trimethoprim, sulfamethoxazole, and ampicillin was 83–94%; use of these antibiotics has been common in Bangladesh poultry farming (Hassan et al., 2021; Ibrahim et al., 2023). Ninety percent of isolates were resistant to the HP-CIA ciprofloxacin, and this correlated with the presence of mutations in the quinolone resistance determining regions of *gyrA* and/or *parC* (isolates with MIC = > 4 mg/L) and with the presence of plasmid mediated quinolone resistance genes of the *qnrS* family (isolates with MIC 0.25 or 0.5 mg/L). High prevalence of ciprofloxacin resistance in *E. coli* from poultry and humans has been previously reported in Bangladesh (Das et al., 2023; Ibrahim et al., 2023). The use in WGS in this study provided new insights by demonstrating that ciprofloxacin resistance is present in a very wide diversity of *E. coli* STs and lineages (Figure 1), rather than confined to specific clades. The widespread and common use of ciprofloxacin in the Bangladesh poultry sector (Parvin et al., 2022; Ibrahim et al., 2023) will likely have contributed to the high prevalence of resistance, which presents a risk to effective treatment of infection in both poultry and humans.

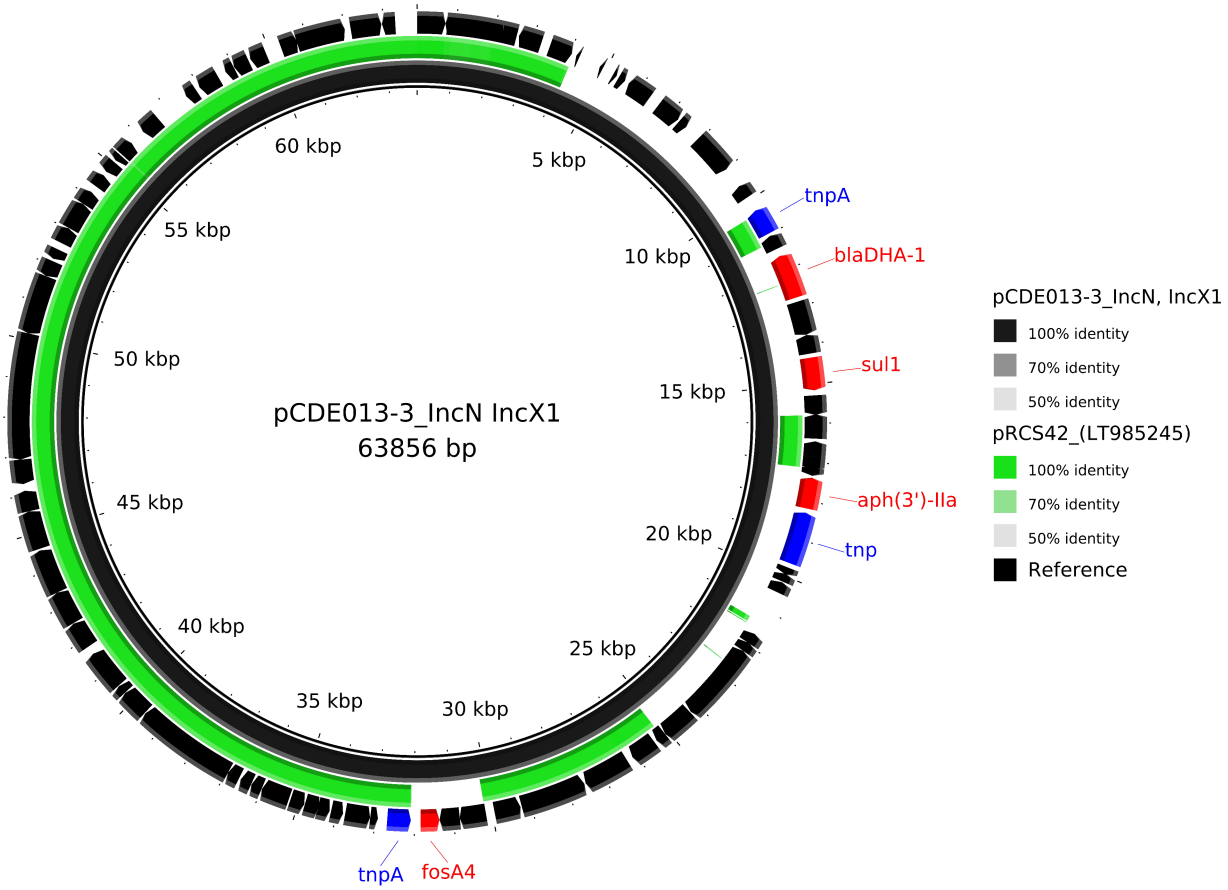

FIGURE 4
Comparative analysis of the IncN/IncX1 hybrid plasmid pCDE013-3 harbouring bla_{DHA-1} (black inner ring) with other published plasmid identified using MOB-suites nearest mash neighbour; LT985245. Each concentric ring displays the nucleotide similarity between pCDE012-2 and the other sequence in the outer ring. The shade of colour indicates the BLAST percentage identity. The arrows around the map indicate deduced Coding Sequences and their orientation. AMR genes are highlighted in red, and transposons highlighted in blue. The image was generated using BRIG.

The high prevalence (47%) of resistance to the Watch group antimicrobial azithromycin was associated with the *mph(A)* gene. In Bangladesh azithromycin has not been commonly used on poultry farms (Ibrahim et al., 2023) and was banned for veterinary use in 2022 (Directorate General of Drug Administration, 2022). However, it remains an important treatment option for invasive *E. coli* infections in humans (Islam et al., 2023a). We note that *mph(A)* confers high level resistance to both erythromycin and azithromycin (Poole et al., 2006) and that erythromycin use in poultry production in Bangladesh is high (Islam et al., 2023a), and therefore consider that this cross-resistance mediated by *mph(A)* may contribute to the high prevalence of azithromycin resistance observed by us and others.

For the 'Reserve' antimicrobial colistin, resistance was detected in 7 isolates (8%), mediated by *mcr1* which has been reported as the dominant *mcr* variant in Bangladesh poultry (Ahmed et al., 2020; Ibrahim et al., 2023). In Bangladesh, restrictions on colistin use in the veterinary sector were introduced in 2019 and it has been fully prohibited since 2022 (Directorate General of Drug Administration, 2022). However, colistin has been reported as commonly used by poultry farmers prior to this ban (Ibrahim et al., 2023) and our study was undertaken before the full prohibition.

We detected four ESBL-producing and two AmpC-producing *E. coli*, giving an overall prevalence of 7% for resistance to the 'Watch' antimicrobials ceftazidime or cefotaxime. This is similar to the 6% cefotaxime resistance prevalence reported by Ibrahim et al. (2023) but lower than the 22% ESBL prevalence reported for animals in a recent review in Bangladesh (Islam et al., 2023b). The ESBL phenotype was associated with $bla_{CTX-M-55}$ (CTX-M group 1) and $bla_{CTX-M-65}$ (CTX-M group 9), which have both been reported in ESBL isolates from human clinical samples in Bangladesh (Mazumder et al., 2021). CTX-M group 1 and group 9 genes have been reported in *E. coli* from Bangladesh poultry (Islam et al., 2023a), but detection was by PCR which precluded the definitive assignment of the gene. There has been limited investigation of AmpC-producing *E. coli* in Bangladesh poultry, and here we describe the presence of bla_{DHA-1} in two isolates. Distinguishing between ESBL-producing and AmpC-producing *E. coli* is important epidemiologically and also clinically as the latter are not affected by ESBLs inhibitors.

Tigecycline is a 'Reserve' antimicrobial and generally only licenced for use in humans, therefore the detection of a tigecycline resistant *E. coli* is significant. Tetracycline has been commonly used in Bangladesh poultry farms (Hassan et al., 2021; Ibrahim et al., 2023) and it has been proposed that the extensive use of tetracycline in livestock has contributed to the dissemination of *tet*(X) variants (Martelli et al., 2022). Tigecycline resistance prevalence in poultry-derived isolates in Bangladesh has been reported at 2 and 27% (Parvin

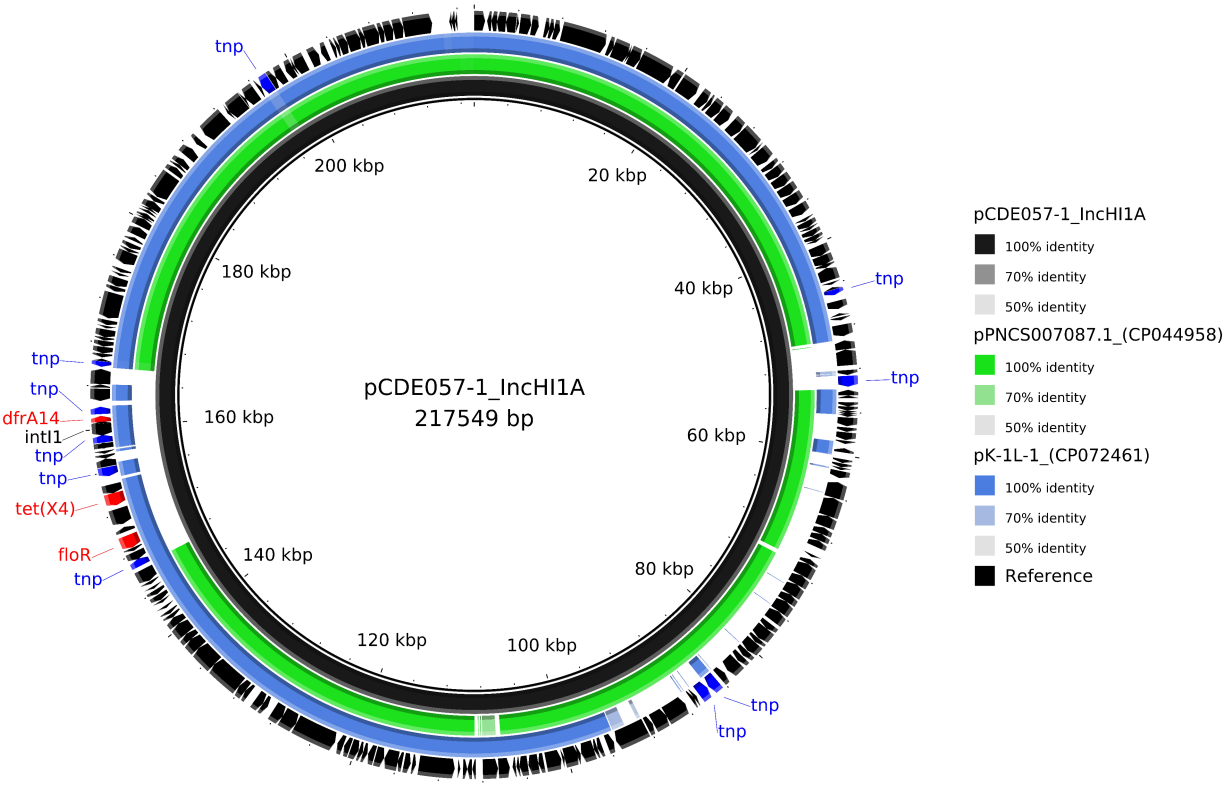

FIGURE 5
Comparative analysis of the IncHI1A hybrid plasmid pCDE057-1 harbouring *tet*(X4) (black inner ring) with other published plasmids present in *Salmonella* and *K. pneumoniae* isolates (accession numbers from inner to outer ring: CP044958 and CP072461). Each concentric ring displays the nucleotide similarity between pCDE057-1 and the other sequences in the outer rings. The most similar plasmid in green, is missing a large area of homology where the AMR genes reside on plasmid pCDE057-1, hence the comparison with the second plasmid in blue, where the AMR gene region shares highest homology, but the rest of the plasmid shares a lower nucleotide identity. The shade of colour indicates the BLAST percentage identity. The arrows around the map indicate deduced Coding Sequences and their orientation. AMR genes are highlighted in red, and transposons highlighted in blue. The image was generated using BRIG.

et al., 2022; Ibrahim et al., 2023), but neither study investigated the genetic basis of resistance. Furthermore, tigecycline is known to be light and oxygen sensitive, which can lead to susceptible isolates being falsely described as resistant (Amann et al., 2021). Our genomic approach demonstrated that for one isolate the tigecycline resistance was conferred by *tet*(X4) residing on an IncHI1A/B-IncFIA mosaic plasmid, which had close homology to plasmids detected in *E. coli* obtained from humans. We believe this to be the first description of the *tet*(X4) gene in *E. coli* from Bangladesh poultry. Emergence of plasmid-mediated tigecycline resistance mechanism threatens the use of tigecycline as a last resort antimicrobial against carbapenem resistant, gramme-negative bacterial infections (Zhang et al., 2022). Importantly, in this study we did not identify any resistance to the carbapenem meropenem (Watch group), either phenotypically or genotypically, although carbapenem resistance genes such as $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-1}}$ have been identified in human clinical isolates from Bangladesh (Jain et al., 2021).

This study has provided a firm foundation for the phenotypic and genomic surveillance of AMR in poultry at LBMs in Bangladesh. In future we would seek to expand on these finding by including more markets, because for logistical reasons it was not possible to sample all LBMs in Dhaka or sample markets in other cities in Bangladesh. It is also possible that the results of this study may not provide a full representation of the smaller live bird markets in Dhaka, which may obtain birds raised using different farming practises with a concomitant impact on carriage of AMR. Additionally, the sampling strategy was impacted by the COVID-19 pandemic. Expanding the number of isolates tested by MIC and WGS will further contribute.

Conclusion

In this study we have demonstrated the value of using phenotypic and genomic approaches in a surveillance programme to define antimicrobial susceptibilities and describe in detail the genetic diversity of isolates. This has highlighted important linkages between public and animal health and provided new insights into the potential for transmission between the two sectors in Bangladesh. Ongoing surveillance, susceptibility testing, and genomic characterisation of *E. coli* in poultry at LBMs will increase awareness of resistance prevalence and enable Bangladesh authorities to assess the impact of interventions designed to address the challenge of AMR, such as improved rational antimicrobial use and the prohibition of certain products in the veterinary sector. The statistical analysis determined that there was a significant increase between the two time periods in the incidence of resistance to three antibiotics (azithromycin, chloramphenicol, and gentamicin), which further highlights the need for continued research and surveillance, according to the National Action Plan for Bangladesh (Alam et al., 2017). Employing similar genomic approaches for human and environmental surveillance will

facilitate assessment of the One Health impact of AMR and identify novel and emerging AMR genes. Measures to increase the public understanding and awareness of AMR, alongside sustainable implementation of protocols to reduce AMU are required to help mitigate the threat of AMR. For consumers of poultry purchased at live bird markets good hygiene and food preparation practises will help minimise risk of exposure to AMR bacteria.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA1100899.

Author contributions

AD: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. TC: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. SA: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. MMHA: Investigation, Methodology, Project administration, Writing – review & editing. MS: Investigation, Methodology, Project administration, Writing – review & editing. SB: Investigation, Methodology, Project administration, Writing – review & editing. MH: Writing – review & editing, Data curation, Formal analysis, Investigation, Methodology, Project administration. MR: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – review & editing. RS: Formal analysis, Writing – review & editing. RC: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. EB: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. MC: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1418476/full#supplementary-material

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The sub-MIC selective window decreases along the digestive tract: determination of the minimal selective concentration of oxytetracycline in sterilised intestinal contents

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Introduction: The administration of antibiotics can expose the digestive microbiota of humans and animals to sub-inhibitory concentrations, potentially favouring the selection of resistant bacteria. The minimal selective concentration (MSC) is a key indicator to understand this process. The MSC is defined as the lowest concentration of an antibiotic that promotes the growth of a resistant strain over a susceptible isogenic strain. It represents the lower limit of the sub-minimal inhibitory concentration (MIC) selective window, where resistant mutants can be selected. Previous studies focused on determining the MSC under standard culture conditions, whereas our research aimed to determine the MSC in a model that approximates *in vivo* conditions.

Methods: We investigated the MSC of oxytetracycline (OTC) in Mueller-Hinton broth (MHB) and sterilised intestinal contents (*SIC*) from the jejunum, caecum and rectum (faeces) of pigs, using two isogenic strains of *Escherichia coli* (one susceptible and one resistant to OTC). Additionally, the MIC of OTC against the susceptible strain was determined to assess the upper limit of the sub-MIC selective window.

Results: Our study took a novel approach, and the results indicated that MIC and MSC values were lower in MHB than in *SIC*. In the latter, these values varied depending on the intestinal segment, with distal compartments exhibiting higher MIC and MSC values. Moreover, the sub-MIC selective window of OTC in SIC narrowed from the jejunum to the rectum, with a significantly closer MSC to MIC in faecal *SIC*.

Discussion: The results suggest that OTC binds to digestive contents, reducing the fraction of free OTC. However, binding alone does not fully explain our results, and interactions between bacteria and intestinal contents may play a role. Furthermore, our findings provide initial estimates of low concentrations facilitating resistance selection in the gut. Finally, this research enhances the understanding of antimicrobial resistance selection, emphasising the intricate interplay between antibiotics and intestinal content composition in assessing the risk of resistance development in the gut.

KEYWORDS

antibiotic resistance, drug binding, gut, low concentration, minimal selective concentration, risk assessment, sub-inhibitory concentration, tetracycline

1 Introduction

Antibiotic resistance represents one of our most urgent global health challenges. The primary goal of administering antibiotics to combat bacterial infections is to achieve the highest possible active drug concentration in the infectious site without inducing toxicity to the patient. This optimises cure rates whilst curbing the emergence of *de novo* resistance amongst pathogenic bacteria within the host. However, the administration of antibiotics leads to concentration gradients within the body, resulting in non-targeted bacteria, including those in the gut microbiota, being exposed to low antibiotic concentrations. Studies have shown that such low concentrations of antibiotics could promote the selection of antibiotic-resistant bacteria (Andersson and Hughes, 2014). Thus, potentially resistant bacteria selected in the digestive tract of treated humans and production animals may enter sewage water, slurry and manure, providing conditions that facilitate the spreading of resistance genes in the environment. Therefore, it is essential to determine the minimum antibiotic concentrations potentially responsible for selecting antibiotic-resistant bacteria *in vivo*.

In the past decade, the concept of minimal selective concentration (MSC) has gained importance in the field of antibiotic resistance, allowing for assessing resistance selection at low concentrations. The MSC refers to the lowest concentration of an antibiotic that confers a competitive advantage to a resistant strain over a susceptible isogenic strain (Gullberg et al., 2011). In this manner, exposure of bacteria to antibiotic concentrations within the range of the MSC and the minimal inhibitory concentration (MIC) against the susceptible bacteria (MIC_s) provides a critical window (sub-MIC selective window) in which resistant bacteria are advantaged whilst still allowing susceptible strains to grow. This phenomenon not only selects and enriches resistant strains but also enhances genetic processes such as horizontal gene transfer, recombination and mutagenesis (Andersson and Hughes, 2014). Furthermore, exposure of bacteria to low concentrations of antibiotics can promote the emergence of resistance through a stepwise accumulation of low-fitness-cost resistance mechanisms, each with minor individual effects (Wistrand-Yuen et al., 2018; Sandegren, 2019). Thus, resistant bacteria with high fitness are selected at low concentrations, which increases the probability of persistence and spread of resistant strains.

Tetracyclines constitute a class of broad-spectrum antibiotics used in both human and veterinary medicine (European Centre for Disease Prevention and Control, 2022; European Medicines Agency, 2022). These antibiotics are primarily administered orally and display highly variable absorption extents, ranging from 25 to 30% for chlortetracycline to over 80% for doxycycline and minocycline. Additionally, certain tetracyclines, including oxytetracycline (OTC), can be eliminated unchanged through biliary or intestinal secretion followed by faecal excretion (Agwuh and MacGowan, 2006). In the gut lumen, the intestinal contents are variable and complex matrices in which antibiotics can bind to various components, leading to a reduction in the concentration of free and active antibiotics along the digestive tract (Vallé et al., 2021). As a result, oral (and even parenteral) administration of antibiotics can expose the intestinal microbiota to sub-inhibitory antibiotic concentrations, either during the treatment as a consequence of incomplete absorption, biliary/intestinal secretion and interactions of the drug with the digestive matrix or after the end of the treatment during the terminal phase of the elimination process.

To date, investigations on the MSC for bacterial species have been conducted in sterile culture broth (Gullberg et al., 2011), culture broth enriched with a pig faecal microbial community (Klümper et al., 2019) and biofilms (Hjort et al., 2022). However, to the best of our knowledge, no experimental study has examined the impact of the intestinal content matrix on the MSC. As reviewed by Murray et al. (2021), several methodological approaches exist to determine the effect of low concentrations of antibiotics. Amongst the diverse approaches, the methodology from Gullberg et al. (2011) presents the advantage of being easily adjustable, permitting it to mimic different environmental conditions. Hence, the present research aimed to establish the MSC of OTC using an adapted protocol based on Gullberg et al. (2011) mimicking conditions encountered in the digestive tract. Specifically, this investigation focused on two under-explored aspects. Firstly, it examined how OTC and bacteria interact with the constituents of the intestinal contents in the context of MSC determination. Secondly, it aimed to assess the extent of the sub-MIC selective window along the digestive tract.

2 Materials and methods

2.1 Bacterial strains and growth conditions

Two isogenic strains of *Escherichia coli*, DA34574 and DA34433, were used in this study. These strains were obtained from the Department of Biochemistry and Medical Microbiology at Uppsala University, Sweden, and both are derivatives of *E. coli* MG1655. Strain DA34574 was susceptible to tetracyclines, whilst strain DA34433 possessed the non-conjugative plasmid pCA24N(gfp-)-tet(G), which conferred resistance to tetracyclines through the expression of efflux pumps (Nicoloff and Andersson, 2016). Cultures of these strains were carried out in 5 mL of Muller-Hinton broth (MHB) at 37°C whilst shaking (180 rpm). For strain DA34433, 6.0 µg/mL of OTC was added to the culture media to preserve the plasmid carrying the antibiotic resistance gene.

2.2 Preparation of sterilised intestinal contents (*SIC*)

In order to take into account individual variation, four White Large pigs aged between 55 and 56 days and weighing between 14.7 and 21.45 kg at the time of the study were used. They had access to water and feed (flour-based growth food [18.0% protein, 7.0% fat, 4.4% cellulose and 3.8% ash], PS2, Solevial, Villefranche de Rouergue, France) *ad libitum*.

After euthanasia, the contents of the jejunum, caecum and rectum were removed and collected in tubes. These tubes were placed on ice and stored at −20°C. The intestinal contents stored at −20°C were thawed at room temperature and pooled. The contents were weighed and placed in a bag with a filter. Mueller-Hinton broth (MHB) was added to achieve an intestinal content:MHB ratio of 1:5 w/v. The resulting filtered suspensions were mixed using a homogeniser blender for 90 s. Then, they were sterilised by autoclaving at 121°C for 20 min to obtain sterilised intestinal contents (*SIC*) from the jejunum, caecum and rectum (faecal content). The obtained *SIC* were stored at −20°C before use.

The French Ministry of Research authorised the experimental protocol (#23387_2019122010002751), and the study was conducted in accordance with local legislation and institutional requirements.

2.3 Evaluation of the bacterial growth

Overnight cultures of strains DA34574 and DA34433 were handled individually. First, each culture was centrifuged, and the supernatant was discarded. Then, the resulting pellet for each strain was rinsed with phosphate-buffered saline (PBS), centrifuged once more, and resuspended in an equal volume of MHB. These suspensions were subsequently diluted to achieve a final concentration of 10^3 CFU/mL in a fresh medium (MHB or *SIC*) without antibiotics and incubated at 37°C for 24 h. During the incubation, aliquots were collected hourly for 8 h, with each aliquot being plated in triplicate on Mueller-Hinton agar (MHA). An additional aliquot was taken after 24 h and plated in triplicate on MHA. The plates were then incubated overnight, and the colonies were counted the following day. This experiment was conducted in triplicate, and the growth kinetics were represented as a curve showing colony-forming units per millilitre over time in hours.

2.4 Determination of the minimal inhibitory concentration (MIC)

The MIC determination was conducted using the microdilution method. Bacterial strains were exposed to different ranges of antibiotic concentrations in MHB or *SIC*, with a two-fold dilution factor between each concentration. For strain DA34574 (susceptible), which had a previously reported MIC of 0.38 µg/mL (Nicoloff and Andersson, 2016), an OTC range of 0.125 to 64 µg/mL was tested. Strain DA34433 (resistant), with an indicated MIC of 64 µg/mL (Nicoloff and Andersson, 2016), was subjected to an OTC range of 1 to 512 µg/mL. Bacterial suspensions of both strains were prepared in MHB, starting from colonies isolated on MHA, and diluted to achieve an approximate concentration of 10^5 CFU/mL in each well of the microplate. Subsequently, the plates were inoculated and then incubated overnight at 37°C.

After incubation, the bacterial suspensions from each well were serially diluted up to a dilution factor 10^{-6}. Next, 10 µL of each dilution was plated in triplicate on charcoal trypticase soy agar. The plates were then incubated overnight, and colony counts were performed the following day. The MIC value corresponded to the highest OTC concentration at which at least 10^5 CFU/mL were observed, indicating a bacteriostatic effect.

2.5 Competition experiments and determination of the minimal selective concentration (MSC)

This experiment aimed to determine the minimum antibiotic concentration that confers a selective advantage to a resistant strain compared to a susceptible isogenic strain. Co-cultures of strains DA34574 (susceptible) and DA34433 (resistant) were conducted in MHB and *SIC* at varying concentrations of OTC, all being lower than the MIC of the susceptible strain DA34574.

An adapted protocol based on Gullberg et al. (2011) was developed. Initially, overnight cultures of both strains were centrifuged, the supernatant was discarded, and the pellet was resuspended in PBS. The pellet of the strain DA34433 was rinsed with PBS to remove any remaining antibiotics. Both strains were co-cultured with an initial bacterial load of 10^5 CFU/mL in either MHB or *SIC* at different OTC concentrations.

Following co-culturing and after 24 and 48 h of incubation, the total bacterial counts (comprising both strains) were determined on MHA, whilst the counts of the resistant strain (DA34433) were determined on MHA supplemented with OTC at 6 µg/mL. The number of susceptible bacteria was calculated by subtracting the counts of resistant bacteria from the total counts. The ratio of resistant to susceptible bacteria was monitored over time, and the selection coefficient was determined using the equation:

$$s = \frac{\ln\left(\frac{R_{(t)}}{R_{(0)}}\right)}{t}$$

Where s represents the selection coefficient, $R_{(t)}$ indicates the resistant to susceptible bacteria ratio after 48 h, $R_{(0)}$ is the initial ratio at the beginning of the co-culture and t is the co-culture time (48 h). The calculated selection coefficients were then plotted as a function of the OTC concentration in each culture. The MSC was determined through interpolation using a polynomial model representing the OTC concentration at which the selection coefficient equals 0.

2.6 Statistical analysis

Bacterial growth data were analysed using three-way analysis of variance (RStudio Team, 2020). The effects of strain (DA34574 and DA34433), medium (MHB and jejunum, caecum and faecal *SIC*) and time points (0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h) and all their interactions on bacterial counts were calculated.

3 Results

3.1 Evaluation of the bacterial growth

In all media (MHB and *SIC*), the susceptible (DA34574) and resistant (DA34433) strains showed identical growth kinetics, confirming the absence of strain-related effects and ensuring that subsequent results were not influenced by variations in growth rates between the two strains (Figure 1A). However, significant variations were observed between the different media conditions ($p < 0.05$). Specifically, slower growth rates were observed in caecum *SIC* (with bacterial counts ranging from 6.6 ± 0.2 to 8.5 ± 0.2 $\log_{10}$ CFU/mL after 8 and 24 h of culture, respectively), whilst faster growth rates were observed in MHB and jejunum *SIC* (where bacterial already reached 8.9 ± 0.1 and 9.1 ± 0.1 after 8 h of culture in MHB and jejunum *SIC*, respectively) (Figure 1B).

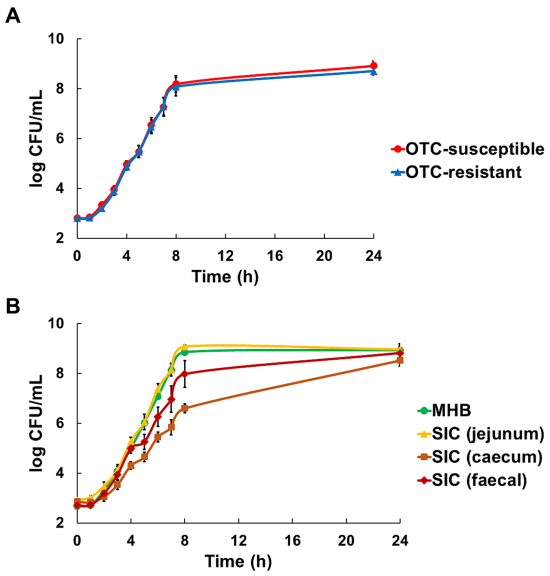

FIGURE 1
Evaluation of the growth of oxytetracycline (OTC)-susceptible (DA34574) and OTC-resistant (DA34433) isogenic *Escherichia coli* strains in Mueller-Hinton broth (MHB) and jejunum, caecum and faecal sterilised intestinal contents (*SIC*) of pigs. Panel **(A)** shows the strain effect [OTC-susceptible (●) and OTC-resistant (▲)] in MHB and jejunum, caecum and faecal *SIC* ($n = 12$). Panel **(B)** shows the medium effect [MHB (●), jejunum *SIC* (▲), caecum *SIC* (■) and faecal *SIC* (◆)] for both OTC-susceptible and resistant strains ($n = 6$). Bars represent standard error.

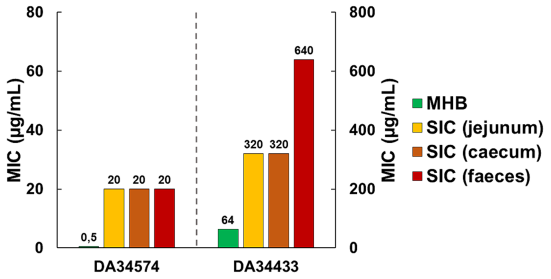

FIGURE 2
Minimal inhibitory concentration (MIC) of oxytetracycline (OTC) against OTC-susceptible (DA34574) and OTC-resistant (DA34433) isogenic *Escherichia coli* strains in Mueller-Hinton broth (MHB) and jejunum, caecum and faecal sterilised intestinal contents (*SIC*) of pigs.

