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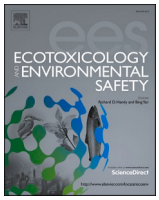

The swine waste resistome: Spreading and transfer of antibiotic resistance genes in *Escherichia coli* strains and the associated microbial communities

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ABSTRACT

The overuse of antimicrobials in livestock farming has led to the development of resistant bacteria and the spread of antibiotic-resistant genes (ARGs) among animals. When manure containing these antibiotics is applied to agricultural fields, it creates a selective pressure that promotes the acquisition of ARGs by bacteria, primarily through horizontal gene transfer. Most research on ARGs focuses on their role in clinical antibiotic resistance and their transfer from environmental sources to bacteria associated with humans, such as *Escherichia coli*. The study investigates the spread of antibiotic-resistant genes (ARGs) through class 1 integrons in 27 Escherichia coli strains from pig manure. It focuses on six common ARGs (*erm*B, *cml*A, *flo*R, *qnr*S, *tet*A, and TEM) and the class 1 integron gene, assessing their prevalence in manure samples from three pig farms. The study found correlations and anticorrelations among these genes, indicating a predisposition of the integron in spreading certain ARGs. Specifically, *cml*A and *tet*A genes were positively correlated with each other and negatively with int1, suggesting they are not transferred via *Int*1. Farm B had the highest int1 counts and a higher abundance of the TEM gene, but lower levels of *cml*A and *tet*A genes. The results underscore the complexity of predicting ARG spread in agricultural environments and the associated health risks to humans through the food chain. The study's results offer valuable insights into the antibiotic-resistant genes (ARGs) profile in swine livestock, potentially aiding in the development of methods to trace ARGs in the environment.

1. Introduction

The last few decades have witnessed an increasing demand for animal protein, and, as a result, global intensive livestock farming has expanded rapidly (Manyi-Loh et al., 2023). Antimicrobials, used to various extents and frequencies at therapeutic and sub-therapeutic concentrations (Massé et al., 2014; Dong et al., 2021) for maintaining a good health status in animals, have exerted a selective pressure on microorganisms, thus being the major driving force behind the emergence and spread of drug-resistance genes among pathogenic and commensal bacteria (Tadesse et al., 2012).

Furthermore, antibiotics used for veterinary purposes, when excreted by animals, end up in manure (Shawver et al., 2021). In case manure is applied as agricultural fertiliser, persistent antibiotics infiltrate through soil and groundwater, thus favouring the dissemination of antibiotic-resistant bacteria (ARB) and their related antibiotic-resistant genes (ARGs) into environments (Kümmerer, 2003).

The occurrence of antibiotics in agricultural settings at sub-therapeutic concentrations might facilitate the acquisition of ARGs or trigger spontaneous mutations in environmental bacteria. Antibacterial resistance can emerge within bacterial populations through the acquisition of external genes in the genome, which are transmitted from cell to cell via mobile genetic elements (MGEs) like transposons and integrons through plasmids, vesicle secretion or infection by bacteriophages or other viruses (Soler and Forterre, 2020; Washington et al., 2021). Horizontal gene transfer (HGT) is one of the most frequently used processes for transferring and rearranging DNA in prokaryotes. Indeed, it has been estimated that more than 25 % of bacterial genomes derive

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from periodic HGT events (Stokes, 2017).

Studying all bacterial species that lead to human disease simultaneously is time-consuming. Therefore, it is necessary to utilize sentinel organisms to track the emergence and dissemination of antibacterial resistance. *Escherichia coli* is a Gram-negative commensal bacterium of humans and animals. It is used as a marker species, namely sentinel, for monitoring the prevalence, types, and movement of resistance genes within and across clinical, farm, community, and environmental settings (Mbelle et al., 2019). Indeed, it has recently been used as a benchmark for tracking antimicrobial drug resistance in faecal bacteria due to its higher prevalence across various hosts. Furthermore, *E. coli* is a naturally competent organism capable of incorporating extracellular DNA under ambient conditions in the surrounding environment.