3.2 Minimal inhibitory concentration (MIC) of oxytetracycline (OTC)

In MHB, the OTC-susceptible strain DA34574 displayed a MIC value of 0.5 µg/mL, whilst the OTC-resistant strain DA34433 exhibited a higher MIC value of 64 µg/mL. In intestinal contents, the OTC-susceptible strain showed a consistent MIC of 20 µg/mL across jejunum, caecum and faecal *SIC*. In contrast, the OTC-resistant strain exhibited varying MIC values depending on the intestinal content, from 320 µg/mL in jejunum and caecum *SIC* to 640 µg/mL in faecal *SIC*. These results indicate a variation in the MIC depending on the specific intestinal content. Overall, the MIC was 40-fold higher in *SIC* compared to MHB for the OTC-susceptible strain and 5- to 10-fold higher in *SIC* compared to MHB for the OTC-resistant strain (Figure 2). In summary, these results show a different impact of the intestinal contents on the MIC of OTC according to the strains.

3.3 Minimal selective concentration (MSC) of oxytetracycline (OTC)

When determining the MSC of OTC in MHB, concentrations of 0, 0.01, 0.02 and 0.04 µg/mL of OTC were selected based on preliminary co-culture tests, which indicated that the selection coefficient approaches 0 within this concentration range. The selection coefficient was calculated over the 48-h co-culture period and yielded the following values for the tested OTC concentrations: −0.034 (0 µg/mL), −0.024 (0.01 µg/mL), −0.009 (0.02 µg/mL) and 0.032 (0.04 µg/mL). These findings suggest that the OTC-resistant strain had a competitive advantage at OTC concentrations above 0.02, as indicated by the selection coefficient becoming positive between 0.02 and 0.04 µg/mL. After plotting the selection coefficient against OTC concentration and interpolation to determine the OTC concentration at which the selection coefficient equals 0, the calculated MSC for OTC in MHB was 0.025 µg/mL (Figure 3A).

Assuming that antibiotics bind to the digestive matrices' contents, and based on preliminary tests, higher OTC concentrations were employed in the co-culture assays using *SIC*: 0, 0.32, 0.64 and 1.28 µg/mL for jejunum and caecum *SIC* and 0, 0.8, 3.2 and 6.4 µg/mL for faecal *SIC*. As anticipated, an increase in the MSC value for OTC was observed depending on the content of each studied digestive compartment. Specifically, the more distal the compartment from which the content was used in the medium, the more the MSC value increased compared to MHB. The MSC values for OTC in jejunum, caecum and faecal *SIC* were 0.26, 0.72, and 2.5 µg/mL, respectively (Figures 3B–D).

4 Discussion

The present study aimed to contribute to the assessment of the risk of selecting antimicrobial resistance at very low antibiotic concentrations that can occur within specific body compartments during antibiotic treatment. More precisely, we evaluated the impact of the intestinal environment on the values of MSC, the determination of which is a prerequisite for implementing strategies to decrease antibiotic concentrations and avoid prolonged exposure of commensal bacteria to sub-MIC levels of antibiotics (Gullberg et al., 2011). Whilst previous research on the selection of resistance at low concentrations of antibiotics in the gut has focused on the role of a natural microbial community on the MSC (Klümper et al., 2019), we were interested in a parameter not studied until now: the digestive matrix. For this purpose, we used *SIC* from different segments of the pig digestive tract to determine the MSC of OCT for *E. coli* strains *in vitro*. The *SIC* were used to evaluate the impact of the physicochemical characteristics of intestinal fluids and contents on the determination of the MSC, as well as the consequences on the sub-MIC selective window along the digestive tract.

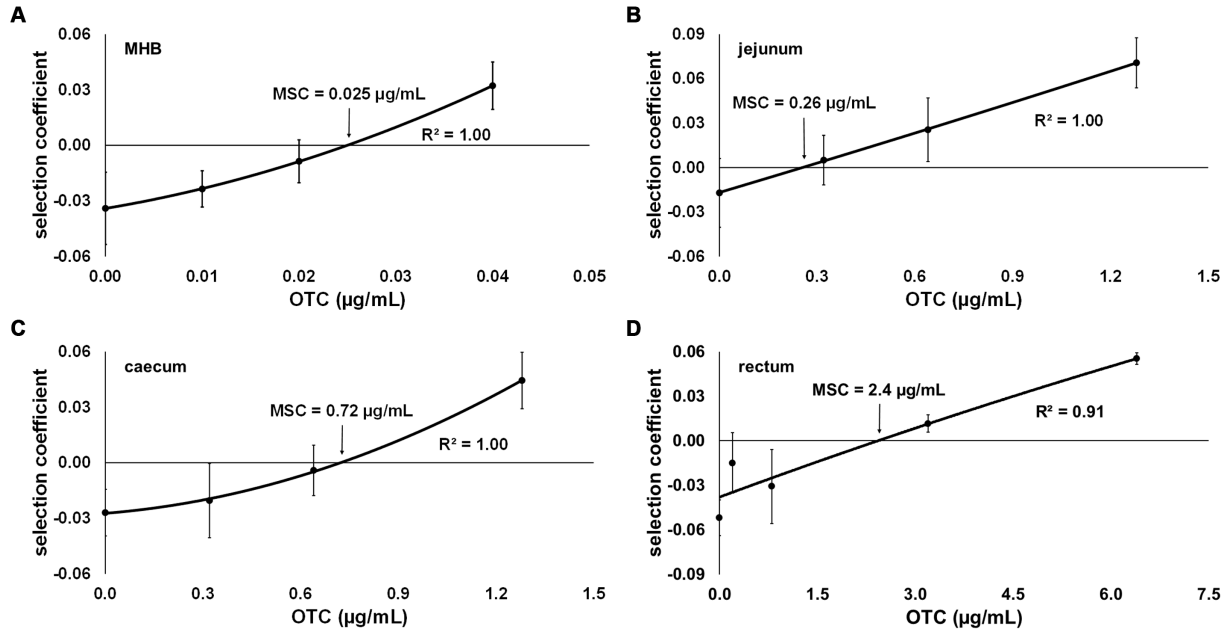

FIGURE 3
Selection coefficient of an oxytetracycline (OTC)-resistant strain (DA34433) against an OTC-susceptible isogenic *Escherichia coli* strain (DA34574) as a function of OTC concentration in **(A)** Mueller-Hinton broth (MHB) and sterilised intestinal content of the **(B)** jejunum, **(C)** caecum and **(D)** rectum from pigs. The OTC concentration at which the selection coefficient is 0 represents the minimal selective concentration (MSC). Bars represent standard error.

The first comparative bacterial growth experiments indicated that the OTC-susceptible and OTC-resistant isogenic *E. coli* strains grew similarly in each medium, namely MHB and jejunum, caecum and faecal *SIC*. These observations confirmed the absence of a potential cause of bias, such as differential growth rates, during the co-culture assays. However, growth rate variations between the strains were observed across the media, with faster growth in MHB and jejunum *SIC* compared to caecum and faecal *SIC*. Several hypotheses can be proposed to explain the observed growth variations. First, it is well documented that the microbiota composition and concentration vary along different digestive tract segments (Sundin et al., 2017), and these variations, along with the digestive metabolism of animals, contribute to the differential breakdown of the constituents of the digestive matrix in each segment (Tan et al., 2018). Consequently, the nutrient composition within the different segments could differ, potentially influencing microbial growth. Additionally, the physicochemical properties of the contents within each segment, such as viscosity (McDonald et al., 2001) and fibre content (Makki et al., 2018), can influence microbial growth. Although not directly tested in our study, it is plausible that the combination of nutrient content variations and the physicochemical nature of the contents within different intestinal segments shaped microbial growth patterns.

The determination of the MIC in MHB and *SIC* revealed an increase in MIC values when determined in *SIC*. This increase in the intestinal contents was expected, as previous studies by Ferran et al. (2013) and Vallé et al. (2021) have demonstrated a decrease in antibiotic activity in the presence of the intestinal matrix. Moreover, Ahn et al. (2018) established that nearly 60% of tetracycline binds to human faeces, as determined through chemical and microbiological assays. Consequently, the fraction of OTC that remains unbound and thus potentially active is lower in *SIC*, necessitating a higher overall concentration of the antibiotic in the intestinal contents to achieve the same bacteriostatic effect as in MHB. Although the binding of OTC to the matrix is hypothetical, and the determination of MIC does not directly inform about the free fraction of the antibiotic, this hypothesis is pertinent in interpreting our results. To illustrate, the 10-fold increase in the MIC for the OTC-resistant strain in faecal *SIC* compared to MHB and the 2-fold increase compared to jejunum and caecum *SIC* can be attributed to the change in matrix. This difference likely results in stronger binding of OTC to the components of distal intestinal segments compared to proximal segments, as observed by Ferran et al. (2013) and Vallé et al. (2021).

Furthermore, we observed a difference in the extent of the increase in MIC values between the OTC-susceptible strain and the OTC-resistant strain during the transition to the intestinal contents. Interestingly, the MIC for the OTC-susceptible strain increased by a factor of 40 across the three different digestive contents when compared to MHB. In contrast, the MIC for the OTC-resistant strain only increased by a factor of five when transitioning from MHB to jejunum or caecum *SIC* and by a factor of 10 in faecal *SIC*. A possible explanation for this result is the saturation of the matrix when determining the MIC at high concentrations. If OTC binds to the matrix, it can be assumed that the interaction continues until saturation occurs. When cultured at high antibiotic concentrations, the matrix becomes saturated more quickly, resulting in a higher proportion of free (potentially active) OTC. Therefore, in determining the MIC for the OTC-resistant strain in faecal *SIC*, it is possible that the matrix reached saturation, leading to a lower ratio of MIC in faecal *SIC* to MIC in MHB for the OTC-resistant strain (10) than observed for the OTC-susceptible strain (40). Finally, we cannot exclude the possibility that although the viable microbiota was eliminated in our study by heat sterilisation, dead bacterial cells could have adsorbed

antibiotics, potentially impacting the concentration available for selection, as confirmed by Podlesek et al. (2016).

The determination of the MSC in MHB and *SIC* revealed an increase in MSC values in *SIC* compared to MHB. This finding aligns with the observations made during the determination of the MIC, suggesting a higher interaction between OTC and the intestinal contents from distal compartments of the digestive tract. However, the variations in MIC and MSC were not proportional across the different intestinal segments. Given that the MSC values are lower than the OTC concentration range where saturation of the intestinal matrix is plausible (as observed with the MIC values), another hypothesis to consider involves the interaction between bacteria and the intestinal contents. This interaction could create an environmental niche, providing some protection against the antibiotic. This hypothesis is supported by a study by Vallé et al. (2021), which demonstrated with another tetracycline, minocycline, that a combination of factors decreasing available antibiotic concentrations and reducing bacterial susceptibility to unbound antibiotic could account for the effects observed in *SIC*.

The MSC and the MIC for the OTC-susceptible strain define a range of concentrations where resistance selection can occur at very low levels. This sub-MIC selective window not only facilitates the horizontal transfer of resistance genes between resistant and susceptible strains but also promotes the emergence of *de novo* mutations (Hughes and Andersson, 2012). Indeed, the selection pressure imposed by low concentrations of antibiotics leads to an increased mutation frequency in susceptible bacteria, enabling their survival. However, these mutations often involve the development of resistance mechanisms with low biological costs, allowing the favoured growth of antibiotic-resistant strains over susceptible strains (Sandegren, 2014). Previous studies investigating the MSC of antibiotics in culture broth and aquatic environments have demonstrated the presence of sub-MIC selective windows encompassing concentrations several tenths-fold below MIC values (Gullberg et al., 2011; Kraupner et al., 2020). In the context of the highly abundant and diverse microbiota within the digestive tract, such sub-MIC selective windows would pose significant concerns. In our study, the MSC in jejunum *SIC* was about 80 times lower than the MIC for the OTC-susceptible strain, whilst it was 28 times lower in caecum *SIC*. However, notably, the MSC in faecal *SIC*, representing the compartment with a significantly more abundant digestive microbiota, was only eight times lower. Consequently, we observed a reduction in the sub-MIC selective window along the digestive tract (Figure 4), providing novel insights into the risk assessment of antimicrobial resistance selection in the gut. However, the lack of previous studies on the MSC in the presence of the digestive matrix prevented a direct comparison of the results of this study.

Besides the interactions between the antibiotic, target bacteria and digestive matrix, the digestive microbiota could also narrow the range of sub-inhibitory concentrations favouring resistance selection. This hypothesis is mainly supported by Klümper et al.'s (2019) study, which explored the impact of a microbial community on the MSC. This study found that resistance selection was less pronounced in the presence of the digestive microbiota, attributable to the complex community's protective effect against antibiotic activity. Consequently, the actual MSC in the digestive tract is likely higher than estimated in our study, where resident microbiota was removed by sterilisation. Thus, employing a dynamic system of bioreactors would be beneficial to reflect both the effects of digestive matrices and the intestinal microbiota and to determine the MSC of OTC under conditions closer to *in vivo*. Such a system would allow for the simulation of various compartments of the digestive tract, controlling physiological aspects like pH, as well as the microflora present in each reactor, as suggested by Dufourny et al. (2019).

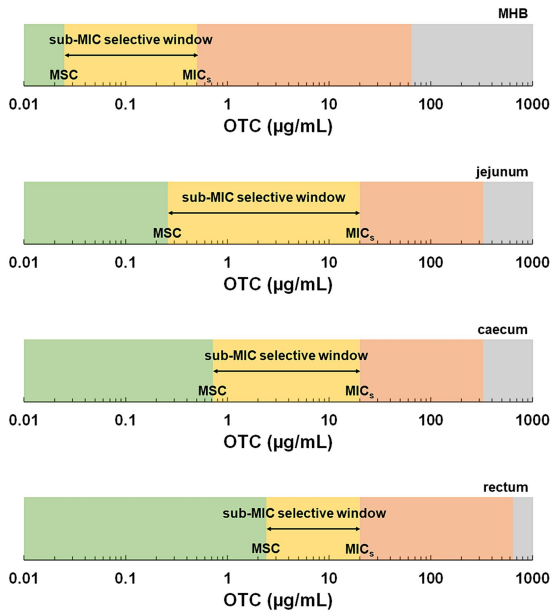

FIGURE 4
Schematic representation of selective windows based on the minimal selective concentrations (MSC) and minimal inhibitory concentrations (MIC) determined in this study as a function of the medium. The green zone represents concentrations below the MSC. The yellow zone represents the sub-MIC selective window, the range of concentrations between the MSC and the minimal inhibitory concentration of the susceptible strain (MIC$_s$). The red zone represents the traditional selective window, the range of concentrations above the MIC$_s$ and below the MIC of the resistant strain, where only the resistant strain can grow. The grey area represents the zone above the MIC of the resistant strain.

It should be highlighted that the physicochemical characteristics of intestinal fluids can vary dramatically between species, influenced by their feeding behaviour and associated digestive physiology, as well as within a species depending on the type of feed, for example. These factors might significantly influence the values of MSC of an antibiotic both between species and within individuals of the same species. Such variability could be seen as a limitation when determining MSC in biological contents, necessitating its determination in specific environmental conditions, as opposed to controlled broths. Whilst the purpose of the present study was not to investigate the variability of the physicochemical characteristics of intestinal fluids, it underscores the importance of considering this factor in the determination of MSC. Moreover, we tested only one antibiotic in this study. The diverse chemical nature of antibiotics may also play a role in the extent of binding, and the fitness cost associated with resistance mechanisms for different antibiotics may influence the minimal antibiotic concentration at which resistant bacteria outcompete susceptible bacteria.

In perspective, further investigations are warranted to fully understand the implications of sub-inhibitory concentrations of

antibiotics on the selection of antibiotic resistance. It is essential to verify whether the active concentrations of OTC in the digestive tract after treatment of infectious diseases in humans or animals align with the concentration window identified in this study. Quantifying the total amount of OTC in the digestive contents and estimating the potentially active fraction (free fraction) in different contents can provide additional elements to assess the risk of antimicrobial resistance selection. Additionally, exploring the influence of the intestinal microbiota on the MSC would be beneficial. The presence of a microbial community is expected to impact the MIC and MSC values, potentially altering resistance selection. Moreover, conducting molecular studies would enable the investigation of the transfer of resistance genes and the selection of associated resistance mechanisms at low concentrations of antibiotics.

In summary, this study provides valuable insights into the emergence of antimicrobial resistance in the gut environment. By analysing the MSC of OTC in different sterilised intestinal contents, we identified the window of sub-inhibitory concentrations contributing to resistance selection and the growth advantage of resistant strains over susceptible isogenic strains along the digestive tract. Furthermore, the methodology developed in this research can be applied to other antibiotics, and the findings obtained may have implications for developing improved guidelines for antibiotic administration.

Data availability statement

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

Ethics statement

The animal study was approved by French Ministry of Research Experimental protocol #23387_2019122010002751. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

PI: Conceptualisation, Data curation, Formal analysis, Funding acquisition, Methodology, Investigation, Project administration, Validation, Visualisation, Writing – original draft, Writing – review & editing. BV: Conceptualisation, Data curation, Formal analysis, Methodology, Investigation, Project administration, Validation, Visualisation, Writing – original draft. NA: Conceptualisation, Data curation, Investigation, Resources, Writing – original draft. BR: Conceptualisation, Data curation, Methodology, Investigation, Resources, Writing – original draft. ED-F: Conceptualisation, Writing – original draft. VD: Conceptualisation, Investigation, Writing – original draft. AF: Conceptualisation, Methodology, Investigation, Writing – original draft. AB-M: Conceptualisation, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – original draft. DB: Conceptualisation, Investigation, Supervision, Writing – original draft.

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Conflict of interest

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

Publisher's note

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Persistence of commensal multidrug-resistant *Escherichia coli* in the broiler production pyramid is best explained by strain recirculation from the rearing environment

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Despite the success of mitigation policies in several countries to reduce the use of antibiotics in veterinary medicine, pathogenic and commensal bacteria resistant to antibiotics are still circulating in livestock animals. However, factors contributing the most to antimicrobial resistance (AMR) persistence in these settings are yet not clearly identified. The broiler production, with its highly segmented, pyramidal structure offers an ideal context to understand and control the spread of resistant bacteria. By taking advantage of an experimental facility reproducing the whole broiler production pyramid, we demonstrate that resistant *E. coli* persist in our system primarily though recirculation of a few commensal clones surviving in the rearing environment. No vertical transmission from hens to offspring nor strain acquisition at the hatchery were detected, while import of new strains from outside the facility seems limited. Moreover, each clone carries its own resistance-conferring plasmid(s), and a single putative plasmid horizontal transfer could have been inferred. These results, observed for now in a small experimental facility with high level of biosecurity, must be confirmed in a commercial farm context but still provide invaluable information for future mitigation policies.

KEYWORDS

***Escherichia coli*, antimicrobial resistance, broiler production pyramid, longitudinal study, whole genome sequencing**

1 Introduction

Bacterial antimicrobial resistance (AMR) is one of the most important global health threats affecting people at any stage of life, as well as the healthcare, veterinary, and agriculture industries (Antimicrobial Resistance Collaborators, 2022). The wide use of antibiotics in humans and livestock animals has contributed to the selection of resistant bacteria and the spread of AMR worldwide (McEwen and Collignon, 2018). To adapt to antibiotic selection pressure, bacteria have accumulated antibiotic resistance genes (ARGs), leading to multidrug resistant (MDR)

phenotypes. Therefore, such ARG accumulation in bacterial pathogens can lead to therapeutic failure (Antimicrobial Resistance Collaborators, 2022). Conjugative elements, such as plasmids, are recognized as a major driver for the spread of ARGs. ARGs can be exchanged between these horizontally-mobile elements by genetic recombination involving insertion sequences, integrons or transposons. Successful plasmids were described to carry numerous ARGs conferring MDR phenotypes including resistances to last generation antibiotics medically-important for public health (e.g., extended-spectrum cephalosporins or carbapenems; Carattoli, 2013; Holmes et al., 2016; Madec and Haenni, 2018; McEwen and Collignon, 2018; Poirel et al., 2018; Leekitcharoenphon et al., 2021). The epidemic success of such MDR plasmids depends both on various co-selections due to antibiotic usage and their spread into and adaptability to different bacterial hosts with which they co-evolve (Madec and Haenni, 2018). Thus, AMR monitoring programs are essential to have comprehensive and reliable information on the emergence and spread of resistant bacteria. They mainly focus on AMR profiles of clinical isolates reported in human and veterinary medicine, but start to routinely screen host-associated commensal bacteria, which may act as ARG reservoir (Madec and Haenni, 2018; Poirel et al., 2018; Racewicz et al., 2022). For instance within the EU, monitoring of AMR in zoonotic and indicator bacteria includes commensal *Escherichia coli* (*E. coli*) from livestock animals and food providing data as an overview, by country and breeding sector (EFSA and ECDC, 2022). *E. coli* is highly-diverse, being commensal as well as pathogenic, and commonly harbors multiple MDR plasmids, hence making it a useful AMR indicator organism (Poirel et al., 2018; Anjum et al., 2021; Leekitcharoenphon et al., 2021). Despite decreasing since few years, MDR *E. coli* isolates in UE are still higher in healthy broilers compared to other livestock animals (38.7% in broilers, 34.2% in pigs and 26.8% in calves in 2018/2019 (EFSA and ECDC, 2022). Particularly, poultry have been shown to play a major role in epidemiology of extend-spectrum β-lactamase (ESBL) and AmpC-type β-lactamase genes, i.e., $bla_{\text{CTX-M-1}}$ and $bla_{\text{CMY-2}}$, respectively (Baron et al., 2018; Madec and Haenni, 2018; Poirel et al., 2018; Apostolakos et al., 2019; Dame-Korevaar et al., 2019; Paivarinta et al., 2020; Mikhayel et al., 2021; Mo et al., 2021).

A recent comprehensive review identified four major transmission routes potentially involved in the dissemination of ESBL/AmpC-producing *E. coli* in the broiler production pyramid: (1) breeders-to-broilers vertical transmission, (2) transmission at hatcheries, (3) horizontal transmission between adjacent or successive flocks due to farm-level strain persistence, and (4) between-farm transmission through external factors (Dame-Korevaar et al., 2019). However, the relative contribution of each of these routes to the global AMR dissemination process could not be determined because of a lack of quantitative results and/or a lack of precision in strains genetic relationships. Moreover, the dissemination of resistance plasmids in addition to resistant strains was not considered. More recent studies included high-resolution typing of resistant isolates using whole-genome analyses, and showed that vertical transmission (Apostolakos et al., 2020), persistence of resistant *E. coli* clones and plasmids in the broiler farms (Paivarinta et al., 2020; Mo et al., 2021), as well as between-farm transmissions are possible factors for the AMR burden in the broiler production pyramid. But again, the relative importance of these transmission routes could not be inferred because quantitative comparisons between the different production steps were missing.

Therefore, the aim of the present study was to quantitatively investigate the dynamics of multi-drug resistant commensal *E. coli* spread in the pyramidal broiler production using whole genome sequencing. AMR transmission without antibiotic pressure was followed over 3 generations of broiler and parental chickens in an experimental facility integrating all steps of the broiler production chain. Four main hypotheses were investigated: (i) familial inheritance (i.e., from hens to offspring), (ii) strain acquisition at the hatchery, (iii) recirculation of resistant strains persisting in the rearing environment, and (iv) strain acquisition from outside the facility (i.e., different for each flock or each generation). Several MDR *E. coli* clonal STs were shown to co-exist and persist between chicken generations raised in the same buildings, with no evidence of vertical transmission. Most of these clonal STs were also detected in the environment after disinfection but not at the hatchery, strongly suggesting that the rearing environment is the primary source of flock colonization in the facility. Most resistance genes were shared between STs, however complete plasmid reconstruction indicated that each clonal ST carried its own MDR plasmid, with only one indication of plasmid transfer event detected between different STs.

2 Materials and methods

2.1 Study design

Resistant *E. coli* strains were monitored from February 2018 to January 2020 in healthy animals and their environments in a French experimental facility integrating all steps of the broiler production chain. The experimental facility is registered for animal experimentation under license number C-37-175-1 of the French Ministry of Agriculture.[1] We followed the chicken line pHu+ designed to study the meat quality on 6 weeks old broilers (Erensoy et al., 2022). The first generation (G1) of the present study corresponded to the 11th of the breeding history and represented 80 pedigree broiler breeder females. Eleven families of chickens were followed over three generations divided into two sibling batches, one for reproduction which are reared in protected houses during 42 weeks and one for broiler production reared in standard conditions during 6 weeks (Supplementary Tables S1, S2; Supplementary Figure S1).

2.2 Sample collection and processing

Individual fecal samples were collected at different weeks of age (1, 3, 6, 20, 32, 42) following standardized protocols specific to age and rearing condition (Baumard et al., 2021). Eggs layed by hens from the followed families were collected at the hatchery during incubation (2 weeks before hatching) of the 2nd and 3rd generations of parental and broiler batches. Additionally, pooled egg shells were collected for each parental hen right after hatching. Environmental samples were collected at the rearing buildings before animal entry using sterile wipes and boot-socks for surfaces, and sterile containers at drinking water supply endpoints. Surfaces and water sprayed to control room moisture at the hatchery were sampled before egg incubation using

1 https://doi.org/10.15454/1.5572326250887292E12

the same protocols. Each batch of animal feed was also sampled before animal feeding. All samples were immediately stored at 4°C upon return to the microbiology laboratory and processed on the same day.

Fecal and surface samples were inoculated in buffered peptone water (bioMérieux, France) and incubated for enrichment at 37°C for 2 h and for 18 h, respectively. Two-hundred ml of each water sample were filtered on 0.45 µm filter and the filter was directly applied on Mac Conkey agar (bioMérieux, France) plates. After 18 h incubation at 37°C, all bacterial colonies of the filter were inoculated in 20 mL buffered peptone water.

One, two, or three incubated eggs from the same hen were placed into a sterile stomacher filter bag with 33 mL, 66 mL or 200 mL of buffered peptone water, respectively. Eggs were crushed gently for the recovery of bacteria from the shell and those located inside. Hatched egg shells were processed in the same way using 50 mL of buffered peptone water. After 5 min of contact, 10 mL were pipetted from the stomacher filter to a sterile tube and incubated overnight at 37°C for enrichment.

2.3 *Escherichia coli* isolation and antibiotic susceptibility testing

Resistant *E. coli* strains were selected on MacConkey agar plates supplemented with ampicillin-tetracycline (50–10 mg/L), ceftriaxone (1 mg/L), enrofloxacin (0.5 mg/L) or sulfonamide-trimethoprim (20–250 mg/L) by plating 100 µL of enrichment cultures. Up to 5 colonies were picked and grown in Luria Bertani broth and stored at - 80°C in 20% glycerol for further analyses. *E. coli* identification was done by specific PCR *uidA* (Heininger et al., 1999) and confirmed for a few isolates by the MALDI Biotyper® system (Bruker Daltonics).

Antibiotic susceptibility was determined by the disks diffusion method on Mueller-Hinton agar (Difco, Sparks, MD), as recommended by EUCAST 2018 guidelines[2] and CA-SFM 2018[3] using the following disks (Biorad, Marne-la-coquette, France): Amikacin (AMK, 30 µg), ampicillin (AMP, 30 µg), cephalexin (CXN, 30 µg), chloramphenicol (CHL, 30 µg), ciprofloxacin (CIP, 5 µg), enrofloxacin (ENR, 5 µg), florfenicol (FFC, 30 µg), gentamicin (GEN, 10 µg), kanamycin (KAN, 30 µg), nalidixic acid (NAL, 30 µg), streptomycin (STR, 10 µg), spectinomycin (SPT, 100 µg), sulfonamides (SUL, 200 µg), tetracycline (TET, 30 µg) and trimethoprim (TMP, 5 µg).

Isolates originating from the same animal or surface at the same sampling date and showing the same resistance profile (whatever the initial antibiotic selection) were considered duplicates and only one of them was chosen for further analysis.

2.4 Whole-genome sequencing

A set of 178 *E. coli* isolates representative of 5 of the 11 chicken families and of the rearing environment, and including the two main antimicrobial resistance phenotypes (AMP-TET or AMP-SUL-TET-TMP), were subjected to whole-genome sequencing (WGS). Briefly, DNA extraction was performed using the Nucleospin 8 tissue kit (Macherey-Nagel, Düren, Germany). DNA samples were sent to Novogene Co., Ltd., for library preparation using the New England Bioloabs Next Ultra DNA library prep kit and WGS on NovaSeq (Illumina) using 2 × 150-bp paired-end sequencing (Novogene, Bejing, China).

A subset of 42 isolates, including at least two isolates for each major *E. coli* sequence type (ST) observed in this study and one for other ST, was subjected to WGS using the Oxford Nanopore technologies (Oxford, United Kingdom). Briefly, libraries preparations were done with the Rapid barcoding sequence kit (SQK-RBK 004) and MinION sequencing runs were performed on standard flow cells (FLO-MIN106).

2.5 *In silico* analyses

Raw Illumina reads quality was assessed using FastQC[4]; all reads had >20 phred scores at every base, suitable for assembly. Residual sequencing adapters were removed using trimmomatic 0.39 (Bolger et al., 2014). Genomes were assembled using SPAdes 3.15.3 (Bankevich et al., 2012) with the best kmer selected among 21, 33 or 55, the careful correction option set to "ON" and no coverage cutoff. Assemblies were then filtered to remove contigs shorter than 200 bp and with a coverage ratio smaller than 0.2 compared to scaffolds larger than 50,000 bp. Sequence types (ST), antibiotic resistance genes, chromosomal mutations conferring resistance, and plasmid replicon markers were detected for each assembled genome using the starAMR tool v.0.8 (Bharat et al., 2022) with mlst, Resfinder, Pointfinder, and Plasmidfinder databases version 2.19.0, 05/24/2022, 02/01/2021 and 11/29/2021, respectively. Parameters for starAMR were set to 'ecoli' for the mlst scheme, 'escherichia_coli' for the pointfinder organism, and 60 and 95% for minimum coverage and identity hit reporting thresholds, respectively. Phylogroups were predicted using ClermontTyping (Beghain et al., 2018) v.1.4.1 with the Mash method, and serotypes were predicted using SerotypeFinder (Joensen et al., 2015) with the same thresholds as above. Maximum-likelihood (ML) phylogenetic trees covering the different isolates were obtained by analysis of the core-genome SNPs using ParSNP v.1.2 (Treangen et al., 2014), with the assembled genomes as input, the MUMi filtering disabled (option -c) and the *E. fergusonii* RHB38-7 genome (accession CP057093) as outgroup. SNP distances between isolates belonging to the same ST were calculated with snp-dists[5] on core-genome SNPs returned by ParSNP (covering 65–76% of the genomes), using default parameters.

MinION sequencing reads with an average quality higher or equal to 7 (phred score) and a minimum size of 500 bp were selected with nanofilt v.2.5.0 and given as input, along with corresponding clean Illumina reads, to Unicycler v.0.4.7 (Wick et al., 2017) with default settings to perform a hybrid assembly. Due to a large amount of raw reads, the quality filter was set to 10 for samples F60, F163, F719, F730, and F1055. Assembled scaffold were then annotated

2 http://www.eucast.org/

3 https://www.sfm-microbiologie.org/boutique/comite-de-lantibiograme-de-la-sfm-casfm/

4 https://github.com/s-andrews/FastQC

5 https://github.com/tseemann/snp-dists

using DFAST v.1.2.14 (Tanizawa et al., 2018) with HMM search on TIGRFAM and CDD enabled and a contig size filter of 200 bp. In addition, Resfinder v4.1, ISfinder, and a home-made plasmid conjugation protein database were used as custom databases with, respectively, 100, 98 and 90% similarity thresholds to increase annotation sensitivity for genes related to antibiotic resistance dissemination. Plasmid sequence types of IncF replicons were identified using the pMLST tool v0.1.0 with standard parameters and database version 2023-04-24 (Carattoli et al., 2014). The IncF plasmid phylogenetic analysis was performed as follows: a ~ 24 Kbp region of IncF plasmids containing genes encoding the conjugative machinery (*tra* genes) was extracted from one representative isolate of each ST and searched against the 34,513 complete plasmids included in the PLSDB database version 2021 (Schmartz et al., 2022) using blastN. The two best matches were kept for each input plasmid and a global alignment of the *tra* region was performed using MAFFT v7.490 (Katoh and Standley, 2013) with default parameters. A maximum likelihood phylogenetic tree was computed from the alignment using IQ-TREE v2.2.0 (Nguyen et al., 2015) with 1,000 bootstrap replicates. All phylogenetic trees were displayed and annotated using iTOL v6 (Letunic and Bork, 2021). Plasmid comparison figures were produced with Genofig v1.1.[6] (Branger and Leclercq, 2024).

2.6 Statistical analyses

The impact of animal family, age, generation and rearing condition on isolates ST was investigated using χ^2 statistics. The 104 tetra-resistant and 33 bi-resistant sequenced isolates originating, respectively, from the 6 and 3 major clonal ST were selected. Only isolates originating from feces were considered. χ^2 tests were performed between each pair of variables (ST, family, age, generation, rearing condition) using R v4.2.2. A Bonferroni correction for multiple tests was applied by multiplying the resulting *p-values* by the number of tests performed (ten). Correlations between the ST and the other variables were considered significant for corrected *p-value* lower or equal to 0.05. The same procedure was applied for tetra-resistant clonal ST isolates associated only to parental animals or broilers with a Bonferroni correction set to six.

3 Results

3.1 Description of the sampling campaign in the facility

Animals were followed for three generations (G1, G2, and G3) in two rearing conditions (Parental and Broiler), and sampling was restricted to 11 hens at G1 and their descendants at G2 and G3 (Supplementary Figure S1). A total of 335 fecal samples were collected from 82 animals evenly distributed among the selected families and among generation, rearing condition, and age (Table 1; Supplementary Figure S1). Sampling started at 20 weeks of age for the first generation, and could not include broiler animals which are slaughtered after 6 weeks in the studied breed (Supplementary Figure S1). Twenty-eight environmental samples were also collected from building surfaces and drinking water the day before animal's entry (Table 1). A total of 613 non-duplicate resistant *E. coli* strains were isolated from these samples using either AMP- TET, ENR or SUL- TMP agar plates (Table 1). Animal feed, surfaces and water at the hatchery, as well as incubated eggs and post-hachting eggshells were also sampled but no AMR *E. coli* could be isolated (Table 1).

3.2 Antibiotic resistance profile of fecal *Escherichia coli* isolates

The 593 *E. coli* isolated from feces were resistant to 4 antibiotics on average, from the set of 15 screened antibiotics. Most isolates were resistant to AMP (88%) and TET (78%), while more than half were resistant to SUL (60%), STR (57%) or TMP (55%). A substantial proportion of isolates were also resistant to the fluoroquinolone CIP (24%) and to a lesser extent to ENR (15%), mainly due to differences in resistance breakpoint definitions between human antibiotics (EUCAST 2018 guidelines) and veterinary antibiotics (CA-SFM 2018 guidelines). Resistances to CHL, FFC or KAN were also observed, but at a much lower rate (<5%). Notably, no resistance was observed to third generation cephalosporins (ceftriaxone, CRO). The average number of resistance per isolate ranged from 3.6 to 4.6 between chicken families but the difference was not statistically significant (Supplementary Figure S2A, Kruskall-Wallis H test, *p-value* = 0.306). At the flock level, strains isolated from the same rearing condition had a similar average number of resistance over the second and third generations (~3.6–4 for parental and 4.5–4.9 for broiler groups), while broiler isolates were statistically more resistant than parental isolates (all Wilcoxon corrected *p*-values <0.05). The level of resistance of parental isolates from the first generation was unexpectedly low compared to the two other generations, with only 2.5 resistance per isolate on average, which led to highly significant differences with all the other flocks (all Wilcoxon corrected *p*-values $<10^{-3}$).

The difference in average resistance between parental and broiler groups was mainly driven by the resistance to (fluoro-)quinolones. Indeed, more than 50% of isolates were resistant to CIP and/or NAL at every sampling date and generation of broiler animals while in the parental group, isolates with these resistances were found almost only in young animals (6 weeks or less) of the second generation (Figure 1). Isolates resistant to CHL or KAN were detected in all sampling dates of the broiler G3 condition and only sporadically in other flocks, but their low prevalence (max 15% of the isolates at the first week of age) did not impact the average number of resistances per isolate compared to the broiler G2 group. Interestingly, the lower average number of resistance in parental G1 isolates is well explained by the total lack of STR resistance otherwise found in all other flocks, and by the lower proportion of isolates resistant to SUL and/or TMP (Figure 1). Apart from the small between-flock differences described above, observed resistances were highly similar between generation and rearing condition, with 77% of all isolates resistant to at least AMP-TET (49–98%, depending on the condition) and 43% to at least AMP-SUL-TET-TMP (21–49% depending on the condition). Finally, no family-specific pattern of resistance was observed (Supplementary Figure S2B).

6 https://forgemia.inra.fr/public-pgba/genofig

10.3389/fmicb.2024.1406854

TABLE 1 Number of samples and corresponding resistant *E. coli* isolates.

		Parental hens (samples/isolates)									Broiler group (samples/isolates)					
		w-2	d-1	w1	w3	w6	w20	w30	w42	Total	w-2	d-1	w1	w3	w6	Total
Feces	G1	–	–	–	–	–	11/10	11/10	11/4	**33/24**	–	–	–	–	–	–
	G2	–	–	15/12	15/20	15/52	14/31	13/29	13/19	**85/163**	–	–	20/14	22/23	23/66	**65/103**
	G3	–	–	18/12	17/21	18/51	14/24	14/19	14/25	**95/152**	–	–	19/40	19/47	19/64	**57/151**
Eggs	G2	16/0	–	–	–	–	–	–	–	**16/0**	5/0	–	–	–	–	**5/0**
	G3	27/0	–	–	–	–	–	–	–	**27/0**	33/0	–	–	–	–	**33/0**
Hatchery surfaces	G2	4/0	–	–	–	–	–	–	–	**4/0**	4/0	–	–	–	–	**4/0**
	G3	3/0	–	–	–	–	–	–	–	**3/0**	8/0	–	–	–	–	**8/0**
Rearing building surfaces	G2	–	7/8	–	–	–	–	–	–	**7/8**	–	4/0	–	–	–	**4/0**
	G3	–	5/7	–	–	–	–	–	–	**5/7**	–	4/4	–	–	–	**4/4**
Drinking water	G2	–	3/1	–	–	–	–	–	–	**3/1**	–	0/0	–	–	–	**0/0**
	G3	–	3/0	–	–	–	–	–	–	**3/0**	–	2/0	–	–	–	**2/0**
Food	G1	–	–	–	–	–	2/0	2/0	–	**4/0**	–	–	–	–	–	
	G2	–	1/0	–	–	1/0	2/0	2/0	–	**6/0**	–	1/0	1/0	–	–	**2/0**
	G3	–	1/0	–	1/0	–	2/0	1/0	–	**5/0**	–	1/0	–	1/0	–	**2/0**

w, week; d, day; G, generation of chickens; –: no sample collected.

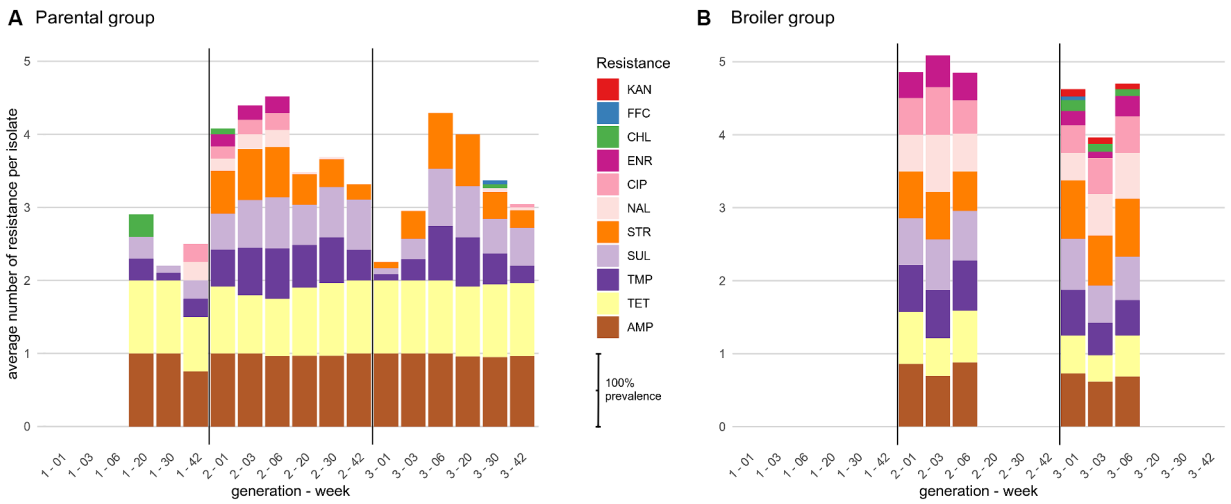

FIGURE 1
Distribution of phenotypic resistances among *E. coli* isolates for **(A)** parental and **(B)** broiler chicken. Bars represent the cumulative prevalence of isolates at each week of age resistant to at least two antibiotics, calculated antibiotic by antibiotic. Broilers are slaughtered at 6 weeks of age. Sampling started at week 20 for generation 1. AMP, ampicillin; TET, tetracycline; TMP, trimethoprim; SUL, sulfonamides; STR, streptomycin; NAL, nalidixic acid; CIP, ciprofloxacin; ENR, enrofloxacin; CHL, chloramphenicol; FFC, florfenicol; KAN, kanamycin.

3.3 Phylogenetic relationship between representative MDR isolates

The widespread prevalence of the tetra-resistance phenotype AMP-SUL-TET-TMP in *E. coli* isolated during this study may indicate the persistence of a dominant MDR clone in the facility, or the efficient dissemination of a resistance plasmid among the facility's *E. coli* population. To investigate these two hypotheses, a subset of 113 fecal isolates showing resistance to at least four antibiotics, representative of the chicken generations and rearing conditions from 5 chicken families, were subjected to a whole genome sequence analysis (Supplementary Table S3). Fourteen isolates from rearing environment surfaces sampled before flock entry and showing the aforementioned tetra-resistance were also included to investigate a potential environmental persistence.

Isolates were distributed among four *E. coli* phylogroups (A, B1, D, and E) with large over-representation of phylogroup A (57%) and B1 (40%). A total of 14 sequence types (ST) was observed but 91.4% of the sequenced isolates clustered in only 6 major ST: ST-453, ST-1611, ST-162, ST-93, ST-206 and ST-2701 (Figure 2). Each of them was represented by a single clonal group (<25 SNPs between each pair of isolates) with two exceptions: (1) ST-162 was separated into two clonal groups differentiated

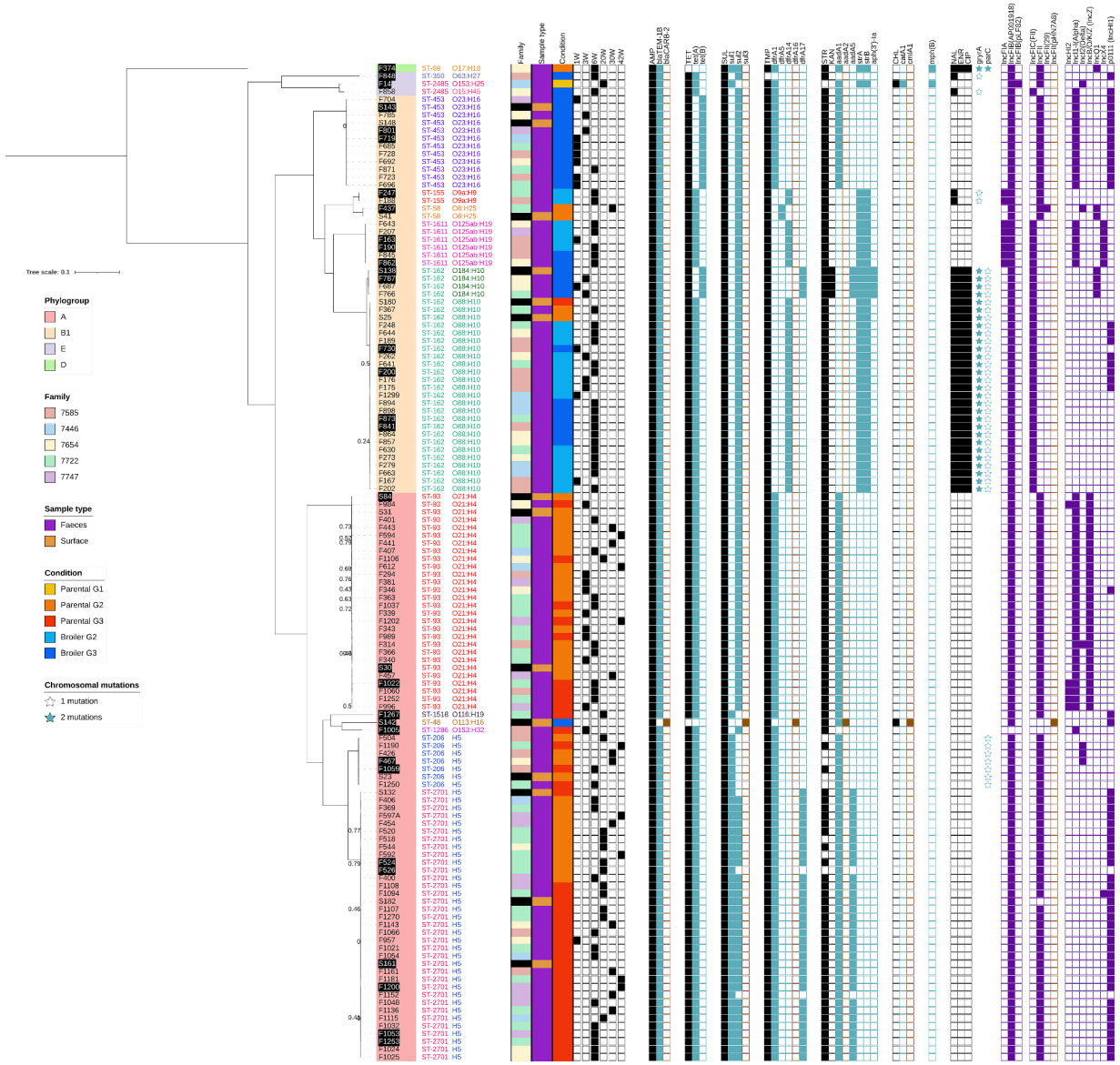

FIGURE 2
Phylogenetic relationship of 127 tetra-resistant *E. coli* strains originating from feces and rearing surfaces before animal's entry. The tree was constructed with a maximum likelihood algorithm from the core genome SNP alignment. *E. fergusonii* was used as an outgroup but is not shown on the figure to improve branch length visibility. Branch confidence values of 0.8 or higher are not displayed. For each antibiotic, the resistance phenotype is symbolized with a black filled square while presence of associated resistance gene(s) is indicated with blue or brown filled squares. Brown squares represent genes detected in surface isolates only. Chromosomal mutations leading to fluoroquinolone resistance are indicated with open or filled stars. Samples highlighted in black were assembled using both short and long reads.

by their inferred serotypes (O184:H10 and O88:H10) and separated by 1866–1875 SNPs, and (2) ST-93 showed a slightly higher diversity (0–188 SNPs, average 38) with two heterogeneous subgroups separated by only 20–50 SNPs. ST-93 can therefore be considered as a single clonal group with a higher mutation rate or originating from an older common ancestor (Duval et al., 2023). The distribution of these seven major clonal groups was highly correlated with the rearing condition (χ^2 test corrected *p-value*: 2.5×10^{-18}): all fecal isolates of the three ST from phylogroup A (ST-93, ST-206 and ST-2701) originated from parental animals, while those of the four ST from phylogroup B1 (ST-453, ST-1611, ST-162_O184:H10, ST-162_O88:H10) originated at 98% (41/42 isolates) from broiler animals. No effect of the family could be detected on the clonal group distribution among animals (χ^2 test corrected *p-value*: 0.81) while a significant effect of animal age and generation was observed (χ^2 test corrected *p-values*: 5.9×10^{-8} and 8.7×10^{-3}, respectively). Impacts of age and generation disappeared when only parental-associated isolates were considered (χ^2 test corrected *p-values*: 0.053 and 0.45, respectively), but were still significant for broiled-associated isolates (χ^2 test corrected *p-values*: 0.01 and 0.005, respectively) probably caused by ST-453 isolates exclusively observed in G3 animals and mostly at week 1 (Figure 2). Interestingly, isolates from all these clonal groups but one (ST-1611) were also detected on surfaces before entry of animals, in the same rearing conditions than their fecal counterparts.

The same analysis was performed on a representative set of AMP-TET resistant isolates originating from feces ($n=47$), surfaces ($n=3$) and drinking water ($n=1$) in order to evaluate if the major

MDR ST described above evolved from less resistant clones already circulating in the facility. These bi-resistant isolates also clustered in a few clonal ST (inter-isolate differences <20 SNPs), whose distribution were again correlated with the rearing condition (χ^2 test corrected *p-value*: 6.8×10^{-7}) but not to any other parameter (Supplementary Figure S3). None of these clonal ST matched to any of the six major tetra-resistant STs, indicating that bi- and tetra-resistant prevalent strains belonged to independent *E. coli* populations.

Overall, the extremely low between-isolates genomic distance within each major ST and their strong association with the rearing condition over generations indicate that the primary factor explaining the persistence of resistant *E. coli* in our facility is a horizontal transmission of a few MDR clones between successive flocks, likely through recirculation from the rearing environment.

3.4 Antibiotic resistance gene content in selected isolates

The ARG content of sequenced isolates was then investigated to characterize the genetic determinants leading to their resistance phenotype. Twenty-five acquired ARGs were detected in total (Figure 2; Supplementary Figure S3), and a very good genotype-to-phenotype match was obtained, except for some ST-206 and ST-2701 isolates sensitive to streptomycin despite the carriage of *aadA1* and/or *aadA5*. $bla_{\text{TEM-1B}}$, *sul2*, and *tet*(A) were the most widely disseminated genes, respectively found in all, six, and five of the seven tetra-resistant clonal groups. The gene *dfrA1* was the most disseminated TMP resistance determinant, being found in four of the seven clonal groups. Streptomycin resistance was conferred either by *aadA*1/*aadA*5 or by *strAB* in the major STs, while the presence of *aph*(3′)-Ia correlated well with the extended aminoglycoside resistance to kanamycin of the ST-162_O184:H10 clonal group (Figure 2). Seven ARGs were detected only in one or two isolates ($bla_{\text{CARB-2}}$, *sul3*, *dfrA16*, *aadA2*, *catA1*, *cmlA5*, *mph(B)*), while the two β-lactamase gene variants $bla_{\text{TEM-1A}}$ and $bla_{\text{TEM-1D}}$ were found only in AMP-TET resistant bacteria (Figure 2; Supplementary Figure S3). Finally, quinolone phenotypic resistance was essentially restricted to ST-162 isolates and a single phylogroup E isolate. ENR/CIP-resistant strains showed typical D87N/D87Y and S83L mutations in *gyrA* and S80I mutation in *parC*, which are the most common (fluoro)-quinolone resistance mechanism in *E. coli* (Fabrega et al., 2009). No plasmid-mediated quinolone resistance genes were detected.

In summary, all isolates from the well-disseminated tetra-resistant clonal groups harbor a similar ARG repertoire, despite large phylogenetic distances between these clones. This situation suggests that the ubiquity of the tetra-resistance profile may be caused by the dissemination of one or two major MDR plasmids or other conjugative elements in the facility.

3.5 Antibiotic resistance gene-carrying plasmids diversity in the various *Escherichia coli* clonal groups

Analysis of plasmid replicon markers detected in our 178 draft assembled genomes suggested the presence of an IncF replicon in 100% of tetra-resistant isolates and in 90% of AMP-TET isolates (Figure 2). IncHI1 and IncI1 replicons were the second and third most abundant replicon types, detected in three of the seven major clonal groups and in several AMP-TET isolates. Seven other replicon types were more sporadically detected although IncZ and IncX4 types were each found in all isolates of one of the major STs. However, no clear ARG-plasmid association could be inferred from Illumina-based assemblies.

Oxford Nanopore sequencing of a subset of 31 tetra-resistant and 11 AMP-TET resistant isolates (black-boxed in Figure 2; Supplementary Figure S3) was therefore performed to link each ARG to its genetic context among the various STs. Within a given clonal group, ARGs were usually carried by almost identical replicons in all isolates (Supplementary Figures S4–S9). Contrary to the high intra-clonal ST homogeneity, ARG-carrying plasmids were much more diverse between clonal STs. All ARGs were located on IncF plasmids in ST-1611 and ST-162 isolates, while most ARGs were located on IncI1 or IncZ plasmids in ST-453 and ST-93, respectively (Table 2). In ST-2701, ARGs were distributed between IncF and IncHI1-related plasmids. Most ARGs were found on different plasmid types, with the exception of *tet*(A) detected only on IncF plasmids in the six major STs. However, *tet*(A) was also carried by IncI1, IncN and IncH1 plasmids in minor STs and AMP-TET resistant isolates (Supplementary Tables S4, S5), ruling out a specific IncF-related dissemination of this gene in the facility. A number of ARGs were also detected at chromosomal positions (Table 2; Supplementary Table S4). For instance, *tet*(B) in ST-453 isolates was part of the Tn10 transposon (Chalmers et al., 2000), within a 60 Kbp region precisely integrated in a threonine tRNA (Supplementary Figure S3), while *sul2* in ST-1611 isolates was part of a truncated CR2-*sul2* unit (Zhang et al., 2022) itself located in a larger mobile region integrated in a phenylalanine tRNA (Supplementary Figure S5). These regions had no large-scale homology to any integrative element reported in the ICEberg database (Liu et al., 2019), but harbored some hallmarks of IMEs (Integrative and Mobilisable Elements) such as genes putatively involved in integration and conjugative transfer.

Since a majority of resistance plasmids were of the IncF incompatibility group, these plasmids were investigated in more details. A plasmid MLST analysis revealed 13 ST among the 22 distinct IncF replicons found in our isolates, indicating a great diversity (Table 2; Supplementary Tables S4, S5). Nonetheless, 6 of the 13 ST showed the B58 allele and could potentially have originated from a unique plasmid which have entered the facility years ago. Publicly available plasmids closest to those of our isolates were therefore collected from the PLSDB complete plasmid database (more than 34,000 replicons at the collection date), and a phylogenetic analysis on the *tra* region was conducted. Most replicons from minor STs and AMP-TET isolates were distantly related to each other, and were intermingled with publicly available plasmids (Figure 3A). IncF plasmids from clonal STs ST-453, ST-93, ST-206, and ST-2701 were extremely closely related (less than 10 mutations over the 25 Kbp aligned region) and formed a well-defined clade with two replicons from minor STs (ST-1518 and ST-2485). They showed a well conserved synteny in their core genome (except a single large inversion in ST-206 and ST-1518 isolates) but their MDR region was highly rearranged (Figure 3B), consistent with the high heterogeneity of ARG content described in Table 2. One exception was the identity between IncF replicons carried by the MDR isolate F1267 (ST-1518) and by ST-206 isolates. Interestingly, ST-1518 isolates from the same serogroup were detected among AMP-TET resistant isolates, but not

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TABLE 2 Genetic context of ARGs detected in the seven major tetra-resistant clonal groups.

Origin	ST	Chr.	IncF	IncF ST	IncI1	IncZ	IncHI1
Broiler	ST-453	*tet*(B)	Ø	F24:A-:B58	bla_{TEM-1B}, *dfrA1, aadA1, sul2*	–	Ø
Broiler	ST-1611	*sul2*	*tet*(A), bla_{TEM-1B}, *dfrA14, strA, strB, sul2*	C4:A-:B58	Ø	–	–
Broiler	ST-162 (O184:H10)	–	*tet*(B), bla_{TEM-1B}, *dfrA17, aadA5, aph(3′)-Ia, strA, strB, sul2*	C4:A-:B58	–	–	–
Broiler and Parental	ST-162 (O88:H10)	–	*tet*(A), bla_{TEM-1B}, *dfrA14, strA, strB, sul2*	C4:A-:B58	–	–	Ø[a]
Parental	ST-93	–	*tet*(A)	F24:A-:B58	Ø	bla_{TEM-1B}, *dfrA1, aadA1, sul2*	–
Parental	ST-206	–	*tet*(A), bla_{TEM-1B}, *dfrA1, aadA1, sul1*	F24:A-:B6	–	–	–
Parental	ST-2701	–	*tet*(A), bla_{TEM-1B}, *dfrA1, aadA1, sul1*[b]	F24:A-:B6	–	–	*dfrA17*, *aadA5*, *sul2*[a,b]

Ø, plasmid present but not carrying any known resistance gene. [a]plasmid not found in all isolates. [b]plasmid cointegrates in some isolates.

carrying any IncF plasmid (Supplementary Table S5). We therefore suspect that this IncF replicon may have transferred horizontally between ST-206 and ST-1518 isolates inside the facility, although MDR and AMP-TET isolates did not originate from the same rearing condition or generation (Figure 2; Supplementary Figure S3). Other plasmids from this clade are unlikely the result of an intra-facility dissemination, as suggested by the divergence in the MDR region and the presence of PLSDB plasmids scattered thorough the clade (Figure 3A). IncF plasmids of the two other major MDR STs (ST-1611 and ST-162) showed similar trends, located in a well-defined phylogenetic clade showing a very conserved core region and a heavily rearranged MDR region (Figure 3B). Again, the clade contained plasmids from PLSDB, ruling out a facility-specific dissemination of these plasmids.

4 Discussion

It is now widely recognized that the gut of healthy animals is a reservoir of antibiotic resistance genes, carried by commensal bacteria (Poirel et al., 2018). However, how animals acquire resistant gut commensals remains unclear. In the broiler production, chicks are bought at one-day old from hatcheries which get eggs to other facilities raising breeder animals. In this system, chicks and hens are never in contact, and eggs are decontaminated upon introduction in the hatchery (Dierikx et al., 2013). Nevertheless, several studies concluded on the possibility of parent-to-offspring bacterial transmission, either through colonized shell or inner egg (Bortolaia et al., 2010; Agerso et al., 2014; Pedroso and Lee, 2015; Ding et al., 2017; Poulsen et al., 2017). Others also highlighted the hatchery as a potential hub for spreading of fluoroquinolone-resistant *E. coli* or bla_{CMY-2}-carrying plasmids in different production farms (Petersen et al., 2006; Baron et al., 2018). The experimental facility used in the present study follows the physical separation of hens and chicks as well as egg decontamination in a dedicated hatchery, and we could not see any effect of the chicken family on resistant *E. coli* carriage. More importantly, parental and broiler animals of the same generation harbored completely distinct *E. coli* populations while originating from the same mothers and the same hatchery. Finally, we were not able to isolate any resistant *E. coli* strain from eggs or hatchery samples collected during the experiment. A recent study demonstrated that APEC strains usually reach the oviduct during systemic infection and in some rare occasion infect layed eggs (Abdelhamid et al., 2024), mirroring what has been described for *Salmonella* (Gantois et al., 2009). Although infected eggs usually do not develop properly, it is not known whether some may still be able to hatch, paving the way for a possible transmission to chicks. Such transmission route is less likely to occur for commensal strains lacking the virulence toolbox necessary for systemic infection, which may partly explains our lack of *E. coli* detection in the 81 tested eggs. Nonetheless, it indicates that even if some vertical transmission or contamination at the hatchery could happen, it had a minor effect on the carriage of resistant *E. coli* in this experimental facility.