To date, several studies have highlighted the presence of ARB in the agricultural environment because of the release of animal-originated antibiotics (Luiken et al., 2020; Shawver et al., 2021). Moreover, it has been pointed out the need to identify genetic markers linked to both the *resistome*, understood as antibiotic resistance profile, and the *mobilome*, which includes transposons and class 1 integron. Additionally, the detection of pathogenic bacteria associated with soils amended with manure is also highlighted (Zhao et al., 2019; Mazhar et al., 2021), because the utilization patterns of antibiotics in livestock farms differ among regions and countries. Following the application of manure contaminated with antibiotics in agricultural practices, the HGT of ARGs from faecal microorganisms to native environmental bacteria becomes a crucial factor in disseminating resistance.

Indeed, antibiotics released by animal manure can provide a positive selective pressure for ARBs and select for antibiotic resistance among commensal and pathogenic bacteria through mobile elements such as integrons (i.e., class 1) (Deng et al., 2015; Gao et al., 2023) and plasmids (in particular, IncQ) (Smalla et al., 2000). For example, in animal faeces and farm soil samples, tetracycline resistance genes (*tet*) were often found in class 1 integrons (Srinivasan et al., 2008). Likewise, quinolone (*qnr*) and macrolide (*erm*) resistance genes were associated with different plasmid groups in wastewater and soil environments. Genes related to β-lactams resistance, i.e., extended-spectrum β-lactamase (TEM) or chloramphenicol transporter nonenzymatic chloramphenicol-resistance gene (*cml*A), as well as a gene related to florfenicol export protein (*flo*R) (Srinivasan et al., 2008), were found in faecal samples of many different livestock species (Rodríguez et al., 2009; Falodun et al., 2020).

However, few studies have been done to date on optimizing the detection of ARGs (Checcucci et al., 2020) and tracking the spread of integron genes in livestock farms (Qian et al., 2018). Furthermore, understanding the dynamics of ARG dissemination among environmental microbial communities could contribute to decreasing their uncontrolled spread.

Considering that ARBs frequently cross environmental and species boundaries, unravelling the potential enrichment and exchange of ARGs within bacteria associated with animals and humans is crucial.

This study provides a comprehensive characterization of the *resistome* and *mobilome*, mainly focusing on class 1 integron and the putative cassette arrays, along with their interactions, within the sentinel bacterium species *E. coli*.

The presence and quantity of six widely diffused ARGs (i.e., *erm*B, *cml*A, *flo*R, *qnr*S, *tet*A, and TEM) and the *Int*1 gene were analysed to potentially predict the persistence of antibiotic resistance in manure, aiming to identify possible correlations and anticorrelations among ARGs and manure samples from different livestock management systems. Furthermore, understanding the dynamics of ARG dissemination in the environment and the relative microbial communities will be crucial for enhancing risk assessment and implementing effective containment measures for ARGs in livestock farms.

2. Material and methods

2.1. Manure samples

Three farms in Northern Italy with different manure management were selected for this study (Fig. S1). All of them are designed to follow pigs from nursery to fattening. Farm A is equipped with a nitro-dinitro treatment plant working on the liquid fraction of manure after centrifugation. In farm B, only the fraction from weaning barns is collected in a storage tank without further treatment, whereas manure from fattening barns feeds a biogas reactor. Farm C's manure from different barns is directly collected in a storage tank. Manure and slurry samples were collected from the three farms at different points of their waste management system. Samples and origins are listed in Table 1.

Liquid manure consisted of pig waste mixed with wastewater used to remove the waste from the pens. Slurry samples from swine farm-reared weaning piglets were collected from approximately 5–10 cm below the pens' surface and shipped to the laboratory within four hours. Samples for microbiological determination were suspended in glycerol broth and then stored at −80°C until analysis.

2.2. Chemical and physical characterization of the manure samples

Pig manure samples were characterised for pH, electrical conductivity (EC), total organic carbon (TOC), total nitrogen (TN), and moisture (Table 2). Due to the heterogeneity of the manure samples, different procedures were adopted to evaluate the chemical-physical parameters.

The pH was measured using an XS Instruments pH meter Mod. pH 510 (Italy) equipped with a Polylite Lab glass electrode (Hamilton, USA). For the liquid samples, the pH was measured on samples left to decant for 15 minutes by immersing the electrode in the supernatant in static mode. Concerning solid manure, the pH was evaluated by adopting the method suggested for fertiliser analysis (http://www.ciec-italia.it): 3 g of dried sample, ground to < 2 mm, was suspended in 50 ml of distilled water and shaken for 30 min. After 10 min of decantation, the pH was measured by immersing the electrode in the liquid phase without shaking.