Several other studies looking at commensal or pathogenic *E. coli* in the broiler production chain also concluded on the lack of vertical transmission, but could not infer the origin of acquired strains (Dierikx et al., 2013; Oikarainen et al., 2019). The most likely route for *E. coli* acquisition in the present study is from the rearing environment. We show evidence that animals from different generations reared in the same building carry the same resistant *E. coli* clones, regardless of the strains carried by their parents. These building-specific clones were also systematically found on the building surfaces before animal's entry despite between-flock

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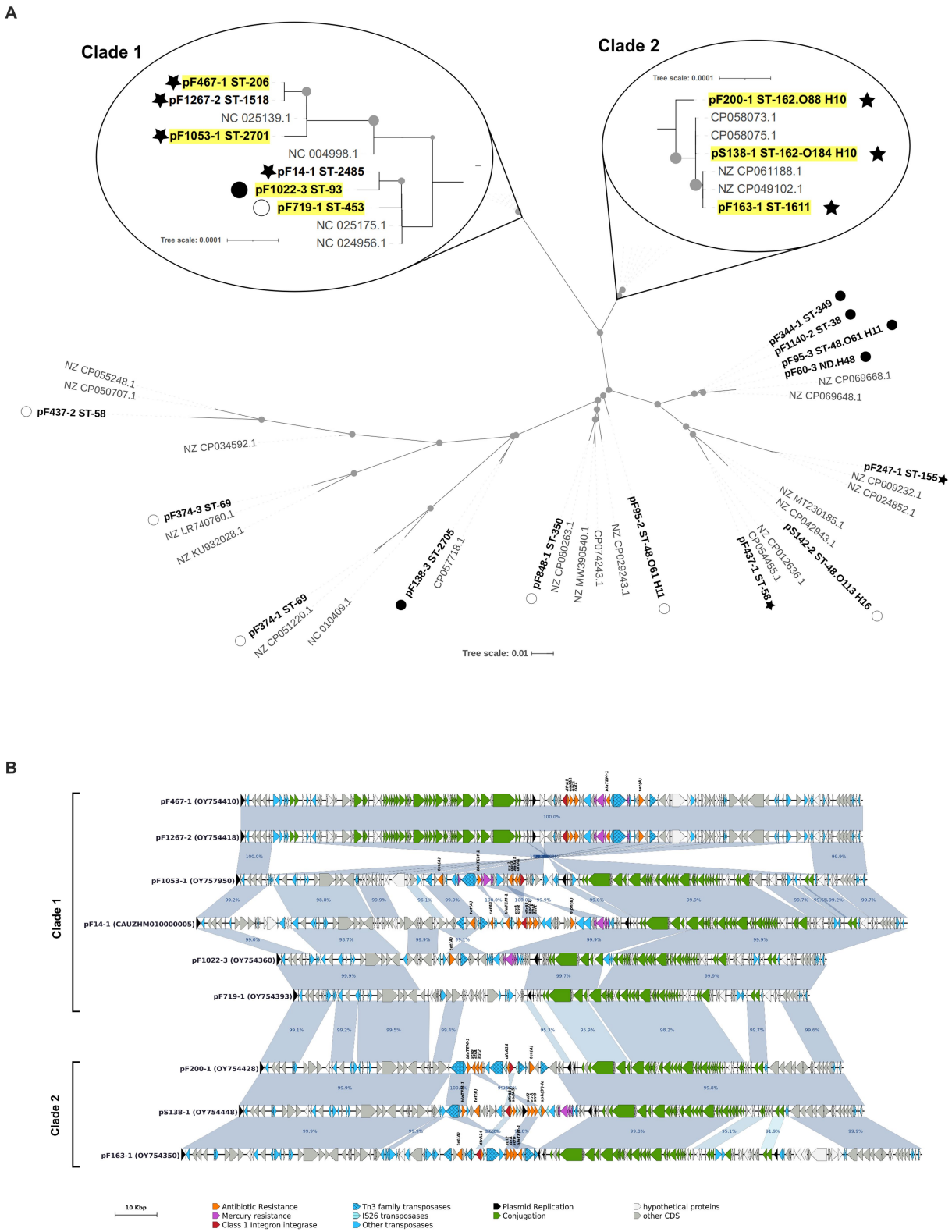

FIGURE 3
(A) IncF plasmids phylogenetic tree. Plasmids from this study are labeled in bold and closest plasmids from the PLSDB database are labeled in gray. Plasmids carried by strains belonging to the six major tetra-resistant ST are outlined in yellow. Only one plasmid by ST is included. Dotted nodes show a bootstrap value >90%. Symbols represent ARG content: star, tetra-resistance determinants; closed circle, AMP and/or TET resistance determinant only; open circle, no ARG detected. **(B)** Graphical representation of IncF plasmids belonging to the two clades zoomed in **(A)**. Antibiotic resistance gene names are not labeled on every sequence to avoid redundancy. Only homologies larger than 2.5 Kbp are displayed.

extensive decontamination, indicating an environmental persistence. Dierikx et al. (2013) also reported the persistence of multidrug resistant *E. coli* in the rearing environment of commercial broiler farms after decontamination and the transmission to the next flock, suggesting that transmission through the rearing environment is probably a common phenomenon. This is in line with Rychlik's observations, showing that *E. coli* is primarily acquired from the environment (Rychlik, 2020). It should be noted that our observations mostly explain farm-level AMR persistence, while rare vertical transmission from breeders or hatcheries, as well as through external factors (transportation equipment, contaminated food, wildlife) should not be neglected as potential factors impacting the spread of AMR between facilities, as proposed for the fast dissemination of APEC O78:H4 strains in parental and broiler farms in Nordic countries (Ronco et al., 2017).

Although no antibiotics was used in the followed breed for the last 10 years, a large amount of multi-resistant *E. coli* was isolated. The genetic determinants of their resistance phenotypes ($bla_{\text{TEM-1}}$, *tet*(A)/*tet*(B), *sul1*/*sul2*, *aadA1* and *dfrA1*) are extremely common in commensal *E. coli* of production animals (Leekitcharoenphon et al., 2021; Szmolka et al., 2021; Gambi et al., 2022) and were already identified as a "common multi-resistance pattern" in this species (Szmolka and Nagy, 2013). These genes confer resistance to "old" antibiotics widely used in livestock prophylactic treatments or as growth promoters in the last 50 years (Samanidou and Evaggelopoulou, 2008). Their presence in the experimental facility devoid of antibiotic pressure is therefore not surprising and reflect what is observed in commercial organic farms (Gambi et al., 2022).

Even if resistance genes were similar between the various isolated *E. coli*, they were carried by distinct, ST-dependent plasmids. Comparisons with the public plasmid database PLSDB indicated that IncF plasmids found in major clonal STs are all more closely related to plasmids outside the facility than to each other, suggesting that they came along with their current *E. coli* hosts rather than spreading in the *E. coli* population of the facility after a single initial introduction. Nonetheless, most resistance plasmids were able to transfer *in vitro* during conjugation assays and a likely transfer event between a major (ST-206) and a minor (ST-1518) clonal ST was observed, ruling out a lack of dissemination because of plasmid non-functionality.

Plasmid persistence in environments without clear selective pressure, dubbed as the plasmid paradox, have long been observed in the lab as well as in nature but the underlying mechanisms are not fully understood yet (MacLean and San Millan, 2015; San Millan and MacLean, 2017; Carroll and Wong, 2018; Wein et al., 2019). Plasmid-encoded toxin-antitoxin and partition systems are efficient in avoiding plasmid loss over time but do not prevent extinction of the plasmid-carrying population to the benefit of plasmid-free populations with a better fitness. Compensatory mutations in the host and plasmid genomes, which reduce the fitness burden of carrying such plasmids, is another mechanism proposed to play a role in plasmid maintenance (Carroll and Wong, 2018). The multiple rearrangements we observed in the MDR region of IncF plasmids could participate in the reduction of fitness burden, for instance by modulating the regulation of resistance genes. The clear host-plasmid association over chicken generations in the present facility is also in favor of host-encoded compensatory mutations which could have resulted in a co-adaptation of both parties and a reduction of plasmid carriage cost.

In conclusion, our experimental design in which the pedigree of every animal was known and our use of whole-genome sequencing approaches allowed us to quantitatively determine the most likely origin of multi-resistant *E. coli* present within the facility. Vertical inheritance through eggs contamination or acquisition at the hatchery seem inexistent in our study, while we pointed out a very strong role of environmental acquisition of building-specific, persistent strains. We also showed that these persistent strains carried their own resistance plasmids, indicating a limited impact of horizontal gene transfer. These results, although providing a clear explanation on how MDR *E. coli* persist in our facility, were obtained in a well-controlled system and without antibiotic pressure. Applying the same methodology in the more open context of commercial farms will be necessary to know if our results can be generalized to the whole poultry production pyramid.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was approved by Ministère Français de la Recherche et de l'Enseignement Supérieur (project n° 00880.02). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SL: Conceptualization, Formal analysis, Investigation, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. PB: Formal analysis, Investigation, Software, Writing – review & editing. IF: Investigation, Resources, Writing – review & editing. YB: Methodology, Resources, Writing – review & editing. AT: Conceptualization, Funding acquisition, Resources, Writing – review & editing. BD: Conceptualization, Funding acquisition, Investigation, Resources, Validation, Writing – original draft, Writing – review & editing. SB: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1406854/full#supplementary-material

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Trends in horizontal gene transfer research in *Salmonella* antimicrobial resistance: a bibliometric analysis

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Horizontal gene transfer (HGT) favors the acquisition and spread of antimicrobial resistance (AMR) genes in *Salmonella*, making it a major public health concern. We performed a bibliometric analysis to provide the current landscape of HGT in research on *Salmonella* AMR and identify emerging trends and potential research directions for the future. Data were collected from the Web of Science Core Collection and limited to articles and reviews published between 1999 and 2024 in English. VOSviewer 1.6.19 and CiteSpace 6.2.R1 software were used to conduct bibliometric analysis and visualize co-occurring keywords. A total of 1,467 publications were retrieved for analysis. American researchers contributed the most articles (n=310). In the meantime, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement have the highest citation/publication rate of 85.6. Recent studies have focused on the application of whole genome sequencing (WGS), *Salmonella* quinolone and colistin resistance, and the biocontrol of *Salmonella* AMR. These findings provide new insights into the role of HGT and help identify new targets for controlling the spread of AMR in *Salmonella* populations.

KEYWORDS

***Salmonella*, antimicrobial resistance, horizontal gene transfer, CiteSpace, VOSviewer**

Introduction

The discovery and use of antibiotics have promoted the prevention and treatment of bacterial infections but have simultaneously resulted in increased antimicrobial resistance (AMR). It is estimated that by 2050, deaths caused by AMR will increase to 10 million per year, resulting in a cost of 100 trillion USD (Despotovic et al., 2023). AMR can arise from mutations in chromosomal DNA or acquisition of AMR genes. Horizontal gene transfer (HGT) is one of the most important mechanisms for the acquisition and spread of AMR genes among bacteria. Among the different HGT mechanisms, conjugation is considered the main driver of resistance gene exchange between bacteria. The majority of medically relevant AMR genes are clustered on mobile genetic elements such as gene cassettes, transposons, genomic islands, and plasmids. Consequently, such resistance genes are easily swapped between bacteria in the same habitat, such as *Enterobacteriaceae* in the intestine of animals and humans (Tao et al., 2022).

Salmonella enterica serovars are prevalent human and animal pathogens that are responsible for gastroenteritis and typhoid diseases. It is responsible for 550 million cases of diarrhea annually, of which 220 million are under the age of five. Many of these cases are life

threatening and deadly. Multi-drug resistance (MDR) *Salmonella* has been classified according to the World Health Organization as a pathogen of high priority, linked to the emergence of fluoroquinolone resistance (WHO publishes list of bacteria for which new antibiotics are urgently needed, 2017). In the context of HGT, *Salmonella* participates either as a donor or recipient of resistance genes, and is therefore implicated in the spread of resistance genes. It has been identified as a major driver of the rapid dissemination of AMR in both humans (Winokur et al., 2001) and animals (Mathew et al., 2009).

Bibliometric analysis is a quantitative research method that involves analysis and evaluation of scientific publications using statistical methods (Ellegaard and Wallin, 2015). This allows for the identification of important authors, institutions, and research trends in the field. By analyzing publication patterns and citation networks, researchers can identify important research gaps, emerging topics, and influential studies. Bibliometric analysis has been applied to the field of AMR (Sun et al., 2022). However, no bibliometric analysis has been conducted on HGT in *Salmonella* AMR. Therefore, it is necessary to provide a quantitative framework for evaluating the growth and impact of research on *Salmonella* AMR and HGT.

In this study, a bibliometric analysis was conducted in the area of *Salmonella* AMR to provide the current landscape of HGT research and to identify emerging trends and potential future research directions.

Materials and methods

Data collection and retrieval strategies

Data were collected from the Web of Science Core Collection (WoSCC) on May 27, 2024. The search query used was ((TS = ("horizontal gene transfer") OR TS = (HGT) OR TS = (conjugation) OR TS = (transduction) OR TS = "mobile genetic element") OR TS = ("mobile element") OR TS = ("conjugative plasmid") OR TS = ("mobilizable plasmid") OR TS = ("integrative conjugative element") OR TS = (ICE) OR TS = ("integrative mobilizable element") OR TS = (IME) OR TS = ("transposable element") OR TS = (transposon) OR TS = (phage) OR TS = (prophage) OR TS = (bacteriophage)) AND TS = (*Salmonella*) AND (TS = ("antibiotic resistance") OR TS = ("antimicrobial resistance") OR TS = (AMR) OR TS = ("acquired resistance gene") OR TS = ("drug resistance") OR TS = ("multidrug resistance")). The results were limited to articles and reviews published in English and indexed in the Science Citation Index Expanded database.

Data analysis

All acquired articles underwent a meticulous screening process to assess their relevance to HGT in *Salmonella* AMR. Variables such as the publication date, document type, affiliation, country, title, and keywords were extracted. Trend analysis was performed to discern patterns in scientific output across the temporal axes, citation indices, and author/institution/country contributions. Bibliometric analyses and network visualizations were performed using VOSviewer version 1.6.19 and CiteSpace version 6.2.R1 (64-bit) to construct networks of co-occurring keywords.

Results

Publication characteristics

The WoSCC database contains a total of 1,467 publications related to AMR/HGT research in *Salmonella* from 1999 to 2024. The number of publications in this field has shown an increasing trend over the years (Figure 1). They were written by 7,028 authors from 1887 organizations in 105 countries. When considering the aging of references in this field, the Price's index (de Price, 1969) and citing half-life were calculated.

$$Price's\ index = \frac{number\ of\ citations\ less\ than\ 5\ years\ old}{total\ number\ of\ citations} \times 100\%$$

The Price's index of these cited references is 17.5%, which means that 17.5% of them were from the last 5 years. The cited half-life is the median age of articles cited in the Journal Citation Report (JCR) year (Garfield, 2001). This indicates the turnover rate of the body of work on a subject. The citing half-life was 8 years. These two indices indicate that the aging of publications in this area is relatively slow, and research is stable and mature. The 1,467 publications received 52,591 citations, and the citation/publication in this area was 35.85. The h-index of retrieved publications was 102.

Authors, countries/regions and institutions cooperation analysis

Table 1 shows the top 10 high-productivity authors and provides an overview of the most productive and cited authors in the field. The top 10 countries that made the most significant contributions to the field of HGT in *Salmonella* AMR are listed in Table 2. The USA scholars have contributed the most research papers in this field (310 papers in total). China follows with 201 publications, but the average citation/publication rate is only 20.8. France has the highest average citation/publication rate (63.8). The institutional analysis corroborates this finding. The distribution of outstanding institutions in this area is relatively equal among countries (Table 3). Three of the institutions were from the USA and contributed 50% of the total publications. The U.S. Food and Drug Administration (US FDA) published the most papers, with a citation/publication of 41.9. The Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) in France contributed only 27 publications but received 2,311 citations, with the highest citation/publication rate of 85.6.

Abbreviations: AMR, Antimicrobial resistance; HGT, horizontal gene transfer; WoSCC, Web of Science Core Collection; JCR, Journal Citation Report; US FDA, U.S. Food and Drug Administration; INRAE, l'Agriculture, l'Alimentation et l'Environnement; PFGE, pulsed-field gel electrophoresis; MDR, multi-drug resistance; SGI1, *Salmonella* genomic island; qnr, quinolone resistance; ESBL, extended-spectrum β-lactamase; WGS, whole genome sequencing.

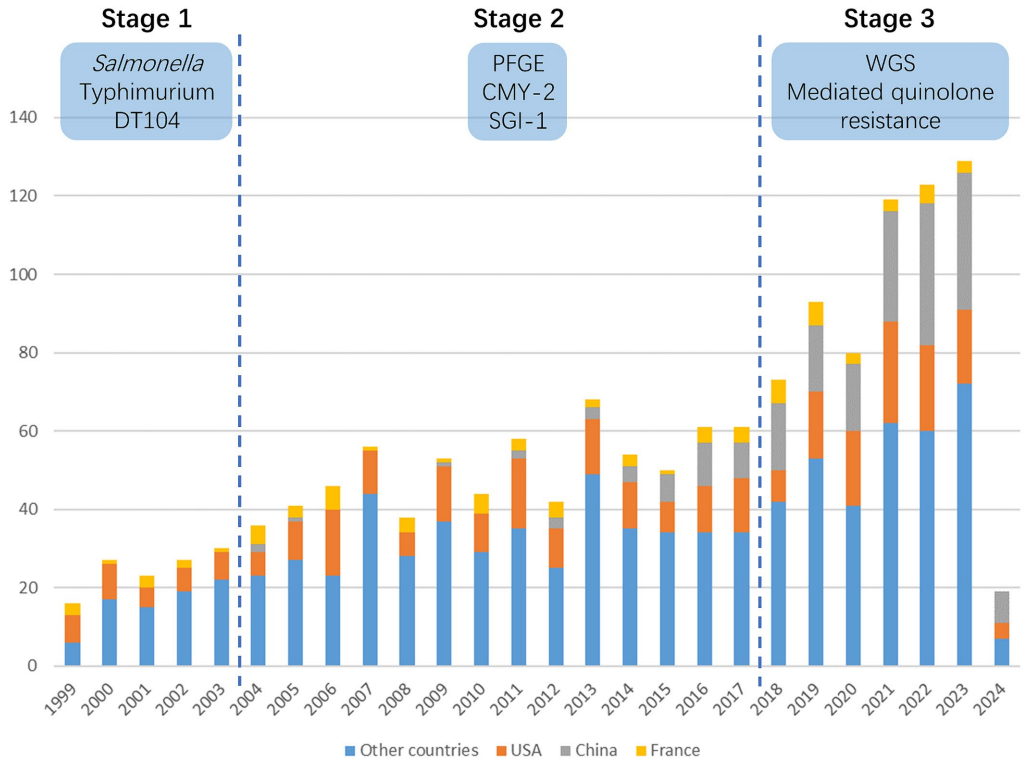

FIGURE 1
Trends in publications on horizontal gene transfer in *Salmonella* antimicrobial resistance. This figure displays the annual count of publications related to horizontal gene transfer in *Salmonella* antimicrobial resistance based on a dataset comprising 1,467 publications gathered from the WoS core collection spanning from 1999 to May 27, 2024 (refer to Materials and Methods for search query details). The figure delineates three distinct stages characterized by varying publication volumes, along with their corresponding research focal points. Additionally, contributions from the USA, China, and France were visually differentiated through distinct color highlights.

TABLE 1 **Top 10 high-productive authors on horizontal gene transfer in *Salmonella* antimicrobial resistance.**

Rank	Author	Country	Publication	Citation	Citation/ Publication	H index
1	Xu, Xuebin	China	19	210	11.1	21
2	Zhao, Shaohua	USA	18	618	34.3	37
3	Fanning, Seamus	Ireland	18	398	22.1	51
4	Foley, Steven L.	USA	17	438	25.8	24
5	Frye, Jonathan G.	USA	16	525	32.8	26
6	Hendriksen, Rene S.	Denmark	15	492	32.8	42
7	Cloeckaert, Axel	France	15	1,009	67.3	36
8	Guerra, Beatriz	German	14	610	43.6	36
9	Dougan, Gordon	England	14	1,245	88.9	82
10	Weill, Francois-Xavier	France	14	720	51.4	48

Highly-cited papers analysis

Table 4 shows the top 10 most-cited papers related to this topic. Most were research articles with only one review. The most cited paper is "Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype" (Kingsley et al., 2009) which has 391 citations. Most of them were published in journals specifically in the microbiology or antimicrobial fields.

Keyword clustering, burst, and evolution analysis

In Figure 2, the five clusters comprehensively map the intricacies of AMR in *Salmonella*, thereby emphasizing the role of HGT. The "Red" cluster deals with the epidemiological spread, including factors from various hosts to environmental transmission. The "Green" cluster focus on specific AMR mechanisms and their impact on clinical treatments. The "Blue" group dissects the

molecular genetics that facilitate resistance, applying high-level sequencing techniques to decode these processes. The "Yellow" cluster is oriented around methodological tools and particular genetic elements like integrons that enable the study of resistance mechanisms. The "Purple" group delves into biocontrol strategies involving bacteriophages and the role of genomics in understanding *Salmonella enterica* in food systems.

Keyword burst detection refers to the significant increase in the frequency of keywords within a short period. The 25 keywords in the field with the strongest citation bursts are shown in Figure 3A. "*Salmonella* Typhimurium DT104" had the highest burst strength (35.17) from 1999 to 2008, followed by "Whole genome sequencing" (12.08) from 2019 to 2023, and "Pulsed-field gel electrophoresis" (PFGE) (10.12) from 2007 to 2013. This evolutionary trend of keywords was further visualized using VOSviewer (Figure 3B). "*Salmonella* Typhimurium DT104" and "Pulsed-field gel electrophoresis" are in purple with an average publication year before 2013 while "Whole genome sequencing" is in yellow with an average publication year after 2016.

Discussion

To date, no bibliometric analysis has been conducted on HGT in *Salmonella* AMR. Therefore, we performed the first bibliometric analysis of this specialized area. The retrieved publications covered various aspects of HGT in *Salmonella* AMR, and these topics have been discussed in both livestock and human (Antunes et al., 2006; Mathew et al., 2009).

Evolution of publications

According to de Price (1969), the Price's index in physics and biology is approximately 60–70%, which is much higher than our result. The low Price's index corresponds to a long cited half-life in this area, signifying a relatively gradual turnover rate. This illustrates the stability and maturity of the knowledge in this field over time. The citation/publication index shows the average quality of all publications, while the H-index reflects more about the number of high-quality

TABLE 2 Top 10 high-productive countries on horizontal gene transfer in *Salmonella* antimicrobial resistance.

Rank	Country	Publication	Citation	Citation/Publication
1	USA	310	13,935	45.0
2	China	201	4,184	20.8
3	England	147	6,666	45.3
4	Spain	96	4,019	41.9
5	France	82	5,232	63.8
6	Germany	80	4,587	57.3
7	Canada	78	3,678	47.2
8	South Korea	72	1,178	16.4
9	Italy	69	3,375	48.9
10	Denmark	61	2,769	45.4

TABLE 3 Top 10 most productive institutions on horizontal gene transfer in *Salmonella* antimicrobial resistance.

Rank	Institution	Country	Publication	Citation	Citations/ Publication
1	U.S. Food and Drug Administration (US FDA)	USA	73	3,061	41.9
2	U.S. Department of Agriculture Agricultural Research Service (USDA ARS)	USA	50	1,710	34.2
3	University Oviedo	Spain	35	1,083	30.9
4	Center for Disease Control and Prevention	USA	29	1,723	59.4
5	Technical University of Denmark	Denmark	29	1,728	59.6
6	Pasteur Institute	France	29	1,364	47.0
7	Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE)	France	27	2,311	85.6
8	South China Agricultural University	China	26	376	14.5
9	Yangzhou University	China	26	164	6.3
10	German Federal Institute for Risk Assessment	Germany	24	1,033	43.0

TABLE 4 Top 10 highly-cited papers on horizontal gene transfer in *Salmonella* antimicrobial resistance.

Rank	Title	Citations	Year	First author	Type	Journal	JCR	IF
1	Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype	391	2009	Robert A Kingsley	Research Article	Genome Research	Q1	7
2	Characterization of multiple-antimicrobial-resistant *Salmonella* serovars isolated from retail meats	342	2004	Sheng Chen	Research Article	Applied And Environmental Microbiology	Q2	4.4
3	Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona	325	2001	David Boyd	Research Article	Journal of Bacteriology	Q3	3.2
4	Molecular characterization of an antibiotic resistance gene cluster of *Salmonella* Typhimurium DT104	272	1999	Connie E. Briggs	Research Article	Antimicrobial Agents and Chemotherapy	Q2	4.9
5	Animal and human multidrug-resistant, cephalosporin-resistant *Salmonella* isolates expressing a plasmid-mediated CMY-2 AmpC beta-lactamase	233	2000	P. L. Winokur	Research Article	Antimicrobial Agents and Chemotherapy	Q2	4.9
6	Epidemic *Salmonella* Typhimurium DT 104--a truly international multiresistant clone	230	2000	E. John Threlfall	Review	Journal of Antimicrobial Chemotherapy	Q2	5.2
7	Antimicrobial resistance in non-typhoid *Salmonella* serotypes: a global challenge	202	2004	Lin-Hui Su	Review	Clinical Infectious Diseases	Q1	11.8
8	Emergence of domestically acquired ceftriaxone-resistant *Salmonella* infections associated with AmpC beta-lactamase	198	2000	Eileen F. Dunne	Research Article	Jama-journal of The American Medical Association	Q1	120.7
9	The *Salmonella* genomic island 1 is an integrative mobilizable element	196	2005	Benoît Doublet	Research Article	Molecular Microbiology	Q2	3.6
10	Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*	194	2006	Kathryn Gay	Research Article	Clinical Infectious Diseases	Q1	11.8

JCR, Journal Citation Report; IF, Impact factor.

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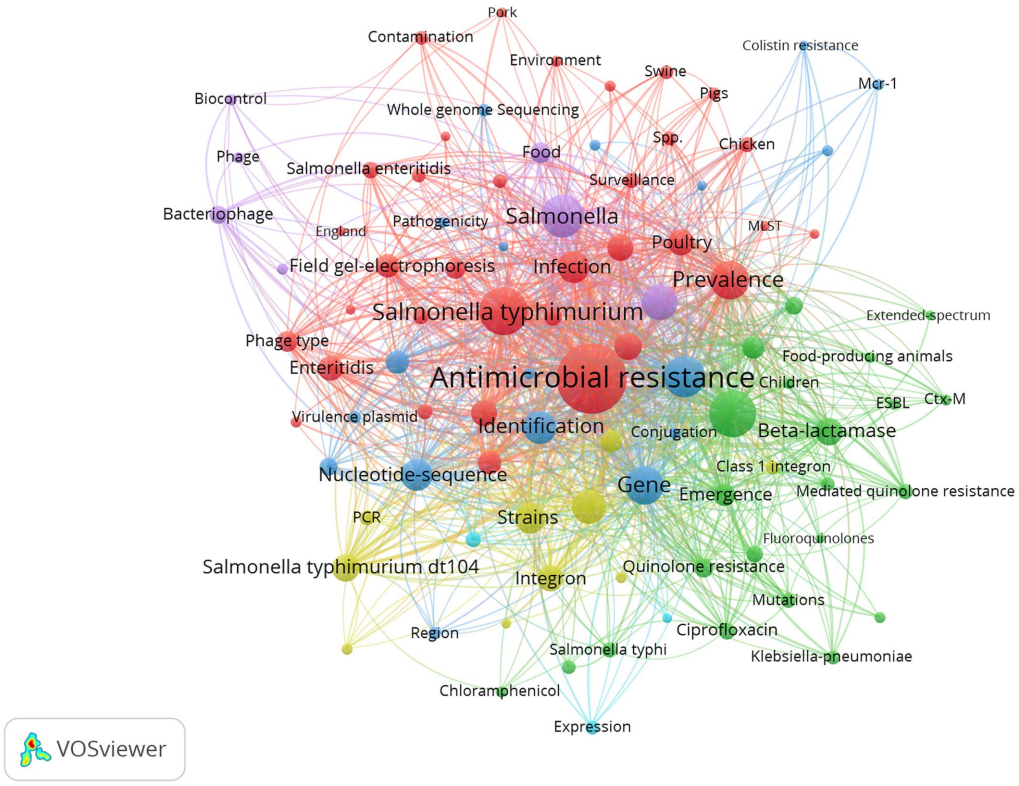

FIGURE 2
The co-occurrence of keywords. The size of each circle corresponds to the number of publications, with larger circles indicating higher publication counts. Connecting lines signify co-occurrence between keywords, with thicker lines signifying more frequent co-occurrences.

publications (Hirsch, 2005). Several bibliometric analyses have focused on other topics in AMR, such as carbapenem resistance (Sweileh et al., 2016), AMR in food-producing animals (Sweileh, 2021), or AMR in the environment (Sweileh and Moh'd Mansour, 2020). In these fields, the citation/publication is approximately 22 (21.47, 22.4, and 22, respectively), which is lower than our result (29.23). However, the h-index for carbapenem resistance and food-producing animals' AMR is 102 and 122, respectively, which are much higher than our results (87). This indicates that the overall quality of publications on HGT in *Salmonella* AMR is above the average level in the field of AMR, but the number of high-quality papers is still inferior to some of the other topics.

The geographic distributions of publications

When we look into highly productive authors, institutions, and countries, the USA, China, and France are the three outstanding countries with different characteristics. The USA is widely recognized as a global leader and holds a prominent position in the scientific research area (These are the 10 best countries for life sciences research, 2020), which is the same in the field of HGT in *Salmonella* AMR. This was proven by the number of publications, citations, and highly productive authors and institutions in the USA. China is second to the USA in terms of the number of publications but with a low number of citations per publication. In recent years, China has significantly increased funding and policy support for scientific research (Marginson, 2022), which has led to an increase in publications from China (Figure 1). However, the emphasis on quantity rather than quality in some academic evaluation systems may lead to higher publication output without a sufficient emphasis on impact and scientific rigor (Wang et al., 2020). To address this, enhancing the quality-oriented evaluation system is important to improve the impact of Chinese research in this field. In contrast, France published only 78 papers in this field but with the highest citation/publication. The INRAE and Pasteur Institution are outstanding in terms of citation/publication. Both institutions are government research centers and have a profound history of bacterial research, which may explain the high quality of their scientific output.

Evolution of research hotspots

When considering publication numbers, highly cited papers, and burstiness of keywords together, we identified three different stages from 1999 to 2024 and observed a clear evolution of research hotspots in the area of HGT in *Salmonella* AMR.

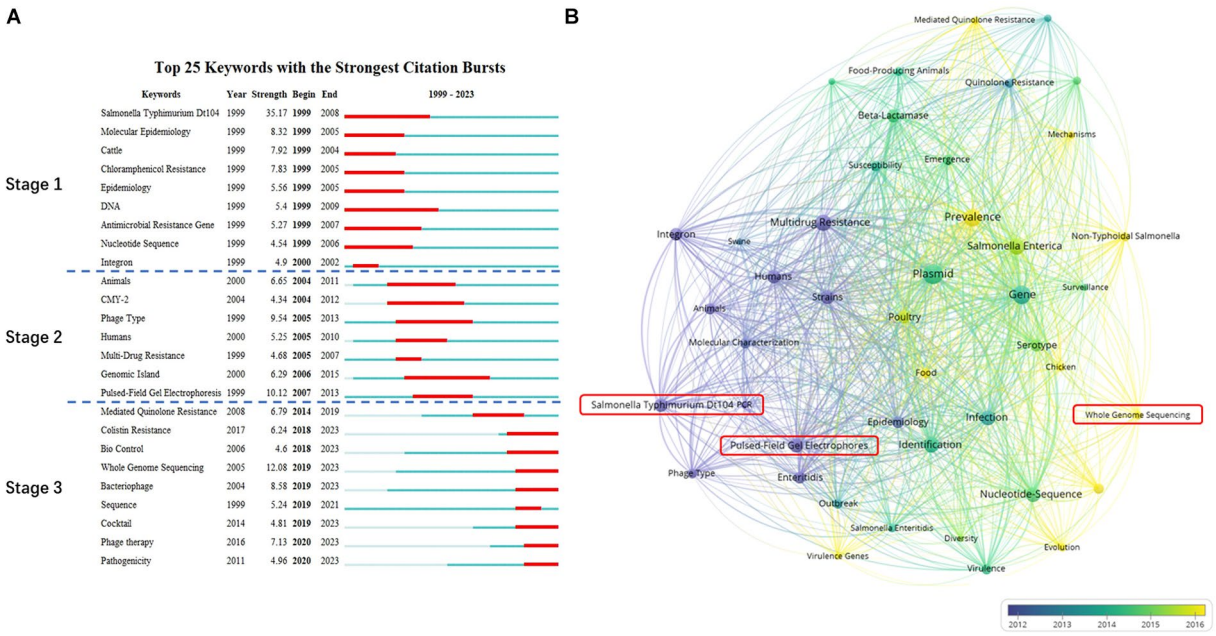

FIGURE 3
The evolution of keywords. **(A)** Top-25 keywords with the strongest citation bursts: "year" represents the first time the keywords appeared. "Begin" and "End" indicate the start and termination point of the period of heightened research activity on a specific keyword. "Strength" refers to the intensity of the burst during this period. **(B)** The co-occurrence and evolution of keywords: Node colors reflect the average publication year of each keyword, offering insight into the temporal distribution of keywords in the network. The size of circular nodes indicates the frequency of occurrence, with larger circles indicating a higher publication count. The thickness of the connecting lines represents the strength of the co-occurrence relationship, with thicker lines indicating a higher number of co-occurrences between two keywords.

Stage 1: understanding of MDR *Salmonella* Typhimurium DT104 (*S.* Typhimurium DT104) (1999–2003)

During this stage, the number of publications was less than 30 per year. According to the strength of the keyword citation burst, the research hotspot during this stage is to genetically explain the MDR mechanism of *S.* Typhimurium DT104 and how it transfers to other strains. MDR *S.* Typhimurium DT104 is known for its ability to cause infections in both humans and animals and has gained significant attention due to its emergence in the 1990s. These resistance genes can be horizontally transferred to other bacteria. Basic molecular methods and Sanger sequencing enabled the characterization of the integrative element *Salmonella* genomic island 1 (SGI1) and its MDR antibiotic gene cluster and demonstrated its potential for HGT.

During this stage, several highly cited publications provided fundamental experience for future research. Threlfall (2000) provided an overview of the international epidemic of MDR *S.* Typhimurium DT104, especially for genetic studies on the HGT of resistance genes. Briggs and Fratamico (1999) cloned and sequenced AMR genes that confer the ACSSuT-resistant phenotype. These genes were grouped within two district integrons and intervening plasmid-derived sequences. This sequence is potentially useful for detection MDR DT104. Doublet et al. (2005) discusses SGI1, which contains an AMR gene cluster found in various *Salmonella enterica* serovars. It demonstrated SGI1's ability to be conjugally transferred and integrated into the chromosomes of recipient strains in a site-specific manner. This sheds light on the mechanisms underlying the spread of AMR genes, and highlights the potential of SGI1 to disseminate MDR among different bacterial populations. Boyd et al. (2001) first reported SGI1 in *S.* Typhimurium DT104 pandemic clone. Several open reading frames showed significant homology with plasmid-related genes, suggesting a plasmid origin for SGI1.

Stage 2: application of PFGE and emergence of CMY-2 related β-lactamase resistance (2004–2017)

From 2004 to 2017, the number of publications per year was 30–70. During this period, PFGE was widely considered the "gold standard" technique for *Salmonella* genotyping (Neoh et al., 2019). After achieving a comprehensive understanding of *S.* Typhimurium DT104, the focus gradually switched to CMY-2 and related β-lactamase resistance. This may be related to the epidemic outbreak of the MDR *Salmonella* serovar Heidelberg from 2002 to 2005 in America and Canada (Zhao et al., 2008). This serovar substantially increased resistance to cephalosporins.

Resistance of *Salmonella* to expanded-spectrum cephalosporins has drawn the attention of the scientific community. *Salmonella* strains carrying $bla_{\text{CMY-2}}$ were first isolated from human, animal, and food samples in the United States in 1996 (Zhao et al., 2001). In 2004, a comprehensive review of AMR in non-typhoid *Salmonella* is highly quoted by researchers (Su et al., 2004). This paper discusses how the gene $bla_{\text{CMY-2}}$, which encodes extended-spectrum cephalosporinases in *Salmonella*, is transferred horizontally through conjugative plasmids, transposons, or integrons. The $bla_{\text{CMY-2}}$ can be acquired

through *in vivo* transfer from other pathogens in the intestines of patients. Additionally, resistance plasmids carrying bla_{CMY-2} can recombine with virulence plasmids to form hybrid plasmids that enhance *Salmonella* survival in drug environments and promote the spread of drug-resistant strains. Two studies reported the appearance of cephalosporin-resistant *Salmonella* expressing a plasmid-mediated CMY-2 AmpC β-Lactamase in 2000 and also received high citations (Dunne et al., 2000; Winokur et al., 2000). It is mainly carried by IncA/C plasmids, which have spread among different *Salmonella* serotypes and *E. coli* in all food-producing animals (Martin et al., 2012). Chen et al. (2004) also report plasmid-mediated transfer of genes encoding CMY-2 and TEM-1-like β-lactamases through conjugation studies. Further comparison showed that 19% of *Salmonella* isolates from retail meats purchased in the USA were resistant or exhibited intermediate susceptibility to ceftriaxone and harbored the bla_{CMY-2} gene. Conversely, all *Salmonella* isolates from China were susceptible to ceftriaxone (and other cephalosporins), and none harbored bla_{CMY-2}. This may be linked to the earlier therapeutic use of cephalosporins in food animals in the USA than in China. In fact, after voluntary withdrawal of ceftiofur in 2005, resistance to ceftiofur declined by 89% in Quebec retail chicken meat (Dutil et al., 2010). This supports the hypothesis that fluctuations in ceftiofur resistance were most likely driven by common exposure (or reduction of exposure) to ceftiofur, rather than simply being secondary to the natural spread and disappearance of a ceftiofur-resistant clone unrelated to ceftiofur use.

Stage 3: application of whole genome sequencing (WGS), emergence of quinolone resistance (*qnr*) and bio-control (2018–2023)

Since 2018, the annual number of publications has increased to over 70, and has reached more than 100 in 2021. "Whole genome sequencing," "quinolone resistance," "bio control" and "phage therapy" became key elements during this stage.

WGS has been increasingly recognized as a promising substitute for *Salmonella* typing (Ibrahim and Morin, 2018). In the post-genomic era, the affordability of the WGS technology has facilitated its widespread adoption in the research community. The analysis of WGS data has significantly enriched our understanding of the population structure, transmission dynamics, and host persistence. In a study with the highest number of citations, Kingsley et al. (2009) utilized WGS to analyze the genetic makeup of the MDR ST313 NTS isolate, D23580, as well as other epidemic ST313 isolates from Malawi and Kenya. Researchers have identified distinct prophage repertoires, composite genetic elements encoding MDR genes, and evidence of genome degradation including pseudogene formation and chromosomal deletions. This suggested that the virulence plasmid may act as a platform for capturing AMR genes, facilitating the exchange of genes collected from other bacteria in the environment. This approach allowed for a comprehensive analysis of the genetic basis of AMR and virulence potential in the studied *Salmonella* Typhimurium strains. Therefore, WGS plays a crucial role in providing detailed insights into the genomic characteristics of the epidemic ST313 NTS isolate and its implications in AMR and pathogenicity.

There has been a growing interest in *Salmonella* plasmid epidemiology due to the emergence of plasmid-mediated *qnr* genes (Gay et al., 2006), the sporadic spread of plasmid-borne ESBL genes (Dor et al., 2020), and the development of plasmid typing methods (Carattoli et al., 2005). In 2006, Gay et al. (2006) first report of plasmid-mediated *qnr* in *Salmonella* isolates from the United States and arouse high citations. This study emphasizes the potential for rapid spread of plasmid-mediated fluoroquinolone resistance and simultaneous resistance to multiple classes of antimicrobial agents. In addition, the presence of multiple *qnr* variants in several *Salmonella* serotypes from widely separated states suggests a broad host and geographic distribution, raising concerns regarding the insidious spread of resistance and the potential for therapy-threatening co-transmission of extended-spectrum β-lactamases.

The serious MDR status of *Salmonella* populations has made "the bio control" an important research area in recent years to prevent *Salmonella* colonization and transmission (Guenther et al., 2012). For example, phage therapy is considered a promising approach for combating *Salmonella* infections (LeLièvre et al., 2019). Bacteriophages specifically target and infect bacteria, hijack the bacterial machinery to replicate, and ultimately lead to cell lysis. However, bacteriophages can act as vehicles for HGT by carrying bacterial DNA during the infection cycle (Borodovich et al., 2022). Thus, HGT may contribute to the spread of AMR in bacterial populations (Colavecchio et al., 2017). The potential of HGT in bacteriophage therapy is an area of active research that requires careful consideration to mitigate any adverse effects, such as the dissemination of AMR genes.

Conclusion

In summary, investigation of HGT in the context of *Salmonella* AMR represents a dynamic and internationally collaborative research domain. The enduring stability and maturity observed in this field underscores its status as a firmly established and influential area of study. Novel methods and technologies such as high-throughput long-read sequencing and advanced bioinformatics tools present exciting opportunities to deepen our understanding of HGT in *Salmonella* AMR. For instance, the use of PacBio long-read sequencing in characterizing 134 *Salmonella* isolates from raw meats and food animals has elucidated the genomic structure and location of resistance genes, significantly contributing to our knowledge of HGT in *Salmonella* (Li et al., 2021). Also, the application of an ISO-certified genomics workflow for identifying and validating antimicrobial resistance in *Salmonella* spp. demonstrates the effectiveness of advanced bioinformatics tools in this field (Sherry et al., 2023). In addition, bio-control methods can act as a novel and effective strategy to combat the rising threat of MDR *Salmonella* strains. For example, the use of bacteriophages in treating antibiotic-resistant *Salmonella* infections has shown promising results, with engineered phage cocktails achieving clinical improvements in a significant number of cases (Khan and Rahman, 2022). Similarly, probiotics such as *Bacillus subtilis* has been found to inhibit the formation of *Salmonella* biofilms, offering an alternative to traditional antibiotic therapies (Jeon et al., 2017).

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

JY: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Conceptualization. BD: Writing – review & editing, Formal analysis, Data curation, Conceptualization. AW: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization.

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Conflict of interest

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

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Bioinformatic analysis reveals the association between bacterial morphology and antibiotic resistance using light microscopy with deep learning

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Although it is well known that the morphology of Gram-negative rods changes on exposure to antibiotics, the morphology of antibiotic-resistant bacteria in the absence of antibiotics has not been widely investigated. Here, we studied the morphologies of 10 antibiotic-resistant strains of *Escherichia coli* and used bioinformatics tools to classify the resistant cells under light microscopy in the absence of antibiotics. The antibiotic-resistant strains showed differences in morphology from the sensitive parental strain, and the differences were most prominent in the quinolone-and β-lactam-resistant bacteria. A cluster analysis revealed increased proportions of fatter or shorter cells in the antibiotic-resistant strains. A correlation analysis of morphological features and gene expression suggested that genes related to energy metabolism and antibiotic resistance were highly correlated with the morphological characteristics of the resistant strains. Our newly proposed deep learning method for single-cell classification achieved a high level of performance in classifying quinolone-and β-lactam-resistant strains.

KEYWORDS

antibiotic resistance, light microscopy, bacterial morphology, deep learning, bioinformatic analysis

Introduction

The emergence of multidrug-resistant bacteria that survive in the presence of multiple types of antibiotics is a global problem, and the spread of these bacterial strains is becoming a threat to public health. Drug resistance is caused primarily by long-term overuse of antibacterial medications. The factors and molecular mechanisms responsible for drug resistance in microbes have been widely reported (Alekshun and Levy, 2007). Accordingly, it has long been known that bacterial cell morphology alters following exposure to antibiotics, with cells undergoing filamentation in response to this stress (Nishino and Nakazawa, 1972; Elliott et al., 1987). In recent years, the effects of antibiotics on bacterial morphology have been studied in terms of bacterial adaptation and survival in response to drug treatments (Monahan et al., 2014; Banerjee et al., 2021). However, the morphology of antibiotic-resistant bacteria in the absence of drugs is not well known.

Laboratory-based evolution is a powerful tool for investigating the dynamics of acquiring drug resistance (Suzuki et al., 2014; Furusawa et al., 2018; Maeda et al., 2020). Using this technique, bacterial cells are exposed to fixed concentrations of drugs, around which cell growth is partially or completely inhibited such that a selective advantage for resistant strains is maintained. Suzuki et al. (2014) performed laboratory-based evolution experiments using *Escherichia coli* under long-term treatment with various antibiotics to obtain resistant strains. Ten antibiotic-resistant strains were identified and transcriptome and genome sequencing analyses were performed to identify gene expression changes and fixed mutations. Because many gene expression changes were observed in the antibiotic-resistant strains, it was hypothesized that these changes may affect bacterial morphology (Suzuki et al., 2014). Using these resistant strains, our laboratory previously reported significant morphological differences between an enoxacin-resistant strain compared with the antibiotic-sensitive parental strain in the absence of the drug, with the changes in cell structure being accurately discernible using deep learning of electron microscopy images (Hayashi-Nishino et al., 2022).

The objectives of the present study were to elucidate the morphological characteristics of the 10 antibiotic-resistant *E. coli* strains using bioinformatics tools and identify the genetic influences on the morphology of the resistant strains. Light microscopy images were used because they are much easier and faster to obtain than electron microscopy images and suitable for analyzing large numbers of living bacterial cells. Moreover, we aimed to discern antibiotic-resistant strains from the antibiotic-sensitive parental strain in the absence of drugs using a newly proposed cell contour-based deep learning method.

Materials and methods

Bacterial strains and culture conditions

Ten laboratory-evolved antibiotic-resistant *E. coli* strains and their parental MDS42 strain (Table 1) (Suzuki et al., 2014) were used in the experiments. A single colony of each resistant strain was firstly obtained from the above original resistant strains described in the following section. Modified M9 medium (Mori et al., 2011) were prepared as described in the Supplementary methods. For morphological observations, bacterial strains were cultured as described previously (Hayashi-Nishino et al., 2022). Briefly, the antibiotic-resistant and parental strains were precultured in M9 medium at 34°C for 23 h with shaking at 432 rpm in Nunc 96-well microplates (Thermo Fisher Scientific Inc.). The cells were then diluted to an optical density $(OD)_{600\ nm}$ of 1×10^{-4} to 1×10^{-8} in 5.0 mL of fresh M9 medium in glass test tubes and further incubated with shaking at 150 rpm in a water bath (TAITEC Corp.) at 34°C until the cultures reached an $OD_{600\ nm}$ in the range of 0.07–0.13, the same range used by Suzuki et al. (2014) for RNA isolation for gene expression analysis. The final $OD_{600\ nm}$ values were determined from 200 μL aliquots of the culture transferred to a Nunc 96-well microplate.

Single colony isolation and determination of minimum inhibitory concentrations

Serial dilutions of each antibiotic were made in 96-well microplates (Thermo Fisher Scientific Inc.) using modified M9 medium and stored at −80°C before use. The range of antibiotic concentrations used for minimum inhibitory concentrations (MICs) was based on two-fold dilution steps up and down from 1 μg/mL, as required depending on the antibiotic (Suzuki et al., 2014). Single colony isolation and determination of MICs were performed as follows:

1 Each resistant strain was cultured on modified M9 agar plates (Supplementary methods) (Mori et al., 2011) at 34°C for two days.
2 Three colonies were chosen and suspended in modified M9 medium (Mori et al., 2011) to yield an initial $OD_{600\ nm}$ of 3×10^{-5}. This suspension was then inoculated into each well of freshly thawed MIC plates to a final volume of 200 μL. The plates were incubated at 34°C for 23 h with shaking 432 rpm on a multimode microplate reader (Infinite M200 PRO, TECAN Ltd.).
3 The $OD_{600\ nm}$ of each well was measured with a microplate reader, and the well with the highest antibiotic concentration that had an $OD_{600\ nm} > 0.03$ was chosen for further MIC determination. A portion of the culture from the selected well was stored in modified M9 medium containing 15% glycerol at

TABLE 1 List of the antibiotic-resistant bacterial strains used in this study.[a]

Antibiotic-resistant strains	Antibiotic name	Class	Cellular target
CPZ	Cefoperazone	Cephalosporin, β-lactam (BL)	Cell wall
CFIX	Cefixime	Cephalosporin, β-lactam (BL)	Cell wall
AMK	Amikacin	Aminoglycoside (AG)	Protein synthesis, 30S
NM	Neomycin	Aminoglycoside (AG)	Protein synthesis, 30S
DOXY	Doxycycline	Tetracycline (TC)	Protein synthesis, 30S
CP	Chloramphenicol		Protein synthesis, 50S
AZM	Azithromycin	Azalide, macrolide (ML)	Protein synthesis, 50S
TP	Trimethoprim		Folic acid synthesis
ENX	Enoxacin	Quinolone (QN)	DNA gyrase
CPFX	Ciprofloxacin	Quinolone (QN)	DNA gyrase

[a]The name of the antibiotic-resistant strains corresponds to the abbreviation of the antibiotics used in the bacterial evolution experiment reported previously (Suzuki et al., 2014).

TABLE 2 Morphological parameters measured in each bacterial strain.[a]

Parameter	Abbreviation	Unit	Definition
Area	–	μm²	Area
Perimeter	Perim	μm	The length of the outside boundary
Major	–	μm	Primary axis of the best fitting ellipse
Minor	–	μm	Secondary axis of the best fitting ellipse
Circularity	Circ	–	$4\pi \times$ Area/(Perimeter)² (A value of 1.0 indicates a perfect circle)
Maximum Feret's diameter	MaxFeret	μm	The longest distance between any two points along the boundary
Minimum Feret's diameter	MinFeret	μm	The shortest distance between any two points along the boundary
Aspect ratio	AR	–	Major/minor
Roundness	Round	–	The inverse of aspect ratio
Solidity	Solid	–	Area/convex area

[a]Ten parameters were measured in each strain of bacteria.

−80°C, and the remaining culture was used for MIC measurement.

4 The remaining cell cultures were diluted in M9 medium to yield an initial $OD_{600\ nm}$ of 3×10^{-5} and inoculated into each well of freshly thawed MIC plates to a final volume of 200 μL. The plates were incubated at 34°C for 23 h with shaking on a multimode microplate reader. The $OD_{600\ nm}$ of each well was measured, and the lowest antibiotic concentration that reduced the growth to an $OD_{600\ nm} < 0.03$ was defined as the MIC.

5 The MICs of the parental and the original resistant strains (Suzuki et al., 2014) were determined as described above. The relative MIC $\log_2$ values were calculated by comparing the MIC values of the original resistant strain to those of the parental strain, and by comparing the MIC values of colonies isolated from the original resistant strain to those of the parental strain. The colony with the MIC $\log_2$ value closest to that of the original resistant strain was selected and used for further experiments.

Image acquisition

Bacterial cell cultures were centrifuged, and the resulting cell pellets were resuspended in phosphate buffered saline (PBS, Sigma-Aldrich) and washed twice. The cell pellets were suspended in 20 μL of PBS and the suspensions were further diluted to a ratio of 1:10 in PBS. Then, 1.2 μL of the cell suspension were mounted on a glass slide and covered with a 22 × 22 mm cover slip (Matsunami, Japan). A phase contrast microscope with a 100× objective lens (Leica Microsystems) was used for observations. Microscopy images were captured using a Leica ICC50 W camera with LAS EZ imaging software (v. 3.4) at a resolution of 96 pixels/inch. Single images were obtained at a resolution of 2,592 pixels × 1,944 pixels in *xy*. The exposure time was 123 ms, with a gain value of 1.0×, a gamma value of 0.60, and a brightness value of 1.0. The images were saved as tiff files.

Microscopy image data were obtained from each antibiotic-resistant strain, which comprised four lines and the parental strain as one set of data and collected three datasets from bacteria cultured on different dates in each set. Approximately 20 images of each bacterial specimen were taken and used for analysis.

Segmentation and feature extraction from cells

As a preprocessing step, denoising of each image was carried out using a Gaussian filter ($\sigma = 4$). Cellular segmentation was then performed using Omnipose v. 0.4.4[1] (Cutler et al., 2022) pretrained for bacterial phase contrast images. Postprocessing involved removing small regions (<96 pixels) to exclude cases of segmentation failures and remnants of dead bacteria, and to fill holes as much as possible.

After segmentation, the following 10 morphological parameters from each region were measured: Area, perimeter (Perim), Major, Minor, circularity (Circ), maximum Feret's diameter (MaxFeret), minimum Feret's diameter (MinFeret), aspect ratio (AR), roundness (Round), and solidity (Solid) (see Table 2 for definitions these features). The upper and lower 1% of the measured parameters were considered outliers and removed from the analysis.

Histogram intersection

Histogram intersections (Swain and Ballard, 1991) were used to examine the similarity between the parental strain and each resistant strain, or between resistant strains, and to examine reproducibility over three experiments. In each histogram, the range of values was divided into 100 parts, or 100 bins, and normalized so that the sum of all bins was equal to 1. The histogram intersection between two histograms h_1 and h_2 is then calculated as follows:

$$d(h_1, h_2) = \sum_{i=1}^{100} \min\left(h_1(i), h_2(i)\right)$$

Cluster analysis

A k-means clustering method was adopted to group bacterial cells into clusters according to the 10 abovementioned morphological

1 https://github.com/kevinjohncutler/omnipose

features so that the cells in each cluster had similar morphological characteristics. The morphological features were standardized so that the mean and the standard deviation for each feature were 0 and 1, respectively, in advance of clustering. A principal component analysis (PCA) of each cluster was conducted, and the results were represented as a biplot depicting both the distribution of samples from each bacterial strain as an ellipse (a normal distribution) and the loading of each feature as an arrow. The biplots can be interpreted as follows: (1) distributions that are close to each other have similar features; (2) features that point in similar directions are highly correlated; and (3) a distribution that is on the same side as a given feature highly contributes to it. Furthermore, to display the representative shape of each cluster of cells, a sequence of contour points representing a cell was evenly interpolated so that the number of points was the same for all contours and then the mean and standard deviation of the coordinates of the points were calculated for each cluster.

Weighted gene correlation network analysis

A network coexpression analysis was performed using the WGCNA R package v.1.72–1 (R v.4.3.0) (Langfelder and Horvath, 2008) to determine correlations between gene expression (Suzuki et al., 2014) and the morphological features of cells cultured as described above and observed in this study. The transcriptome data of resistant strains obtained by Suzuki et al. (2014) were utilized for the WGCNA. The dataset included 2,829 genes with expression levels exceeding a log-transformed threshold of 300 to account for background noise, as described in Suzuki et al. (2014). During data cleaning, the gene *rrsG*, which exhibited the same expression levels across all strains, was eliminated. First, a coexpression network was constructed wherein the nodes corresponded to gene expression profiles and the edges between genes were determined by the absolute value of the correlation coefficient, with soft-thresholding between the nodes as follows:

$$a_{ij} = \left|s_{ij}\right|^{\beta}, s_{ij} = \left|cor\left(x_i, x_j\right)\right|$$

where x_i is the i-th gene expression profile and β is the soft-thresholding parameter. A scale-free topology analysis was applied to choose an appropriate soft-thresholding power. Then, modules were identified as clusters of highly interconnected genes by hierarchical clustering with an average linkage method and the Dynamic Tree Cut method on the basis of interconnectedness defined by the topological overlap measure with a minimum cluster size of 30, a deep split of 2, and no respect of dendrogram. Those modules that were closely related with each other were merged according to a correlation threshold of 0.25. The gene expression profiles of each module were summarized by an eigengene that was defined as the first principal component of the expression matrix. Finally, the modules (genes) most correlated with each morphological feature were identified for further analysis.

Gene ontology annotation and enrichment analysis

Genes found using the WGCNA were annotated according to the EcoCyc (Keseler et al., 2021) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al., 2016). Then, gene ontology (GO)-term annotation and enrichment analyses were performed using the PANTHER classification system (v.18.0[2]), operated by the GO Consortium, which provides the largest free biological databases for a variety of species (Ashburner et al., 2000; Thomas et al., 2022; Aleksander et al., 2023). The parameters used were as follows: analysis type: PANTHER Overrepresentation Test (Released 20231017); annotation version: GO database DOI: 10.5281/zenodo.7942786 (Released 20230105); reference list: *Escherichia coli* (all genes in database); test type: Fisher's exact. Multiple testing was corrected by calculating the false discovery rate (FDR) and FDR $p < 0.05$ was considered statistically significant.

Deep neural networks for cell classification

For classification of single cells between the parental strain and each resistant strain, we developed a deep neural network, taking a sequence of cell contour points as the input. Cell regions were segmented from microscopy images by Omnipose as described above. A sequence of the coordinates of the contour points was then extracted from the segmented region of each single-cell and aligned so that the major axis was horizontal. The number of contour points differs among single cells depending on their sizes and shapes and should be the same as the input to the contour-based classifier models. An aligned sequence of the coordinates of contour points, therefore, was linearly interpolated on an equally spaced grid. The number of contour points was set to 128 in the following experiments.

The Residual Network (ResNet) architecture (He et al., 2016) incorporates shortcut connections to allow deeper networks without the degradation of training accuracy and is widely adopted as a backbone for state-of-the-art neural networks. Circular convolution (Peng et al., 2020) was proposed to extract effective features from object boundaries and mainly applied for instance segmentation and object detection. We integrated circular convolution layers into the ResNet architecture to learn the discriminative features of bacterial cell morphology in an end-to-end fashion. A circular convolution layer was created using the coordinates/features of neighboring points as the input, and the convolution operation with a one-dimensional kernel was performed, sliding all the way around a cell contour, to produce outputs representing the morphological properties of the bacterial cells.

Classifier models were trained from scratch for 100 epochs using a stochastic gradient descent algorithm (LeCun et al., 2012) with a weight decay of 0.0001, a momentum of 0.9, and a batch size of 128. The initial learning rate was set to 0.0001 and decreased following the cosine schedule (Loshchilov and Hutter, 2016). Data augmentation was conducted by adding a Gaussian noise with a sigma value of 0.01 to the coordinates of the contour points, smoothing by the Savitzky–Golay filter (Savitzky and Golay, 1964) with a window length of 5 and a polynomial order of 2, and randomly shifting the starting point.

Experiments were conducted using threefold cross-validation—because the microscopic image acquisition procedures were repeated three times, two of three datasets were used as a training set and the remaining dataset served as a test set. The classification performance

2 http://www.pantherdb.org

of our proposed method was evaluated by the mean and standard deviation of the area under the receiver operating characteristic curve (AUC), sensitivity, and specificity over three datasets. Sensitivity, specificity, and accuracy were defined as follows:

$$\text{Sensitivity} = \frac{\text{TP}}{\text{TP} + \text{FP}} \quad (1)$$

$$\text{Specificity} = \frac{\text{TN}}{\text{TN} + \text{FN}} \quad (2)$$

$$\text{Accuracy} = \frac{\text{TP} + \text{TN}}{\text{TP} + \text{FP} + \text{TN} + \text{FN}} \quad (3)$$

where TP denotes true positives (correctly classified resistant cells), FP denotes false positives (parental cells that were classified as resistant cells), TN denotes true negatives (correctly classified parental cells), and FN denotes false negatives (resistant cells that were classified as parental cells), respectively. We also conducted experiments to discriminate patches extracted from the microscopy images to compare our proposed approach with the original ResNet model with respect to its performance in classifying the parental strain and each resistant strain. A patch (224 × 224 pixels) was extracted from the center of a cell region and discarded if the area overlapping with another patch, which was evaluated by the intersection over union, was greater than 0.5 (50 percent). The intersection over union (IoU) is defined as follows:

$$\text{IoU} = \frac{|A \cap B|}{|A \cup B|}$$

where A and B are the areas of two patches, the denominator represents the area of union, and the numerator represents the area of intersection, respectively. Data augmentation was conducted by randomly flipping and rotating patches.

Results

Morphological variations exhibited by antibiotic-resistant *Escherichia coli* strains

Single colonies were isolated from 10 strains of antibiotic-resistant *E. coli*, as listed in Table 1, for microscopy-based observation of cell morphology. Most isolates, comprised of four lines from each resistant strain, exhibited resistance in accordance with the original strain (Suzuki et al., 2014) (Supplementary Figure S1). These isolates were then cultured in the absence of antibiotics and observed under phase contrast light microscopy.

The resistant strains showed a variety of morphologies that differed from the structure of the rod-shaped, sensitive, parental strain (Figure 1). For example, CPZ-, CFIX-, and CP-resistant cells appeared shorter or smaller than the parental cells, whereas the ENX-and CPFX-resistant cells exhibited rounder or fatter morphology compared to the parental cells, in agreement with our previous study (Hayashi-Nishino et al., 2022). On the contrary, some of the TP-and AMK-resistant cells showed slightly elongated morphology, although this varied among the four lines. The majority of the antibiotic-resistant strains showed different cell morphologies from the parental strain; however, the NM-, DOXY-, and AZM-resistant strains were difficult to evaluate qualitatively. We therefore proceeded with a quantitative evaluation of the morphologies of the resistant and parental strains.

Quantitative differences in cell morphology between the parental and antibiotic-resistant strains

A morphometric analysis was performed to evaluate the quantitative differences in cell morphology between the antibiotic-resistant and parental strains. We obtained microscopy image data from each resistant strain and the parental strain as one set of data and collected three datasets from bacteria cultured on different dates in each set to evaluate data variance caused by differences in the experiment. Then, single cells were extracted from the microscopy images using Omnipose, a CNN-based image segmentation tool applicable to various bacterial species and morphologies (Cutler et al., 2022). Examples of segmented resistant and parental cells are provided in Supplementary Figure S2. Several thousands of cells were segmented from each bacterial strain in a single dataset. These segmented cells were collected from the three datasets and used for analysis (Supplementary Table S1). Then, 10 morphological parameters (Area, Perim, Major, Minor, Circ, MaxFeret, MinFeret, AR, Round, and Solid) were measured from the extracted contours of the cells.

We included the four lines of each resistant strain without dividing them to find common morphological characteristics in the resistant strain and identify differences from the parental strain. Quantitative differences in each morphological feature between the parental and resistant strains were examined by comparing the mean values obtained from the three datasets (Supplementary Figure S3). As a result, the parameters of most features in the resistant strains showed significant differences ($p < 0.001$) from the parental strain in all datasets. The mean values for many features differed slightly among the resistant strains, although the Solid values showed greater similarity. Standard deviations were relatively large for the majority of features, possibly reflecting variations in cell shape and size in the cell population, a different experiment date, and differences between lines. To further evaluate the variations in features between resistant strains, the ratio of change from the parental strain was calculated using median values. Decreases in Area, Perim, Major, and MaxFeret were observed in most resistant strains, while the changes in both Minor and MinFeret were slightly different between the resistant strains (Figure 2). In addition, the majority of the resistant strains showed increases in both Circ and Round, and a decrease in AR, but the AZM-and TP-resistant strains showed an opposite tendency from other resistant strains. For Solid, a small change ratio was seen in all resistant strains. These results suggested that most of the drug-resistant strains tested displayed morphological differences from the parental strain.

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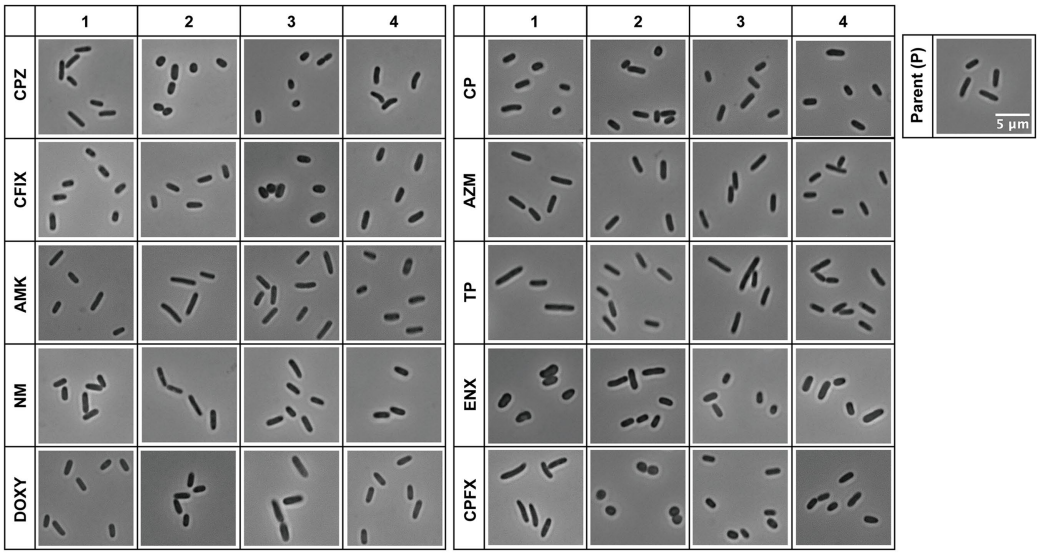

FIGURE 1
Light microscopy of antibiotic-resistant strains. Representative images of antibiotic-resistant *Escherichia coli* strains and the sensitive parental strain (P) from one of three datasets (Dataset 1) are shown. Four lines were isolated from each resistant strain, and the numbers at the top indicate the line of the resistant strain. The scale bar shown in the image of the parental strain applies to all panels. AMK, Amikacin; AZM, Azithromycin; CFIX, Cefixime; CP, Chloramphenicol; CPFX, Ciprofloxacin; CPZ, Cefoperazone; DOXY, Doxycycline; ENX, Enoxacin; NM, Neomycin; TP, Trimethoprim.

Comparison of cell morphology between parental and antibiotic-resistant strains

Quantitative differences in each morphological feature between the parental and resistant strains were further examined using histogram intersections to consider the variability in cell populations.

First, the similarities between three datasets in terms of the morphological parameters in each bacterial strain were examined to evaluate the reproducibility of the data between the datasets. The overall mean similarity was about 0.9, ranging from 0.83 to 0.93 between parameters and from 0.88 to 0.92 between bacterial strains, suggesting that the data variation was quite low between the datasets (Supplementary Table S2). Thus, we included the three datasets in the histogram intersection and asked whether the similarity of each feature between the parental and resistant strains was lower than the similarity between datasets (<0.83). In Figure 3A, histograms displaying the values obtained for each feature of the resistant strains are overlayed with the corresponding histogram for the parental strain. The histograms for Area, Perim, Major, and MaxFeret in the majority of the resistant strains were shifted slightly to the left of the parental strain, suggesting that these features were smaller in the resistant strains. In contrast, the histograms for Solid in the majority of the resistant strains were shifted slightly to the right of the parental strain. Surprisingly, the shapes and distributions of the histograms for Minor and MinFeret differed largely among the resistant strains, with some being broader than those of the parental strain. To a certain extent, these features affected the distributions of the Circ, AR, and Round histograms. While the histogram distributions and shapes differed from those of the parental strain for the majority of the resistant strains, the AZM-and TP-resistant strains showed striking overlaps with the parental strain for most features except Minor and MinFeret.

The histogram intersection was calculated to examine the mean similarity values between the parental and resistant strains (Figure 3A, insets). For some features, these values were < 0.8, with the values for MinFeret and Minor being particularly low (Figure 3B), suggesting that these features were related to the large differences in the shapes of the histograms for the parental and resistant strains (Figure 3A). The CPZ-, CFIX-, CP-, and CPFX-resistant strains, and the ENX-resistant strain in particular, exhibited the lowest similarity to the parental strain (Figure 3C). Conversely, the AZM-and TP-resistant strains showed particularly high similarity to the parental strain.

Clustering of the antibiotic-resistant strains according to morphological features

The findings described above suggested that the resistant strains had different degrees of morphological similarity to the parental strain. Thus, we considered what morphological tendencies seen in the resistant strains differed from those seen in the parental strain, although the histogram distributions suggested that all resistant strains showed variation in cell shape but many of the cells resembled the parental strain (Figure 3A). Therefore, we attempted to extract morphological characteristics that were shared by the resistant strains. This was done by dividing the cell shapes into different clusters using the k-means clustering method.

First, k-means clustering was conducted for all feature data obtained from the bacterial cells, with the number of clusters set to six. To determine the number of clusters, observations from the microscopy images and the results of the histogram analysis were used to group the bacterial cells into different types with respect to shape and size. A PCA was then applied to each cluster to reveal the characteristics of the clustered cell features. Furthermore, the average

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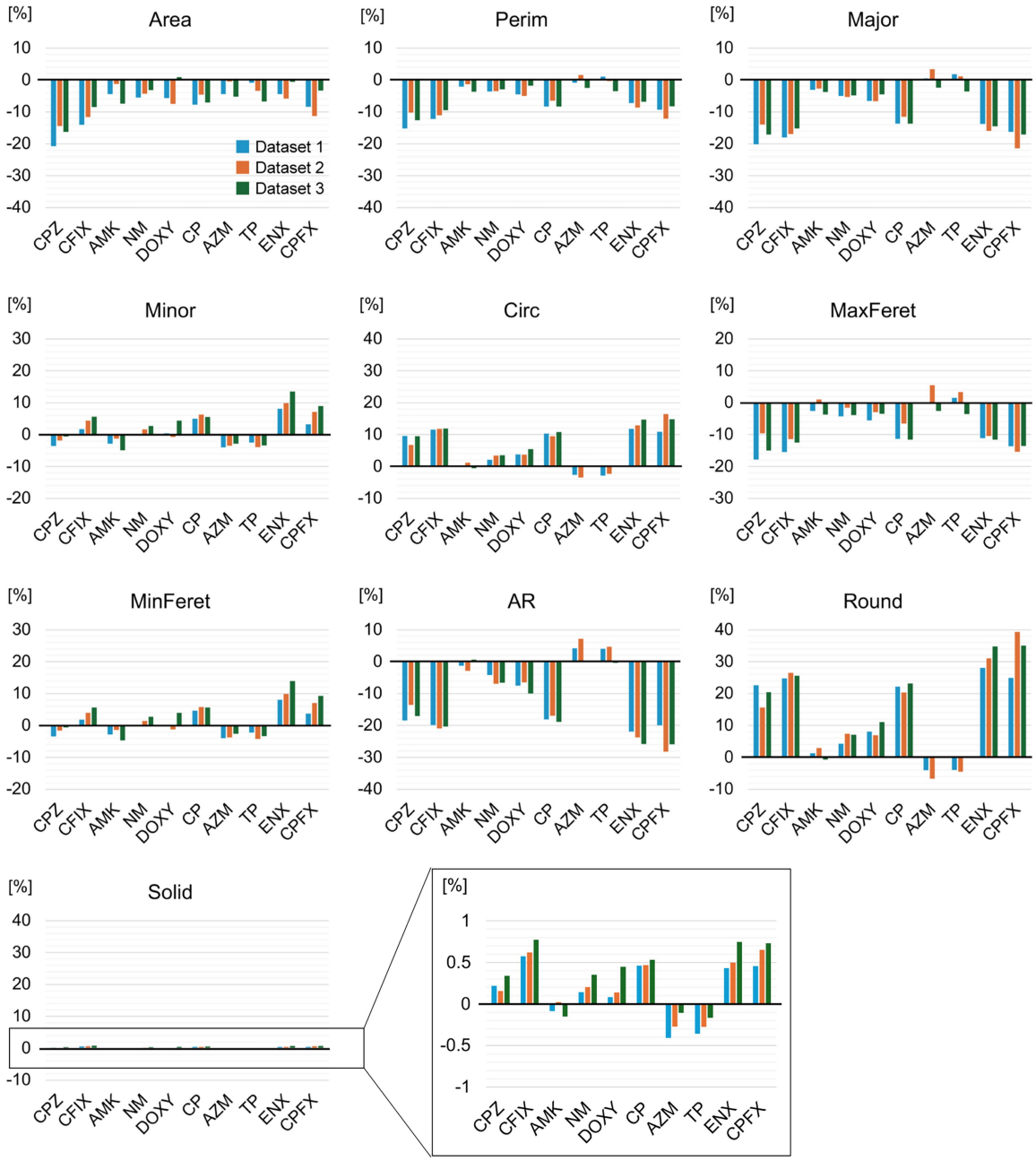

FIGURE 2
Changes in morphology between the parental and antibiotic-resistant strains. The change ratios of the median values for each morphological feature of the indicated drug-resistant strain against the parental strain are shown. The vertical axis represents the percentage change [%]. Values from the three replicate datasets obtained during the experiment are included. The y-axis of the black square box in the Solid graph has been enlarged and is displayed on the right-hand side of the graph. AMK, Amikacin; AZM, Azithromycin; CFIX, Cefixime; CP, Chloramphenicol; CPFX, Ciprofloxacin; CPZ, Cefoperazone; DOXY, Doxycycline; ENX, Enoxacin; NM, Neomycin; TP, Trimethoprim; AR, aspect ratio; Circ, circularity; MaxFeret, maximum Feret's diameter; MinFeret, minimum Feret's diameter; Perim, perimeter; Round, roundness; Solid, solidity.

shape of the cells in each cluster was visualized by calculating the mean and standard deviation of the contour point coordinates.

The results of the clustering analysis are presented in Figure 4 and the numbers of cells in each cluster are provided in Supplementary Table S3. The proportion of the bacterial strain in each cluster is visualized using a pie chart. The parental strain was classified in most clusters; however, it was largely excluded from Cluster-4, and surprisingly, the proportions of QN-, BL-, and CP-resistant strains was larger in this cluster. Notably, the proportions of the TP-, ML-, and tetracycline (TC)-resistant strains in Cluster-4 was very small, suggesting that there might be a peculiar morphological characteristic shared by the QN-, BL-, and CP-resistant strains causing them to be clustered together. In addition, clusters containing the most parental strain included less of the QN-, BL-, and CP-resistant strains. However, the presence of both the TP-and ML-resistant strains coincided with that of the

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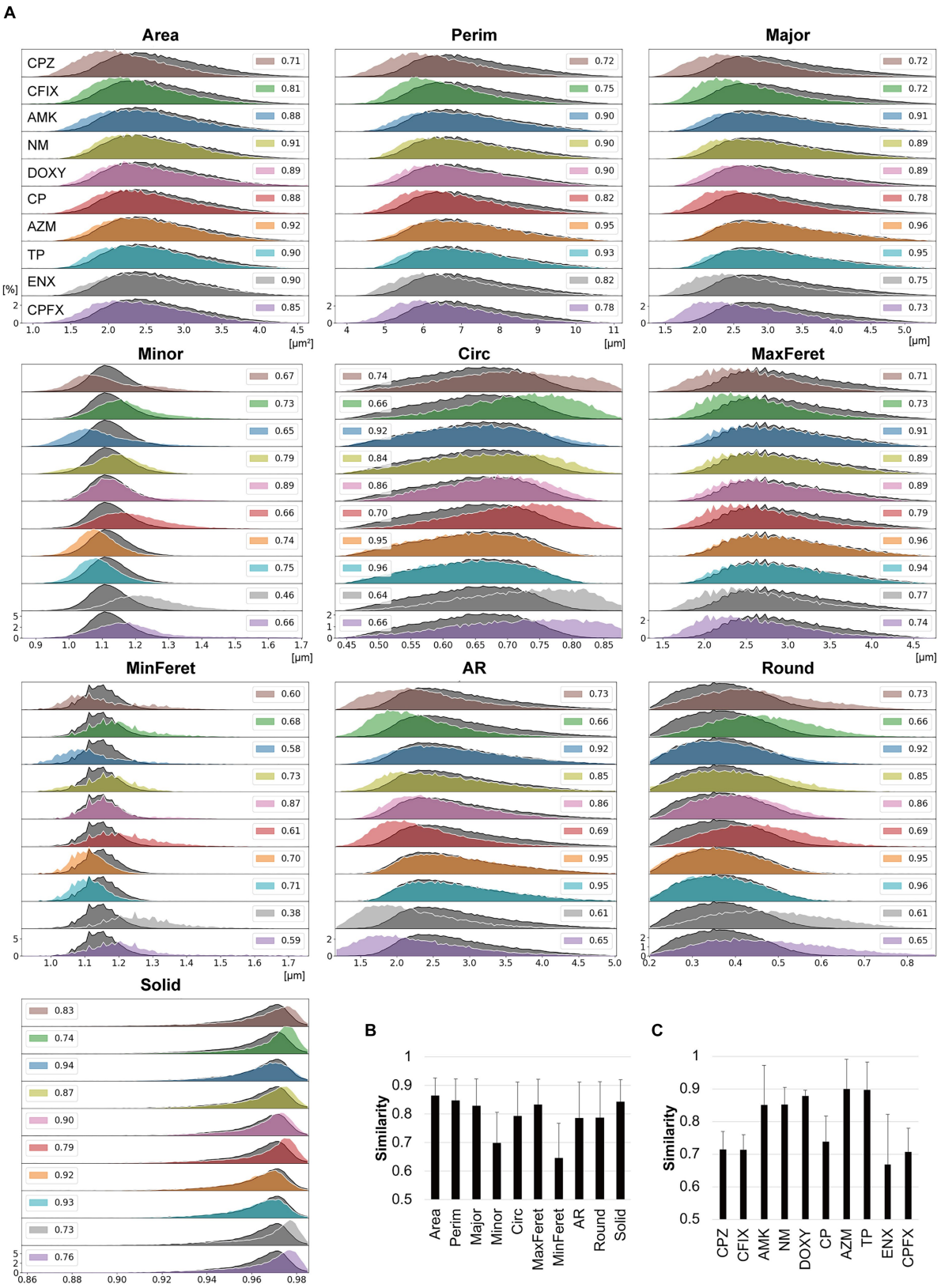

FIGURE 3
Comparisons between the morphological features of parental and antibiotic-resistant strains using histogram intersections. **(A)** Histograms for each morphological feature measured in the antibiotic-resistant strains are shown in color and outlined in white. The histograms of the parental strain are overlayed in dark gray with black outlines. The numbers provided in the insets indicate the mean similarity values between the parental and resistant strain calculated from the histogram intersections. The horizontal axis indicates the unit value of the measured parameter, and the vertical axis

(Continued)

FIGURE 3 (Continued)
indicates the frequency [%]. **(B,C)** Mean similarity between the parental and antibiotic-resistant strains for each morphological parameter (**B**) and between the parental strain and each antibiotic-resistant strain **(C)** calculated using the histogram intersections. The error bars indicate standard deviation. AMK, Amikacin; AZM, Azithromycin; CFIX, Cefixime; CP, Chloramphenicol; CPFX, Ciprofloxacin; CPZ, Cefoperazone; DOXY, Doxycycline; ENX, Enoxacin; NM, Neomycin; TP, Trimethoprim; AR, aspect ratio; Circ, circularity; MaxFeret, maximum Feret's diameter; MinFeret, minimum Feret's diameter; Perim, perimeter; Round, roundness; Solid, solidity.

parental strain, implying that their morphological characteristics were similar. All clusters contained the aminoglycoside (AG)-resistant strain in similar proportions. The results of the PCA suggested that the Minor and MinFeret features in Cluster-4 were most strongly correlated with the first principal component, while Round was correlated with the second principal component, which was in agreement with the morphological characteristics found in this cluster. The average shapes of the cells differed in each cluster, suggesting that the various cell morphologies of the different bacterial strains were classified into distinct clusters. For example, the average shapes of the cells in Cluster-2, -4, and -6 were rod-like, with different widths or lengths. Cells were fattest in Cluster-4. Cells in Cluster-1, -3, and -5 were elongated and slightly concave around the center. The PCA showed the correlation with Solid was strongest in these three clusters, suggesting that they might contain cells at the onset of division.

The proportions of the six clusters for each bacterial strain were also examined (Supplementary Figure S4). The results revealed large proportions of the parental strain were made up of Cluster-1 and Cluster-6 cells, and a similar tendency was seen in both the TP-and ML-resistant strains. However, the other resistant strains contained smaller proportions of these two clusters. Interestingly, although very small proportions of the parental strain, TP-, and ML-resistant strains were comprised of Cluster-4 and -5, larger proportions of the other resistant strains, particularly the QN-, CP-, and BL-resistant strains were composed of these clusters. The proportion of Cluster-2 cells in most of the resistant strains was slightly larger than in the parental strain.

Genes correlated with morphological characteristics in the antibiotic-resistant strains

The results of the cluster analysis revealed an increased proportion of fatter or shorter cells in the resistant strains and suggested that they contained a subpopulation of cells with morphological characteristics different to those of the parental strain. Therefore, we investigated the genes associated with the morphological characteristics of the resistant strains.

A WGCNA (Langfelder and Horvath, 2008) was performed to determine the correlation between gene expression (Suzuki et al., 2014) and the morphological features analyzed in this study. As a result, six groups (modules) of genes were found, each of which was most highly correlated with the corresponding morphological feature (Figure 5A). Intermediate results and details of the WGCNA are presented in Supplementary Figure S5 and Supplementary Table S4. A list of genes contained in the six modules is given in Supplementary Table S5.

GO enrichment analysis (Ashburner et al., 2000; Thomas et al., 2022; Aleksander et al., 2023) for the term biological processes in the six modules was performed to determine the tendencies that affected resistant strain morphology. Among the significantly enriched GO terms (FDR < 0.05), the top 10 GO terms with the highest gene ratios are shown in Figure 5B. The observed tendencies in the GO enrichment results suggested that the genes highly correlated with some morphological features were related to cellular energy metabolism. For example, the GO terms in the module "greenyellow," which was most highly correlated with Major, MaxFeret, and AR, suggested enrichment in energy metabolism, particularly ATP synthesis. Similarly, the GO terms in the module "pink," which was most highly correlated to Perim, suggested enrichment in cellular processes related to energy reserve metabolism of sugars. Additionally, the GO terms in the module "midnightblue," which was most highly correlated with Solid, suggested enrichment in the regulation of phosphate metabolism and the cellular events associated with cell division. The GO enrichment results from the other two modules, "gray" and "magenta," which were most highly correlated to Area and Circ and Round, respectively, suggested enrichment in specialized cellular processes, such as an adaptive metabolic network to efficiently utilize available nutrients and respond to environmental changes, and ion transport and metabolism, which is essential for specific enzymatic functions and important for bacterial survival and adaptation. Although no GO term was enriched in the module "lightgreen," which was most highly correlated with Min and MinFeret, we focused on this module because it contained genes that are important for antibiotic resistance. In fact, several genes encoding drug efflux pumps or transporters appeared in the module; the *acrA* and *acrB* genes encode proteins that comprise the AcrAB-TolC drug efflux system, known to be a major cause of both intrinsic and acquired resistance to many compounds, including antimicrobials (Okusu et al., 1996), the *mdtG* gene encodes a multidrug efflux pump thought to be involved in resistance to fosfomycin (Nishino and Yamaguchi, 2001), and the *mdlA* and *mdlB* genes encode proteins annotated as putative multidrug resistance-like exporters (Saier et al., 2016). In addition, *acrR*, *soxS*, *soxR*, and *rob,* which are thought to play roles in the regulation of expression of these transporter genes (Ma et al., 1996; Seo et al., 2015; Blanco et al., 2016) appeared in the same module (Table 3).

Overall, these results strongly suggested that the morphological features of the resistant strains were correlated with changes in the expression of genes involved in cellular energy metabolism and multidrug resistance.

Single-cell classification between the parental and resistant strains

Recently, deep learning approaches have been applied to microscopy images of bacterial cells and cutting edge algorithms have been developed for automatic cell segmentation, tracking, and antimicrobial susceptibility testing (Lugagne et al., 2020; Cutler et al.,

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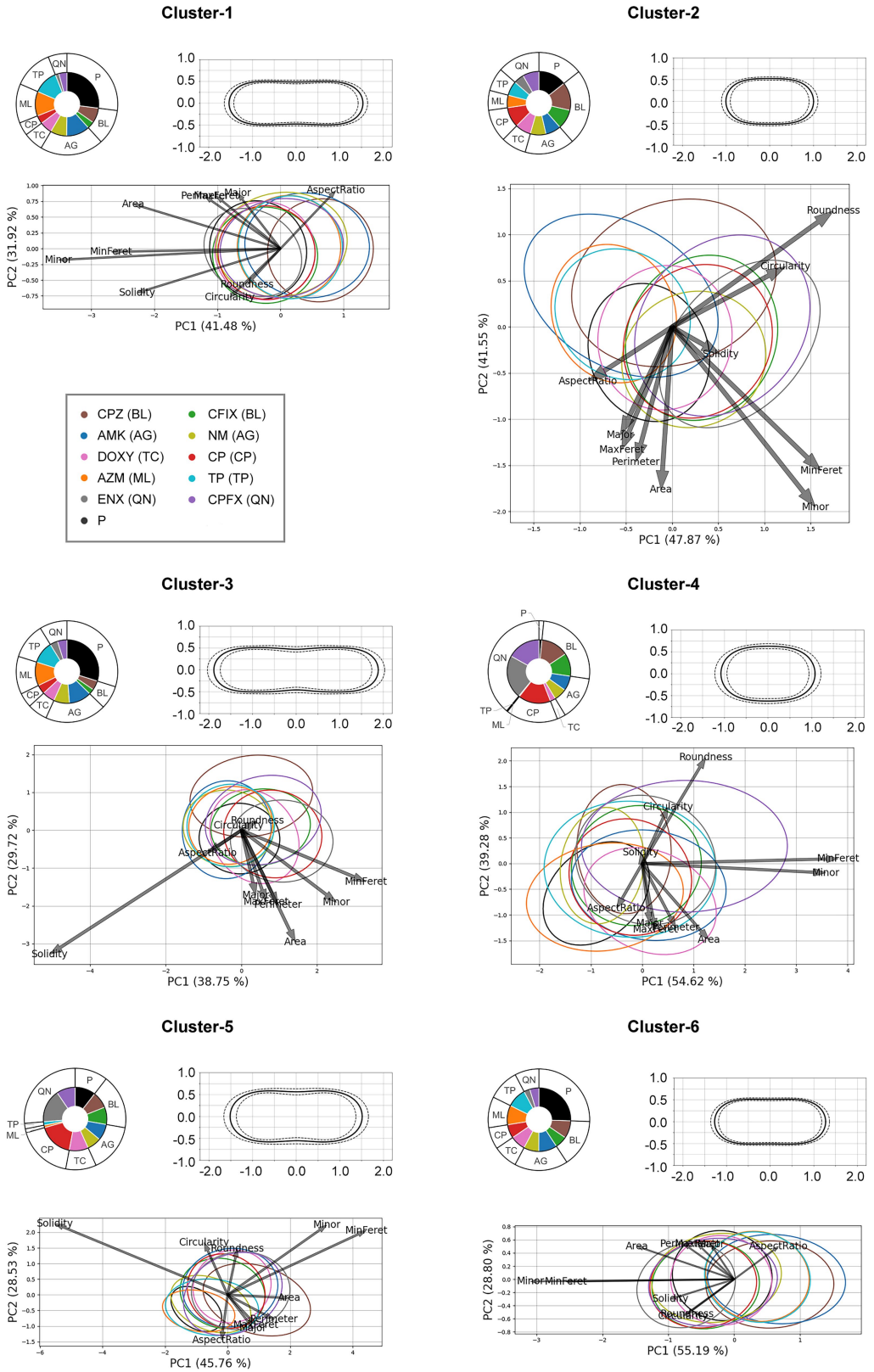

FIGURE 4
The results of k-means clustering and principal component analysis (PCA) of each cluster. *Upper left panel for each cluster*: The pie charts indicate the proportion of the cluster occupied by each bacterial strain. *Upper right panel for each cluster*: The average shape of the cells in the cluster is shown; the solid line represents the mean and the dotted lines represent the standard deviation. The vertical and horizontal axes are in μm. *Bottom panel for each cluster*: The results of the PCA for each cluster are shown. The ellipse contains approximately 68% of the cells from each strain. The arrows represent the loadings of each feature. P, the parental strain; BL, β-lactam-, AG, aminoglycoside-, TC, tetracycline-, CP, chloramphenicol-, ML, macrolide-, TP, trimethoprim-, QN, quinolone-resistant strains; MaxFeret, maximum Feret's diameter; MinFeret, minimum Feret's diameter.

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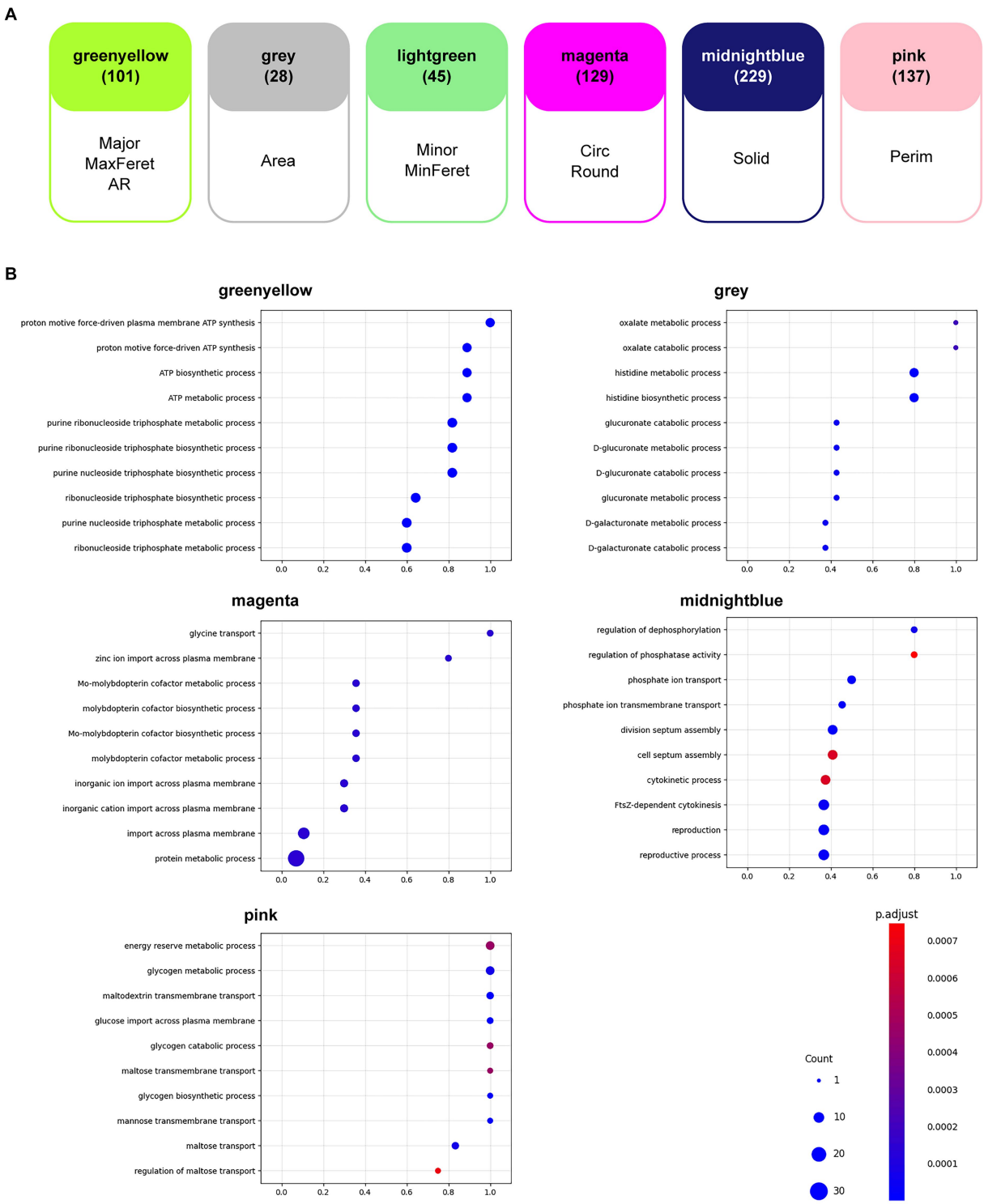

FIGURE 5
Weighted gene correlation network analysis (WGCNA). **(A)** Gene modules that were most highly correlated with the morphological features analyzed in this study are shown. The number of genes found in each module is shown in parentheses. AR, aspect ratio; Circ, circularity; MaxFeret, maximum Feret's diameter; MinFeret, minimum Feret's diameter; Perim, perimeter; Round, roundness; Solid, solidity. **(B)** The results of the gene ontology (GO) enrichment analyses for each of the six gene modules are shown in the bubble charts. The horizontal axis of each chart represents the gene ratio, the size of the bubbles represents the number of genes, and the color of the bubbles represents the false discovery rate (FDR)-adjusted *p*-value.

2022). However, to the best of our knowledge, no computational methods for identifying drug-resistant bacteria without using drugs have been investigated to date. As the parental and drug-resistant strains presented different cell morphologies even in the absence of drugs, we investigated whether the resistant strains were discernible from the parental strain using deep learning approaches.

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TABLE 3 List of genes in the module "lightgreen".[a]

Gene	Description
yaaY	DUF2575 domain-containing protein YaaY
lpxC	UDP-3-O-acyl-N-acetylglucosamine deacetylase
dgt	dGTP triphosphohydrolase
panE	2-dehydropantoate 2-reductase
ybaO	DNA-binding transcriptional activator DecR
mdlA	ABC transporter family protein MdlA
mdlB	ABC transporter family protein MdlB
acrB	Multidrug efflux pump RND permease AcrB
acrA	Multidrug efflux pump membrane fusion lipoprotein AcrA
acrR	DNA-binding transcriptional repressor AcrR
rnk	Nucleoside diphosphate kinase regulator
lipA	Lipoyl synthase
fur	DNA-binding transcriptional dual regulator Fur
fldA	Flavodoxin 1
seqA	Negative modulator of initiation of replication
ssuA	Aliphatic sulfonate ABC transporter periplasmic binding protein
pqiA	Intermembrane transport protein PqiA
pqiB	Intermembrane transport protein PqiB
ymbA	Intermembrane transport lipoprotein PqiC
mdtG	Efflux pump MdtG
ribA	GTP cyclohydrolase 2
nhoA	Arylamine N-acetyltransferase
fumC	Fumarase C
zwf	$NADP^+$-dependent glucose-6-phosphate dehydrogenase
nfo	Endonuclease IV
yeiI	Putative sugar kinase YeiI
ypeB	PF12843 family protein YpeB
ligA	DNA ligase
fldB	Flavodoxin 2
ygfZ	Folate-binding protein YgfZ
yggX	Putative Fe^{2+}-trafficking protein
mltC	Membrane-bound lytic murein transglycosylase C
kdsC	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase KdsC
yicM	Purine ribonucleoside exporter
yieP	DNA-binding transcriptional dual regulator YieP
frvX	Peptidase M42 family protein FrvX
sodA	Superoxide dismutase (Mn)
kdgT	2-dehydro-3-deoxy-D-gluconate:H^+ symporter
fpr	Flavodoxin/ferredoxin-$NADP^+$ reductase
soxS	DNA-binding transcriptional dual regulator SoxS
soxR	DNA-binding transcriptional dual regulator SoxR
ryjA	Small RNA RyjA
yjjW	Putative glycyl-radical enzyme activating enzyme YjjW
yjjI	DUF3029 domain-containing protein YjjI
rob	DNA-binding transcriptional dual regulator Rob

[a]Genes appearing in the module "lightgreen," which was most highly correlated with the parameters Minor and MinFeret are listed in ascending order of the gene annotation number (Blattner et al., 1997).

We proposed a neural network based on the ResNet architecture and built by replacing all of the two-dimensional convolutions with circular convolutions (Figure 6A). In our preliminary experiments, we employed the original ResNet models for patch classification and found that the classification performance worsened between the training and test phases (Supplementary Figure S6; Supplementary Table S6). These results suggested that the image-based models were substantially affected by the inconsistency in the characteristics seen in the microscopy images between the training and test phases. In the present study, we used cell regions from which the coordinates of contour points were extracted and interpolated as inputs to the ResNet architecture (Figure 6A; Supplementary Table S7) and demonstrated that our contour-based models achieved higher classification performance without suffering from such inconsistency (Figure 6B). As shown by the results of the AUC obtained from the test sets, our classifier models achieved high performance for ENX-, CPFX-, CPZ-, CP-, and AMK-resistant strains (Figure 6C). In addition, the sensitivity, specificity, and accuracy results, defined as Equations 1–3 respectively (see Materials and Methods), showed that the classification performance of our models was higher for specificity than sensitivity in all resistant strains, suggesting that there were fewer false positives than false negatives (Figure 6C). Our classification models showed high performance for sensitivity in the ENX-and CPZ-resistant strains. One of the reasons for the lower performance for sensitivity compared with specificity was that many resistant cells were of a similar shape to the parental cells, which may have made it more difficult for classifier models to learn the differences in shape between resistant and parental cells, therefore increasing the false negatives in the test phases.

Discussion

Previous studies on bacterial drug resistance have generally focused on changes in the resistance potential caused by specific gene mutations in drug-resistant cells. Similarly, morphological changes associated with particular genes have been described (Hirota et al., 1968; Wachi et al., 1987). In recent years, the analysis of transcriptional data has revealed that the expression patterns of many genes are altered in laboratory-evolved antibiotic-resistant cells, and it is becoming possible to predict drug-resistant bacteria from gene expression profiles (Suzuki et al., 2014; Maeda et al., 2020). Because the expression patterns of multiple genes are altered in antibiotic-resistant strains, it is plausible that these changes may have a complex effect on the morphology of bacterial cells. Studies on the relationship between antibiotic resistance and bacterial morphology have included observations of bacterial cells treated with drugs, and a recent study suggested that changes in bacterial cell shape are important for adaptation to antibiotics and drug resistance (Ojkic et al., 2022). However, the morphology of resistant bacteria in the absence of antibiotics is not well known.

In this study, we showed that the antibiotic-resistant *E. coli* strains that evolved in the laboratory maintained their morphological changes even in the absence of drugs. The strains that evolved more prominent changes in their cell morphology showed higher resistance to the corresponding drug (Supplementary Figure S1), but the relationship between the degree of morphological change and the minimum inhibitory concentration values require further investigation.

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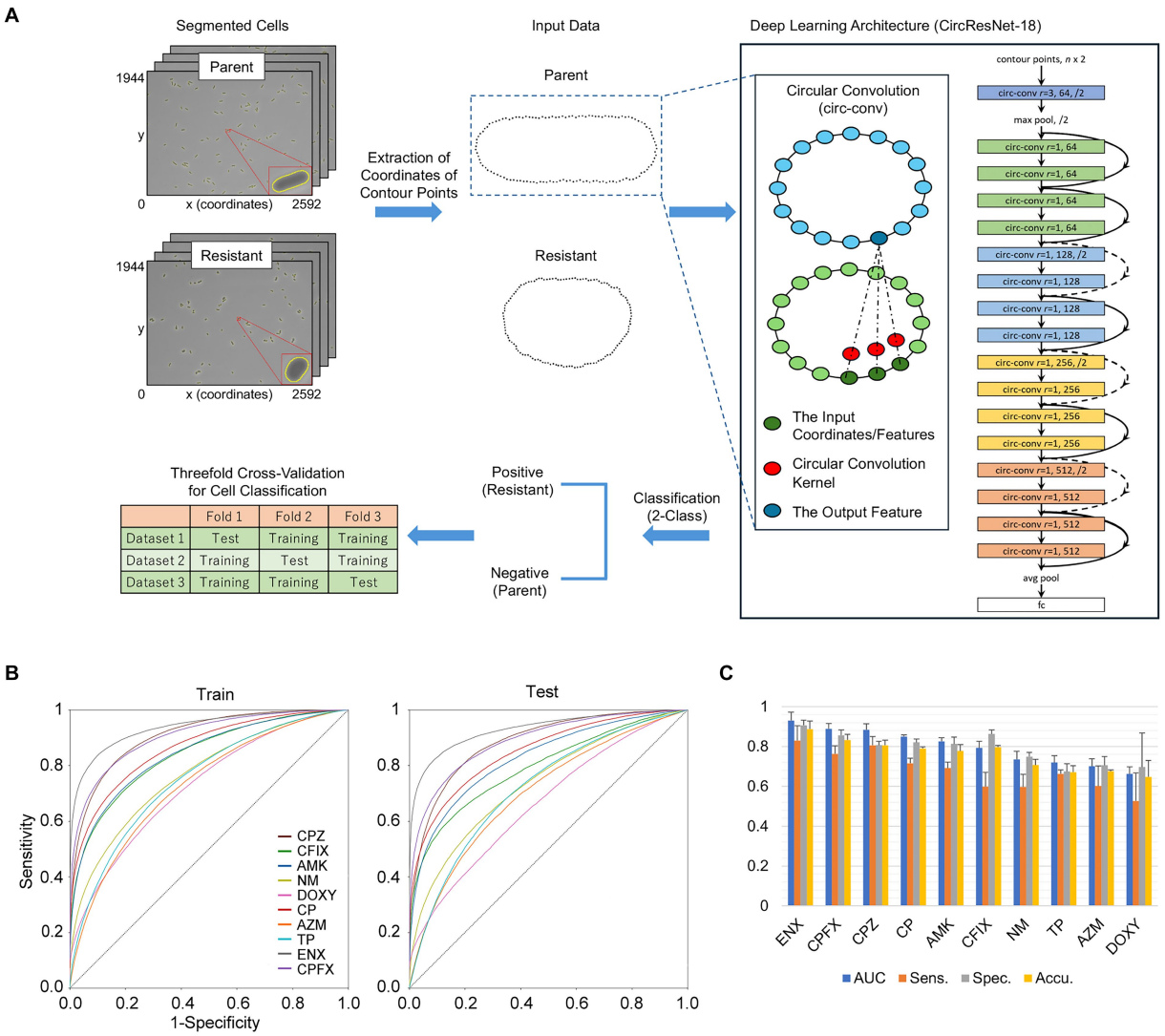

FIGURE 6
Single-cell classification of parental and antibiotic-resistant cells using deep neural networks. **(A)** The classification workflow. The insets show enlarged views of the contours of the cell region extracted from microscopy images of the parental and resistant (ENX) strains. The corresponding cell contours aligned horizontally and evenly interpolated to 128 points are shown as these formed the input data. A cartoon of the deep learning architecture shows a circular convolution (circ-conv) integrated into the ResNet architecture. For the circ-conv layer, r represents the radius or neighborhood size, resulting in a kernel size of $2r+1$, followed by the number of channels and optionally a stride. The circ-conv layers are followed by batch normalization and rectified linear unit functions. The curved arrows depict a shortcut connection, and the dashed lines indicate the increase in the number of channels. The term fc stands for a fully-connected layer. Threefold cross-validation was conducted for evaluation of the classifier models. **(B)** The receiver operating characteristic (ROC) curve for the contour classification. Each curve shows an average of the ROC curves obtained from the threefold cross-validation. The vertical and horizontal axes represent the values of sensitivity and 1 – specificity, respectively. **(C)** Performance of the network in classifying resistant strains. The classification results (mean values) from the test sets in the threefold cross-validation are presented as bar graphs with standard deviations. The resistant strains are listed in descending order of the area under the curve (AUC). Sens., sensitivity (correctly classified resistant cells); Spec., specificity (correctly classified parental cells). Accu., accuracy (collectly classified parental and resistant cells); AMK, Amikacin; AZM, Azithromycin; CFIX, Cefixime; CP, Chloramphenicol; CPFX, Ciprofloxacin; CPZ, Cefoperazone; DOXY, Doxycycline; ENX, Enoxacin; NM, Neomycin; TP, Trimethoprim; AR, aspect ratio; Circ, circularity; MaxFeret, maximum Feret's diameter; MinFeret, minimum Feret's diameter; Perim, perimeter; Round, roundness; Solid, solidity.

Previous reports have indicated that there is a relationship between cells with smaller morphological features, such as persister cells, and drug tolerance (Shah et al., 2006; Lewis, 2010). A relationship between the morphological characteristics of the drug-resistant *E. coli* strains and the genes involved in cellular energy metabolism, cell division, and antibiotic resistance is suggested. The drug-resistant *E. coli* strains have a slower growth rate than the parental strain under antibiotic-free conditions (Suzuki et al., 2014), and this growth characteristic was observed in the present study (Supplementary Figure S7). We observed a similar morphology in resistant strains across different antibiotic mechanisms (QN, BL, CP), suggesting a potential common bacterial survival response. We identified genes highly correlated with morphological features that exhibited significant expression changes in drug-resistant *E. coli* strains (Supplementary Table S8). Notably, *ompF*

was present in strains resistant to QN, BL, and CP. Given that *ompF* significantly contributes to drug resistance in laboratory-evolved *E. coli* (Suzuki et al., 2014; Maeda et al., 2020), it is possible that this gene is also associated with both cell morphology and survival response observed in strains resistant to these antibiotics. Considering that some of the genes that regulate persister formation also appeared in the group of genes that were correlated with some of the morphological features measured in this study (Supplementary Table S5), it is possible that changes in the expression of genes that play a role in cellular functions such as those described in this study may have a complex effect on the morphology of drug-resistant strains under long-term antibiotic stress, and this morphology may be maintained in subpopulations even after removal of the stress. The resistant strains used in the present study were obtained through long-term exposure to the corresponding antibiotics, and when the morphological changes took place is currently unknown; moreover, the mechanism by which subpopulations with revised cell morphologies are generated from single colonies of drug-resistant strains is unclear. They may be generated through uneven division of cells that are similar in shape to the parental strain, or possibly cells with distinct morphology are preferable and therefore maintained as the subpopulation.

It should be noted, however, that the interpretation of the results is limited by the sole use of experimentally-evolved strains and morphological information obtained from light microscopy images; the internal structures of most of these resistant cells have yet to be studied. Future investigations are required to clarify whether the acquisition of drug resistance is generally associated with changes in cell morphology and what genes are responsible, using various drug-resistant bacterial strains obtained under other environmental conditions such as clinical isolates.

Finally, our proposed single-cell classification demonstrated a high level of performance in characterizing some of the drug-resistant strains. The classification results generally coincided with the findings from the morphological analysis in that the accuracy in discrimination between the parental and resistant strains reflected both the similarities in cell shape and the minimum inhibitory concentration values. A future challenge will be to develop an algorithm that enables the classification of cells in a heterogenous population.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

MI: Formal analysis, Investigation, Methodology, Writing – original draft. KA: Formal analysis, Investigation, Methodology, Writing – original draft. MH-N: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft. CF: Conceptualization, Resources, Writing – review & editing. KN: Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Towards harmonized laboratory methodologies in veterinary clinical bacteriology: outcomes of a European survey

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Introduction: Veterinary clinical microbiology laboratories play a key role in antimicrobial stewardship, surveillance of antimicrobial resistance and prevention of healthcare associated-infections. However, there is a shortage of international harmonized guidelines covering all steps of veterinary bacterial culture from sample receipt to reporting.

Methods: In order to gain insights, the European Network for Optimization of Veterinary Antimicrobial Treatment (ENOVAT) designed an online survey focused on the practices and interpretive criteria used for bacterial culture and identification (C&ID), and antimicrobial susceptibility testing (AST) of animal bacterial pathogens.

Results: A total of 241 microbiology laboratories in 34 European countries completed the survey, representing a mixture of academic (37.6%), governmental (27.4%), and private (26.5%) laboratories. The C&ID turnaround

varied from 1 to 2 days (77.8%) to 3–5 days (20%), and 6– 8 days (1.6%), with similar timeframes for AST. Individual biochemical tests and analytical profile index (API) biochemical test kits or similar were the most frequent tools used for bacterial identification (77% and 56.2%, respectively), followed by PCR (46.6%) and MALDI-TOF MS (43.3%). For AST, Kirby-Bauer disk diffusion (DD) and minimum inhibitory concentration (MIC) determination were conducted by 43.8% and 32.6% of laboratories, respectively, with a combination of EUCAST and CLSI clinical breakpoints (CBPs) preferred for interpretation of the DD (41.2%) and MIC (47.6%) results. In the absence of specific CBPs, laboratories used human CBPs (53.3%) or veterinary CBPs representing another body site, organism or animal species (51.5%). Importantly, most laboratories (47.9%) only report the qualitative interpretation of the result (S, R, and I). As regards testing for AMR mechanisms, 48.5% and 46.7% of laboratories routinely screened isolates for methicillin resistance and ESBL production, respectively. Notably, selective reporting of AST results (i.e. excluding highest priority critically important antimicrobials from AST reports) was adopted by 39.5% of laboratories despite a similar proportion not taking any approach (37.6%) to guide clinicians towards narrower-spectrum or first-line antibiotics.

Discussion: In conclusion, we identified a broad variety of methodologies and interpretative criteria used for C&ID and AST in European veterinary microbiological diagnostic laboratories. The observed gaps in veterinary microbiology practices emphasize a need to improve and harmonize professional training, innovation, bacterial culture methods and interpretation, AMR surveillance and reporting strategies.

KEYWORDS

veterinary clinical bacteriology, bacterial culture, bacterial identification, antimicrobial susceptibility testing, harmonization, methodologies, ENOVAT

Introduction

Microbiological diagnostic laboratories play an important role in antimicrobial stewardship, since results may impact the decision to treat with an antimicrobial agent and the drug selected. Optimization and standardization of the diagnostic process, including all steps from sample collection to reporting of the results, are key factors to obtain reproducible and reliable results that can support evidence-based therapeutic decisions by clinicians.

In human clinical microbiology, several international manuals (UK Standards for Microbiology Investigations [UK SMIs], 2023) and standards have existed for many years. For instance, the ISO 15189 standard developed by the International Organization for Standardization that specifies requirements for quality and competence in medical laboratories is recognized and implemented throughout the world (ISO 15189:2022(en), 2022). The ISO/IEC 17025:2017 standard (ISO/IEC 17025:2017(en), 2017) setting the general requirements for the competence of testing and calibration laboratories and Good Laboratory Practice (GLP) policies may be implemented by veterinary microbiology laboratories for demonstration of competency to carry out high quality and accurate laboratory testing. However, with a few notable exceptions such as the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (WOAH, 2023) and standards for AST of veterinary pathogens (CLSI VET02, 2021; CLSI VET01, 2024; CLSI VET01S, 2024; CLSI VET09, 2024), similar standards and guidelines have not been developed specifically for veterinary diagnostic laboratories. Although standards developed for human microbiology can be routinely used by veterinary diagnostic laboratories (Cornaglia et al., 2012; Leber and Burnham, 2023), they are not directly transferable and applicable to veterinary laboratories, for instance due to the existence of animal pathogens with specific growth requirements (Guardabassi et al., 2017).

Adding to this problem, standardized training of veterinary clinical microbiologists is still in its infancy. The establishment of the EBVS recognized European College for Veterinary Microbiology (ECVM)[1] in 2016 was a major step forward that should lead to more well-trained veterinary microbiologists. Also, VetCAST (Toutain et al., 2017; EUCAST, 2015) (subcommittee of EUCAST) and CLSI-VAST (Feßler et al., 2023; Veterinary Antimicrobial Susceptibility Testing [VAST], 2024) (subcommittee of CLSI) are producing veterinary-specific manuals and clinical breakpoints. However, clinical breakpoints for many antimicrobial agent - animal species - body site - pathogen combinations are still lacking and other steps of the diagnostic process beyond AST,

1 www.ecvmicro.org

such as bacterial culture and identification methods, selection of relevant isolates for AST and reporting approaches, remain poorly addressed for veterinary diagnostic laboratories.

Taken together, these shortcomings in veterinary diagnostic microbiology, combined with any technical errors that may conceivably occur in routine bacteriological diagnostics (Rampacci et al., 2021; Cuénod et al., 2023), impose a risk of poor-quality results and potential large differences between laboratories, which may lead to suboptimal treatment and subsequent selection of antimicrobial resistance in animal patients. Furthermore, the lack of surveillance harmonization poses considerable challenges to accurately quantify AMR, understanding its dynamics and implementing effective strategies to mitigate its impact.

As part of the Cooperation in Science and Technology (COST) actions, the European Network for Optimization of Veterinary Antimicrobial Treatment (ENOVAT—CA18217)[2] has been established. A key aim of ENOVAT was to refine and harmonize veterinary microbiological diagnostic procedures. This survey-based ENOVAT study, was intended to gain insight into the current practices used by European diagnostic laboratories, with a focus on the methodologies and interpretative criteria used for bacterial culture and AST of animal clinical specimens. Information about such practices provides the foundation for identifying knowledge gaps and areas to prioritize during future works and to harmonize veterinary microbiology procedures.

Materials and methods

Survey design and distribution

An ethical approval for undertaking an online survey was secured through the University of Liverpool Research Ethics and Integrity Office (VREC958). The survey was designed by a panel of ENOVAT members including veterinary clinicians, microbiologists and epidemiologists representing seven different countries. The survey was designed and distributed via the online tool SurveyMonkey (SurveyMonkey Inc., USA). The survey included 37 questions (Supplementary material), divided into four topics: laboratory information (Section A), methodology related to bacterial culture, identification and susceptibility testing (Section B), results interpretation and reporting (Section C), and surveillance, laboratory data management and further developments (Section D).

Prior to its launch, the survey was piloted among ENOVAT members to pinpoint essential concerns, detect any potentially confusing questions and incorporate additional suggestions obtained during this pilot phase.

The survey was distributed in February 2021 via the ENOVAT Network as a weblink advertised on the ENOVAT webpage, social media and sent via the consortium channels. Country representatives from the ENOVAT Network have been actively involved in dissemination of the survey in their respective countries through engagement with veterinary national bodies, agencies and partners. Participation to the survey was on a voluntary basis, and responses were collected until mid-August 2021.

2 https://enovat.eu/

The presented data have not been adjusted according to parameters like the accurate number of laboratories per participating country, scale of animal production, number of companion animals and other factors. Rather, we aimed to present the raw data gathered from the participating laboratories, as described below. As the number of participating laboratories (Supplementary Table 1 and Figure 1) greatly varied between countries but information on the number of veterinary laboratories in each country is inconsistent, country-specific comparisons and conclusions could not be investigated.

Methodology for inclusion and analysis of complete and partial responses in the survey

Answers for each question were analysed and responses from laboratories providing full and partial answers were used if appropriate. Partial answers were included under the assumption that some participants were either reluctant or unwilling to disclose data, or uncertain of the answer. Working under this assumption, each question was analysed separately. Partial answers were included in the analysis, except when an answer was not in agreement with a previous response. To exemplify the latter scenario, one participant reported failure to participate in external quality assurance programs and despite this selected one of the optional assurance schemes.

Statistical analysis

Survey results were exported with the SurveyMonkey export tool and imported to IBM SPSS Statistics 28.0.1.0 (IBM, USA). Descriptive statistics and figures were generated for each survey response if appropriate. Microsoft Excel (Microsoft, USA) was used for certain data sorting tasks if needed. R programming language was used with the RStudio (Allaire, 2012) software for visualization of certain graphs and figures.

Results

Representatives from 290 laboratories commenced the survey. Nineteen laboratories were automatically excluded, as they declined to consent to participate in the survey (n = 4) or indicated that they did not offer bacterial culture and AST (n = 15). An additional 30 participants were excluded as they did not answer any further questions despite completing the inclusion criteria. Overall, 241 respondents completed the survey, at least partially; this variability in the number of responses was acceptable as not all questions relating to methodologies or available facilities were applicable to all respondents.

Respondents were from laboratories in a variety of European and two neighbor countries (i.e. Morocco and Israel), most frequently the United Kingdom (27/241, 11.2%), France (26/241, 10.7%), Croatia (15/241, 6.2%), Italy (15/241 6.2%), Germany (13/241, 5.4%) and Romania (11/241, 4.6%) (Figure 1).

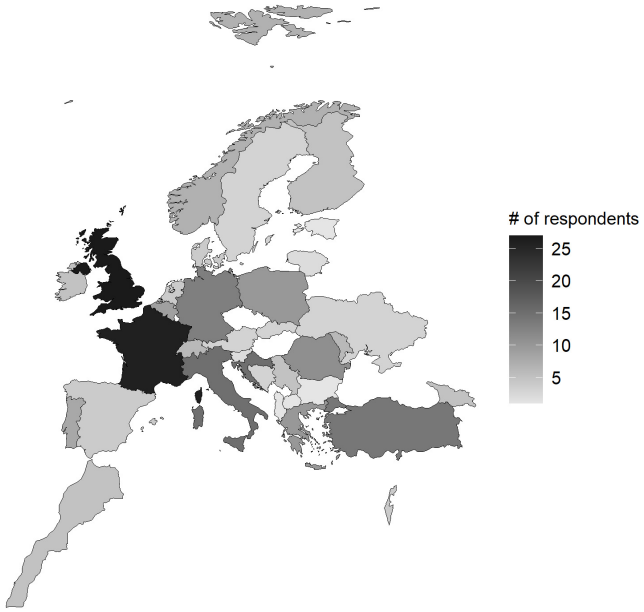

FIGURE 1
Geographic distribution of survey responders across Europe.

The complete list of participants by country is available in Supplementary Table 1.

Section A: laboratory information

An overview of responses to questions concerning laboratory settings and processes is provided in Supplementary Table 2, and a summary is provided in the following text. Most laboratories were situated in academic settings (37.6%), followed by governmental (27.4%) and private (26.5%) settings (Supplementary Table 2). Within the private sector, most laboratories were commercial (65.6%) followed by in-house veterinary practice/hospital laboratories (26.2%). The majority of participating laboratories (90.7%) processed clinical samples from animals, whereas 23.1% and 18.5% processed food or feed (i.e. animal derived food for human consumption or animal feed) and environmental samples, respectively. The majority of laboratories in all settings processed fewer than 3,000 samples per year (Figure 2).

In most laboratories, the microbiology diagnostics team consisted of technical staff (84.9%) and veterinary microbiologists (77.3%). More than half of the laboratories were headed by a veterinary microbiologist (55.7%), followed by a veterinarian/clinician (13%) and microbiologist of non-veterinary background (9.2%) (Supplementary Table 2). The free text responses included in the "Other" category (7%) indicated diverse backgrounds for this leader role, such as veterinary parasitologist, pathologist, engineer, chemist, or financial manager.

Most laboratories (75.1%) provide guidance for optimal specimen collection and management via various routes including: telephone (72.3%), e-mail (57.2%), website information (47.2%) and sample submission forms (41.5%) (Supplementary Table 2).

Quality assurance (QA) was by far less common in academic laboratories compared to laboratories in other settings (Supplementary Table 2). As for the type of QA, 70.5% of all laboratories indicated that they conducted internal QA, primarily in the form of Standard Operating Procedures (90.1%), equipment maintenance and calibration (85.6%), and use of quality control strains (85.6%). Participation in external QA was less common (59.6%) and occurred mostly by taking part in national proficiency testing (76.6%), accreditation from a recognized QA system (e.g. ISO) (68.5%), and external audits (67.6%).

Most of the laboratories reported 1–2 days turnaround time for both bacterial culture and identification (77.8%) and AST (62.7%). Longer turnarounds were less commonly reported by laboratories for both C&ID (20% and 1.6% for 3–5 and 6–8 days, respectively) and AST (32.4% for 3–5 and 4.3% for 6–8 days, respectively).

Section B. Methodology (bacterial culture, identification and susceptibility testing)

An overview of responses to questions concerning methodology used by laboratories is provided in Figure 3 and Supplementary Table 3. Nearly all laboratories (97.8%) offered aerobic culture, followed by anaerobic (89.3%), microaerophilic (77.0%), and 5–10% CO_2 enriched culture (71.3%). Only 41.6% of laboratories provided selective culture for one or more of the following target species/phenotypes: *Salmonella* spp., methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus pseudintermedius* (MRSP), *Campylobacter* spp., *Listeria* spp., *Yersinia* spp., *Bartonella* spp., *Brucella* spp., *Brachyspira* spp., and *Dermatophilus* spp.

Only three laboratories (1.7%) reported that they do not attempt to identify bacterial isolates at species level, whilst others performed this in all (57.3%), most (28.7%) or some (12.4%)

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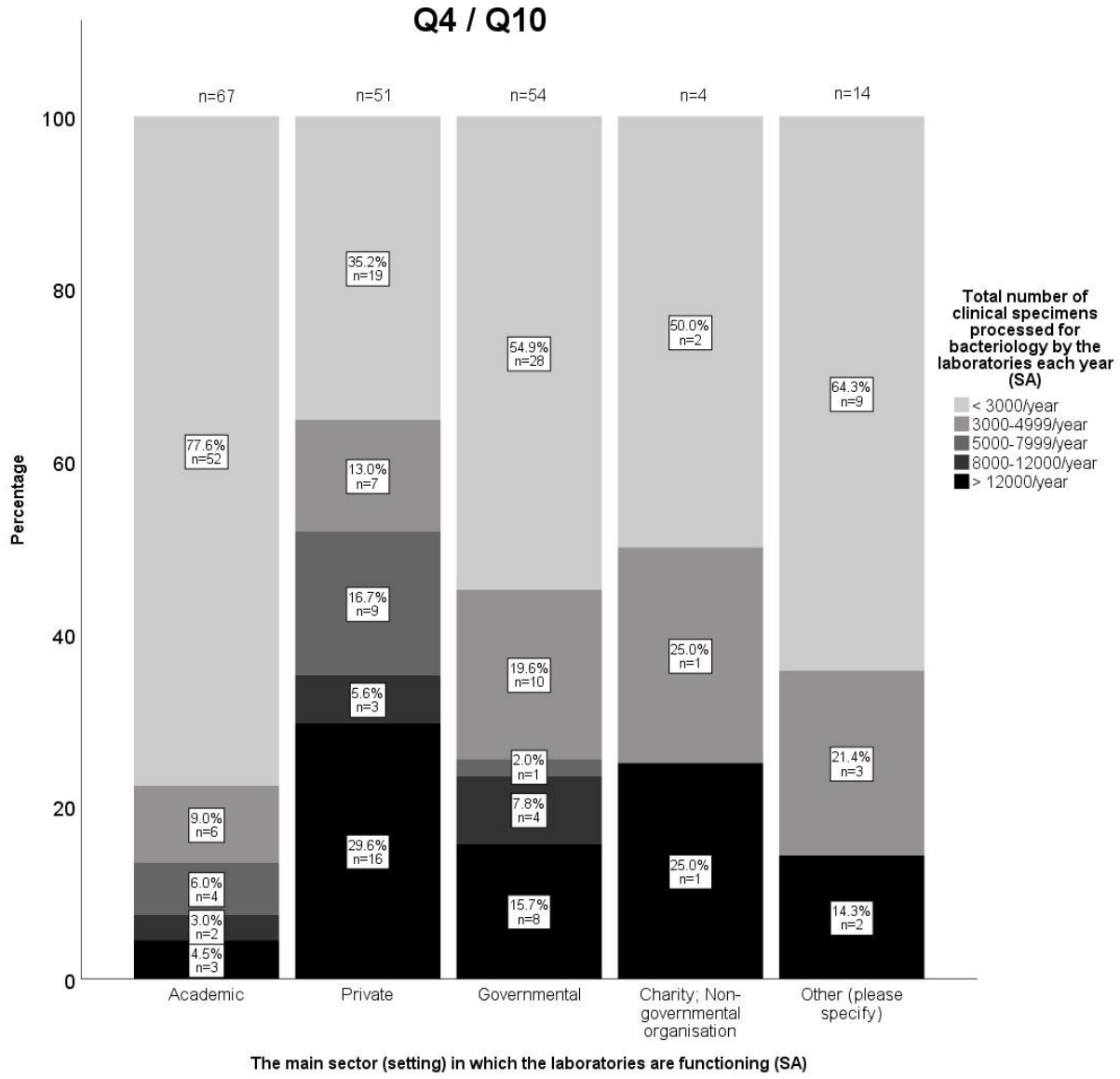

FIGURE 2
Number of clinical specimens processed for bacteriology by the laboratories based on the main sector in which they are functioning.

cases. Individual biochemical assays (e.g. catalase, oxidase) for bacterial identification were employed in 77% of laboratories, followed by API kits or similar (56.2%), PCR (46.6%), MALDI-TOF MS (43.3%), and VITEK 1 or the VITEK 2 automated ID/AST instruments (25.3%) (Supplementary Table 4).

For AST, by default most laboratories (43.8%) reported using Kirby-Bauer disk diffusion (DD), followed by minimum inhibitory concentration (MIC) determination (32.6%). In addition, some laboratories only provide DD (28.7%) or MIC (15.2%) results upon request. The most commonly used approach to perform MIC testing was VITEK automated ID/AST instruments (39.6%), followed by in-house broth microdilution (30.7%), Trek Sensititre (27.7%), and gradient test strips (26.7%) (Supplementary Table 4).

More laboratories reported using a combination of EUCAST and CLSI clinical breakpoints (CBPs) for interpreting AST results than using solely EUCAST or CLSI breakpoints. This trend was observed for both DD (41.2%) and MIC testing (47.6%). In the absence of animal species - body site—pathogen specific CBPs for one or more antimicrobials, AST interpretation most commonly employed human-specific CBPs (53.3%) or veterinary CBPs for another body site, organism or animal species (51.5%). Less commonly, laboratories would report no interpretation (30.9%) or test not possible/not performed (22.4%). Alternatively, they would use epidemiological cut-offs (ECOFFs) (21.2%) (Supplementary Table 4).

Most laboratories reported screening for MRSA/MRSP (48.5%) and ESBL-producing *Enterobacterales* (46.7%), followed by pAmpC (36.4%), carbapenemase-producing *Enterobacterales* (CPE) (32.1%), inducible clindamycin resistance in Gram-positive bacteria (26.7%) and vancomycin-resistant *Enterococcus* (VRE) species (20.0%) (Figure 4 and Supplementary Table 6). Detection of these resistance mechanisms was mostly performed by DD followed by MIC and molecular testing. The use of chromogenic

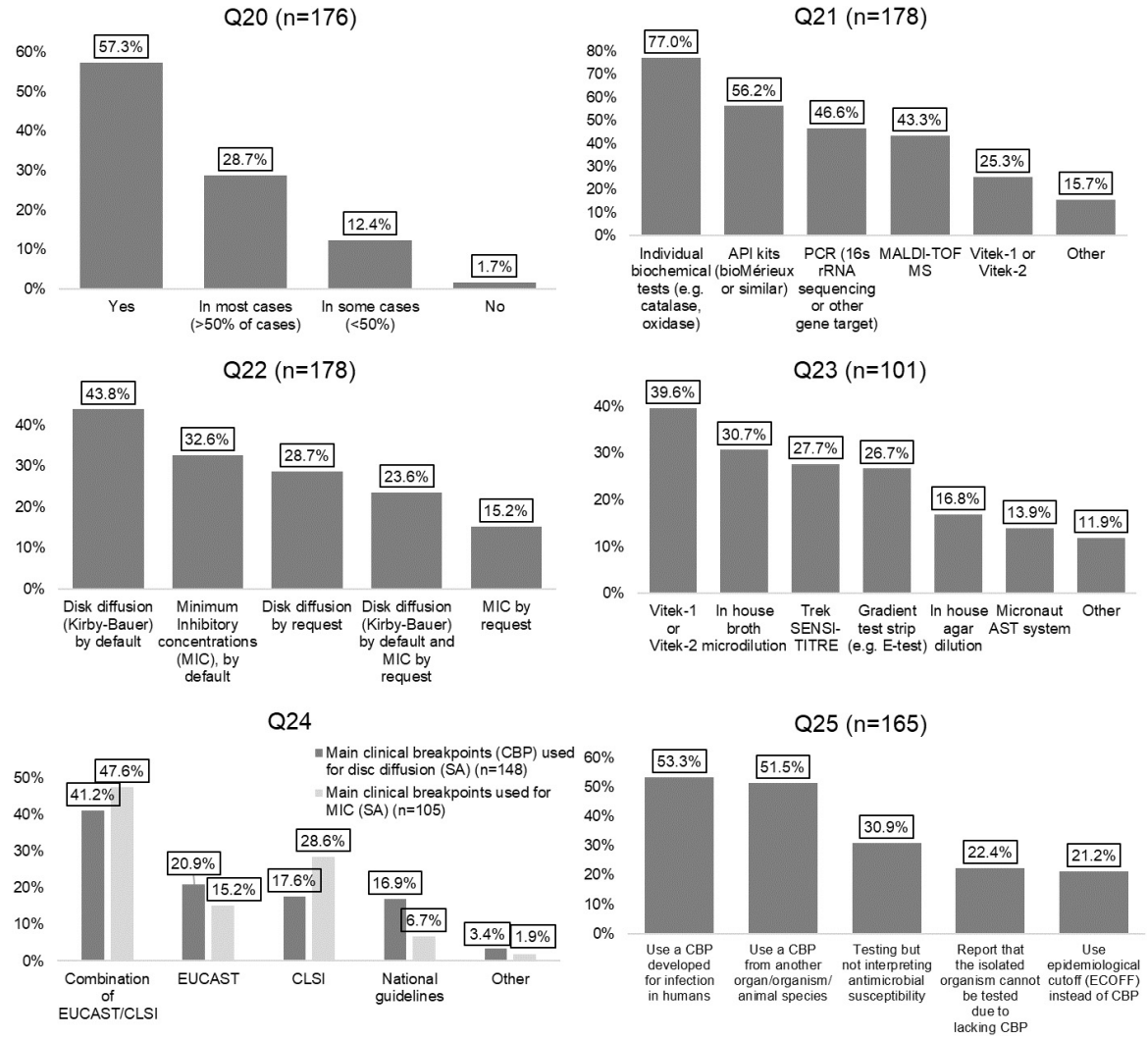

FIGURE 3
Figures for questions Q20–Q25. Responses regarding the methodology employed by the participating laboratories for bacterial culture and antimicrobial susceptibility testing. Percentages are based on the number of responses to individual questions. MA, multiple answer question; SA, single answer question. Q20–Identification to species level (SA); Q21–Methods for bacterial identification (MA); Q22–AST method provided (MA); Q23–MIC method provided (MA); Q24-1–Main clinical breakpoints (CBP) used for disc diffusion (SA), Q24-2–Main clinical breakpoints used for MIC (SA); Q25–Approach when no species–specific CBPs (MA).

media and sending isolates to reference laboratories were used to a lesser extent (Supplementary Table 5). Screening for ESBL production, pAmpC, methicillin resistance and inducible clindamycin resistance was reported as mostly performed for therapeutic guidance, whereas screening for CPE and vancomycin resistance in *Enterococcus* species was primarily performed for epidemiological surveillance (Figure 4 and Supplementary Table 6).

Section C. Interpretation of results and reporting

To determine the clinical significance of bacterial isolates obtained upon culture, most laboratories (59.2%) reported always using knowledge on the most common organisms known to cause infections at the sampling site concerned. This was followed by knowledge of sampling method/site (56.7%), the duration/mode of sample transport (39.5%) and identification of the organism at species level (47.8%). Cytology reports and Gram-stained smears were less often used with 32.5% and 15.9%, respectively, of respondents claiming to never use these options (Supplementary Table 7).

The laboratories use different approaches for selection of isolates for AST, depending on whether cultures originate from normally sterile or non-sterile body sites (Supplementary Table 8). The most remarkable difference was that 31.2% of respondents selected all phenotypically distinct isolates for AST from cultures of normally sterile sites, whereas only 4.5% of respondents would do the same from non-sterile body sites.

Concerning reporting to clients, less than half of respondents generally add comments in their diagnostic answers to indicate if obtained bacterial isolates can be regarded as clinically significant (48.4%), likely commensal/resident flora (41.4%) or likely opportunistic bacteria (30.6%) (Supplementary Table 9). Two

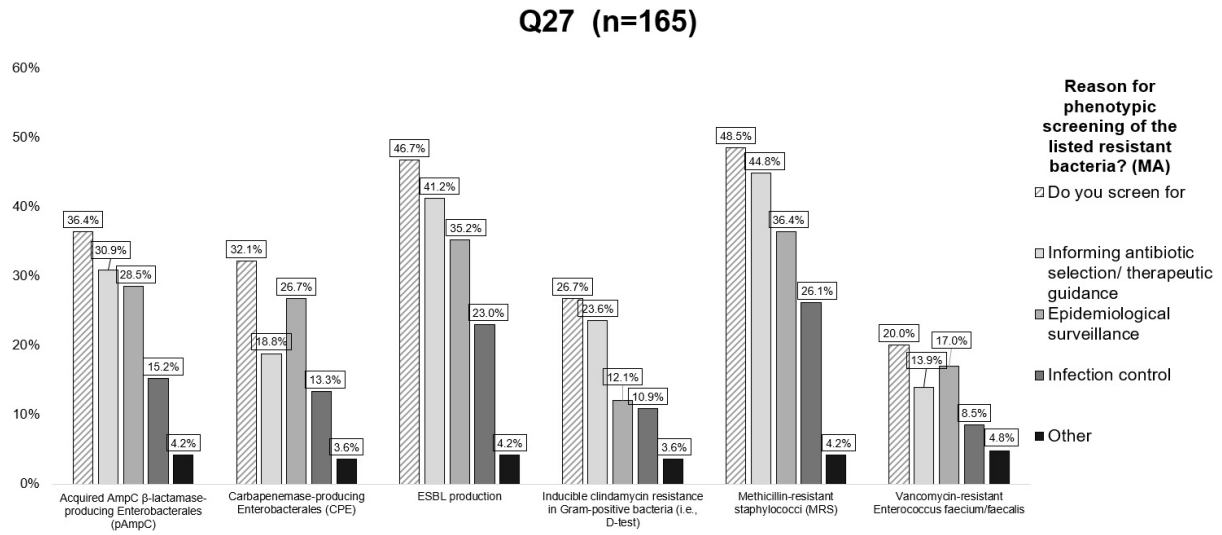

FIGURE 4
Responses to question Q27 on reasons for phenotypical screening of various resistant bacteria. For each type of resistant bacteria listed, multiple answers were possible. Valid answers were considered if participants interacted with at least one of the questions in a row of the table (see Supplementary Table 3). MA, multiple answers.

questions (Q31 and Q32) concerned the provision of information to clients concerning AST result and measures to promote prudent antimicrobial use. There was a tendency to rarely provide such information, although selective reporting of antimicrobials (i.e. excluding highest priority critically important antimicrobials from AST reports) was fairly common with 39.5% of respondents claiming to do that (Supplementary Tables 10, 11).

The survey revealed variety of approaches in reporting AST results, where most laboratories (47.9%) only included the qualitative interpretation of the result (S, R, and I), whilst others provided the actual MIC value with (25.3%) or without (36.5%) displaying the CBPs used for interpretation (Supplementary Table 12). Moreover, a couple of laboratories took a more complex approach, e.g., by indicating the ratio between the CBP and the MIC.

Section D. Surveillance, laboratory data management and further developments

The survey identified that most laboratories had a data management system for sample recording (86.1%) and reporting (86.0%). Additionally, the majority of respondents claimed to be able to archive and extract culture and AST results from this system (91.3%), and to extract data for analyzing AMR trends (77%) (Supplementary Table 13).

Many respondents indicated that they participated in pathogen or AMR surveillance programs. Almost 60% of laboratories participated in *Salmonella* reporting and 44.4% participated in broader zoonotic pathogen surveillance. Participation in AMR surveillance programs was generally poor, with a higher participation rate in farm animal (53.6%) compared to companion animal (40.1%) schemes. (Supplementary Table 14).

Overall, laboratories strongly supported the development of specific guidelines, especially guidelines for interpreting and reporting of AST results (Supplementary Table 15).

Discussion

The survey identified broad variability in practices between laboratories, underscoring the well-recognized problem of veterinary diagnostic laboratories not adopting uniform microbiological procedures (Guardabassi et al., 2017; Timofte et al., 2021). In the survey, this shortcoming in the veterinary microbiology laboratories practices was observed both within a country (if more than one laboratory answered the survey) and between countries, adding to the conclusion that the lack of standardized microbiology practices is a general problem that needs to be addressed. These differences can be attributed to various factors including training background, lack of broad consensus for the use of a common methodology in veterinary laboratories with multiple standards (e.g. EUCAST, CLSI or national committees) adopted depending on local factors and choices, access to resources and new technologies, reporting methodology etc.

Besides the lack of inharmonious methodology, the survey identified diverse training backgrounds and expertise of the microbiology diagnostic teams. Although our survey did not address the issue of how "veterinary microbiologist" training was achieved, only 55.7% of laboratories reported to have a head of service/director with a primarily veterinary microbiology-based background. This seems to be a wider issue, which is also affecting human clinical microbiology (Humphreys et al., 2010). Interestingly, a recent US-based publication has highlighted the need for well-trained and qualified medical microbiologists as directors of clinical microbiology laboratories (Samuel et al., 2021). In addition, the Infectious Disease

Society of America (IDSA) guideline is recognizing the value of medical microbiologists as core members of any antimicrobial stewardship programme, and their potential to significantly impact antibiotic usage (Dellit et al., 2007). The establishment of the European College of Veterinary Microbiology (ECVM) in 2016 promoting standardized training and recognition of the professional identity of veterinary microbiologists will help building specific expert capacity for veterinary microbiology laboratories in the future.

Laboratory performance can also be impacted by financial capacity or willingness to invest in technologies, which can greatly facilitate faster and more accurate results. In that regard it is worth noting that whilst 77% of responding laboratories still used individual biochemical identification assays for various reasons, 43.3% of laboratories reported having access to a MALDI-TOF MS for bacterial/fungal species identification. This is encouraging, as MALDI-TOF has revolutionized clinical microbiology by greatly improving the turnaround time and accuracy of bacterial and fungal identification at species level (Patel, 2015; Van Driessche et al., 2019). The rapid and accurate identification offered by MALDI-TOF significantly impacts how bacterial cultures are interpreted and which isolates are selected for AST, as laboratories need to ensure that their bacterial culture and AST results are clinically relevant. Since MALDI-TOF equipment constitutes a big economic investment, use of conventional phenotypic tests is unlikely to be replaced in small laboratories and/or limited resource settings. On the other hand, MALDI-TOF is more cost-effective for large clinical microbiology laboratories due to its low cost per sample, reduced reagent costs, labor, and turnaround time, as described by several studies (Bizzini and Greub, 2010; Thompson, 2022; Calderaro and Chezzi, 2024).

Bacterial culture interpretation is a valuable and complex skill, which makes use of a plethora of clinical and paraclinical information to identify isolates likely associated with an infection. In this context, the survey showed that, for instance, knowledge of organisms likely to be aetiological agents at the infection site was commonly taken into consideration (59.2%). The use of Gram-stained smears from clinical specimens has been shown to be a key step in interpretation of bacterial culture results as well as for guiding empiric antimicrobial therapy (Musher et al., 2004; Stone and Steele, 2009). However, it is surprising that Gram-stained smear findings or evidence of inflammation from cytology reports was an identification criterion used consistently by only 24.8% and 15% of labs, respectively. This may reflect lack of expertise for smear staining or interpretation, laboratories being too busy or not having enough personnel resources for these informative, but time-consuming evaluation tools. This finding is related to another important issue concerning selection of clinically relevant isolates for AST: our data showed that 23.4% of laboratories claimed to always select only pure growth isolates from non-sterile body sites (e.g., skin, mucosal surfaces) for AST, whilst 62% of laboratories would select up to two or three isolates. Such large variation between laboratories reflects that there is not yet international consensus on how to select isolates from such samples, which may indeed be difficult and should also rely on other factors such as the relative proportion of colonies and sample type and origin. Nevertheless, this result suggests that training and guidance in this area is acutely needed, as isolate selection for AST is a key element directly impacting antibiotic use. Remarkably, whilst several standards describe how to perform AST (CLSI, EUCAST, WHO, etc.) (OIE, 2020; CLSI VET01, 2024; CLSI VET01S, 2024 EUCAST, 2024), there is little guidance on which isolates to select for AST. It is therefore encouraging that some recently published training resources from human medicine (CDC, 2020), are more specific about the need for laboratory procedures to reflect best practices for the workup of clinical specimens and emphazising the importance of isolate selection for AST. Guidelines in this area could reduce the risk of performing AST on commensal or contaminating organisms (CDC, 2020). To this end, ENOVAT organized two consecutive training schools focused on bacterial culture interpretation of veterinary clinical specimens and isolate selection for AST.

Maybe unsurprisingly, the survey also showed that communication between the laboratory and clinicians was rare (22.3% only). This might reflect the distancing of clinical microbiology services from the patient, as is the case for most commercial laboratory settings. We do however encourage regular communication between the laboratory and clinician, since requisition schemes often lack important patient- or sample-related information that could be used to guide the approach taken in the laboratory. Communication is also valuable from the perspective of the clinician who may benefit from help with interpretation of AST results, and consequently with selection of appropriate treatment.

One of the most important findings of this study was the identification of combined approaches being used for interpreting AST results, with a notable proportion of responding laboratories using a combination of CLSI, EUCAST (47.6% for MIC and 41.2% for DD) and national guidelines (16.9% for DD and 6.7% for MIC) for interpretation of results. On the contrary, few respondents (as low as 15.2% for MIC and 17.6% for DD) used either EUCAST or CLSI guidelines exclusively. The combination of multiple standards for interpreting AST in veterinary medicine can be considered a necessity, as CBPs are missing for several antimicrobial agent—animal species—body site—pathogen combinations. This is also reflected in our finding that 51.5% and 53.3% of laboratories reported the use of CBPs developed for other body sites/organisms/animal species or for humans, respectively, when a specific CBP is missing. While realizing that the shortage of specific CBPs cannot be solved in the short term, a guideline on how to prioritize among non-specific CBPs has been recently developed by CLSI (CLSI VET09, 2024). Despite providing useful support, this guideline also emphasizes that the clinical validity of non-specific CBPs is often questionable. One additional downside of using many different interpretive criteria for AST is the complexity of comparing AMR data across laboratories unless raw data (MICs or inhibition zone diameters) are provided. In that regard, several recent studies have analysed the discrepancies in results interpretation when applying either CLSI or EUCAST CBPs to various pathogen-antimicrobial combinations, supporting the claim for a globally harmonized AST system that would be both practical and freely available (Delgado-Valverde et al., 2017; Cusack et al., 2019; EFSA Panel on Animal Health and Welfare [AHAW] et al., 2021; Maganga et al., 2023). At the same time, there are profound differences in the ways CLSI and EUCAST are set up and governed which can generate practical differences

between their methodologies and interpretative criteria, making the systematic harmonization of the two committees challenging (Kahlmeter, 2015).

Reporting AST results effectively is crucial for guiding clinical decision-making and promoting antimicrobial stewardship. In that context the laboratories can employ a number of approaches such as selective and cascade reporting of AST results (Guardabassi et al., 2017). The survey results demonstrated that selective reporting of antimicrobials was the most common approach taken by the laboratories when reporting AST results (39.5%), meaning not reporting the results for the highest priority critically important antimicrobials. However, 37.6% of laboratories do not take any specific approach when reporting AST results. Therefore, it's crucial to encourage practices in AST reporting that align with the principles of antimicrobial stewardship, so the clinicians can be directed towards prescribing narrower-spectrum or first-line antibiotics. This strategy aids in conserving broader-spectrum antibiotics and those critical for human health, reserving them for instances where they are indispensable.

The determination of MIC of an antimicrobial agent offers valuable information in terms of drug choice, dose and frequency of treatment. The survey results indicated that the laboratories using MIC-based tests have a variety of strategies when reporting i.e. reporting only the susceptibility, both susceptibility and the MIC value, as well as susceptibility, MIC value and the breakpoints values. More detailed and comprehensive information reported by the veterinary laboratories like the breakpoint-MIC ratio is important in guiding the drug selection since the distance of the MIC from the breakpoint is relevant for the level of susceptibility of the pathogen to the selected antimicrobial. Additionally, the selection of an antibiotic should not solely depend on the MIC value. It is important to consider a variety of factors including the distance of the MIC from the breakpoint, infection site, the species, age and health status of the animal, drug's pharmacokinetics, as well as the route and frequency of administration. Due to factors like pharmacokinetics and poor tissue penetration, which can result in subtherapeutic drug levels at the site of infection, using an antimicrobial with a lower MIC relative to the breakpoint is recommended. Additionally, for drugs with an MIC near the breakpoint, increasing the dosage or frequency of administration may be necessary to achieve sufficient drug levels at the infection site reducing the risk of treatment failure (Allerton and Nuttall, 2021). In this context, guidance to the clinicians is crucial in the interpretation of the MIC test results, which will facilitate appropriate antibiotic selection, improve treatment outcome, promote antimicrobial stewardship and ultimately tackle AMR.

The lack of specific guidelines for detection of AMR mechanisms is reflected by the plethora of responses when laboratories were asked if, and for what reason, they screened bacterial isolates for resistance mechanisms (e.g., epidemiological surveillance, informing antibiotic therapy or infection control). In this context, it is remarkable that only 48.5% of laboratories screened for methicillin resistance (MR), and only 26.7% also screened for inducible clindamycin resistance in staphylococci. Even fewer labs screened for CPE and VRE. This is probably due to the fact that these antimicrobial classes (carbapenems and glycopeptides) are not registered for use in animals meaning that for therapeutic guidance this screening is not needed. However, for epidemiological surveillance it would be very interesting to screen for these primarily human-relevant resistance mechanisms in the scope of One Health. Surveillance of such resistance types was also recommended by the European Antimicrobial Resistance Surveillance Network (EARS-Vet), even though the primary focus of this network is AMR in clinical isolates of animal origin (Mader et al., 2022a).

Survey respondents clearly indicated their desire to have access to common guidelines for bacterial culture, isolation and identification, and to be recommended preferred guidelines to follow for interpretation of AST. Consequently, and unsurprisingly, there is a need for standardization of the bacteriological diagnostic process from sample collection, processing, pathogen identification, selection of isolates for AST, and reporting in laboratories across all veterinary diagnostic providers. Some national initiatives, such as RESAPATH (the French network for surveillance of AMR in bacteria from diseased animals, available at),[3] offer an example of achieving common methodology for national harmonization of AMR surveillance. At European level, work led by the EARS-Vet framework mapped national monitoring systems for AMR in bacterial pathogens of animals (both companion and food-producing) among 27 countries, reviewing their structure and operations and generating useful information for countries planning to build or improve their AMR systems (Mader et al., 2022b). These authors showed important gaps in the current landscape of AMR surveillance in animals, and they proposed a pragmatic AST harmonized strategy. Similarly, data accumulated via the current survey highlights gaps to be addressed for optimizing and harmonizing veterinary diagnostic laboratory practices and will serve as the foundation for tackling the main gaps identified. As such, a subgroup of ENOVAT members is working towards building an archive of Veterinary Microbiology Protocols (initially focusing on companion animal clinical specimens) as the first steppingstone towards achieving the long-held goals of harmonization of bacteriological diagnostic procedures across veterinary microbiology laboratories in Europe and beyond.

The findings from this study have some limitations. First, due to the extent of the ENOVAT Network (> 300 members from > 40 countries) who were asked to disseminate the link to the survey, we cannot know exactly how many laboratories were reached and invited to participate. This means that we cannot be certain that the findings represent the laboratory approaches to bacterial culture and AST in all these countries. Another limitation is that there was a large variation in laboratory settings making it hard to compare responses. For instance, governmental laboratories mainly focus on screening for specific zoonotic pathogens or resistance phenotypes in defined sample types from farm animals (e.g., faeces), whereas commercial laboratories may receive a large variety of clinical samples from companion animals. Additionally, data from the survey was not adjusted for the number of laboratories in each participating country and other country-specific metrics, which does not allow for comparisons between countries and country-specific conclusions. As accurate information on the exact number of practising laboratories in each participating country was not available, future studies looking to capture these data and using

3 https://resapath.anses.fr/

country-specific metrics coupled with statistical analysis will be beneficial to draw unequivocal country-specific conclusions.

Conclusion

We identified a broad variety of methodologies being used for bacterial culture and AST in European veterinary microbiological diagnostic laboratories. Although some responses (e.g., failure to identify bacteria to species level) are against the general perception of good diagnostics, the overall variation in responses was expected, since internationally acknowledged veterinary-specific guidelines are lacking for many steps of bacterial culture beyond AST (from sample receipt to reporting).

The diversity in methodologies for C&ID, lack of consensus on isolate selection for AST, combined use of multiple guidelines for interpreting AST results and variation in AST reporting practices emphasize the need for an internationally harmonized approach in veterinary clinical microbiology. Furthermore, the inconsistent screening for AMR mechanisms requires development of standardized protocols, especially for epidemiological surveillance in a One Health context.

We therefore call for the development of specific guidelines and standards for processing clinical specimens to support veterinary laboratories. Furthermore, resources need to be dedicated to ensuring that laboratory staff are trained appropriately, and technical facilities are available to support them. Clinical staff also need to be trained to interpret the results and to communicate regularly with laboratories, thereby ensuring the best foundation for diagnostic-driven antimicrobial stewardship.

Data availability statement

The original contributions presented in this study are included in this article, further inquiries can be directed to the corresponding author.

Author contributions

TK: Formal analysis, Writing – original draft, Data curation, Visualization, Writing – review and editing. IC: Data curation, Formal analysis, Writing – original draft, Conceptualization, Writing – review and editing. FZ: Writing – review and editing, Investigation, Methodology. SB: Formal analysis, Investigation, Methodology, Writing – review and editing. SC: Investigation, Methodology, Writing – review and editing. PK: Investigation, Methodology, Writing – review and editing. CH: Formal analysis, Investigation, Methodology, Writing – review and editing. ZŠ: Investigation, Methodology, Writing – review and editing. SK: Investigation, Methodology, Writing – review and editing. JG: Formal analysis, Investigation, Methodology, Writing – review and editing. IZ: Investigation, Methodology, Writing – review and editing. EP: Investigation, Methodology, Writing – review and editing. AL: Writing – review and editing, Visualization, Data curation, Writing – original draft, Formal analysis. DS: Writing – review and editing. FA: Writing – review and editing. EB: Conceptualization, Writing – original draft, Writing – review and editing, Funding acquisition, Investigation, Methodology, Project administration, Supervision. PD: Conceptualization, Funding acquisition, Methodology, Writing – original draft, Writing – review and editing, Investigation, Project administration, Supervision. DT: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Writing – original draft, Writing – review and editing, Funding acquisition, Investigation.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1443755/full#supplementary-material

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