The EC was measured using a conductometer SAT (Halosis, Italy) equipped with a Pt electrode (cell constant of 1 cm^{-1} CRISON, 5292). The EC of the liquid samples was measured after filtration through Whatman 42 filters GE Healthcare UK). For the solid samples, the following procedure was adopted. After pH evaluation, the suspensions were left to stand for 24 h; the supernatants were filtered through

Table 1
Pig manure sampling on the farms A, B and C (REFLUA project partners).

Acronym	Sample	Collection position
Farm A		
M_A	Manure	Treatment plant inlet
LP_A	Liquid phase from M_A	Liquid phase tank
SP_A	Solid phase from M_A	Solid phase collecting area
STOC_Dep_A	LP_A after treatment	Treatment plant tank
MW_A	Manure	Weaning farm
MG_A	Manure	Growing farm
MF_A	Manure	Fattening farm
Farm B		
MW_B	Manure	Weaning farm
MF_B	Manure	Fattening farm
DIG_B	MF_B digested in biogas plant	Outlet biogas plant
LP_B	Liquid phase from DIG_B	Liquid phase tank
LS_B	Solid phase from DIG_B	Solid phase collecting area
STOC_B	Manure	Storage tank
Farm C		
MW1_C	Manure	Weaning farm
MW2_C	Manure	Weaning farm
MF1_C	Manure	Fattening farm
MF2_C	Manure	Sow farm
STOC_C	Manure	Storage tank

Table 2
Chemical characterization of manure samples from Farm A, B, and C. Abbreviations: EC = Electrical conductivity; TOC = Total Organic Carbon; TN = Total Nitrogen. Values are indicated ± standard deviation.

Sample.	pH	EC (mS cm^{-1})	TOC (g L^{1})	TN (g L^{-1})	Moisture (g kg^{-1})
M_A	7.27 ±0.03	17.06 ±0.18	7.1±0.7	1.7±0.1	986.66±0.2
LP_A	7.41 ±0.04	15.98 ±0.91	9.1±2.9	2.1±0.6	988.02±0.2
SP_A	8.47 ±0.05	1.63 ±0.04	408.2 ±7.4*	12.8 ±1.5*	718.35±0.6
STOC_Dep_A	8.27 ±0.04	5.92 ±0.16	0.5±0.1	0.10 ±0.02	988.45±1.0
MW_A	5.54 ±0.02	18.22 ±0.36	14.0±1.3	2.2±0.2	983.24±0.2
MG_A	6.39 ±0.05	19.48 ±0.47	13.0±3.6	2.1±0.5	979.63±0.3
MF_A	7.87 ±0.11	25.09 ±1.75	8.6±1.8	3.0±0.7	986.76±0.2
MW_B	6.63 ± 0.01	5.04 ± 0.05	1.24 ± 0.17	0.51 ± 0.02	991.12 ± 1.3
MF_B	7.49 ± 0.02	14.05 ± 0.10	3.24 ± 0.32	1.56 ± 0.11	984.23 ± 1.3
DIG_B	11.45 ± 0.05	5.75 ± 0.21	362.31 ± 3.28*	35.68 ± 0.32*	944.14 ± 1.1
LP_B	NQ	NQ	2.6 ± 0.4	0.4 ± 0.1	946.77 ± 0.9
LS_B	8.57 ± 0.07	2.11 ± 0.01	395.4 ± 0.62*	19.56 ± 0.18*	684.00 ± 4.2
STOC_B	7.86 ± 0.02	11.00 ± 0.29	1.48 ± 0.09	0.80 ± 0.07	990.23 ± 0.1
MW1_C	6.07 ± 0.02	29.71 ± 1.28	17.06 ± 0.78	3.80 ± 0.19	927.4 ± 2.8
MW2_C	6.65 ± 0.05	26.16 ± 0.79	8.12 ± 0.57	2.27 ± 0.16	955.0 ± 2.7
MF1_C	8.17 ± 0.04	23.94 ± 3.55	1.22 ± 0.15	1.86 ± 0.13	921.1 ± 2.5
MF2_C	8.17 ± 0.02	19.47 ± 0.84	1.16 ± 0.3	1.84 ± 0.09	922.5 ± 1.0
STOC_C	8.09 ± 0.04	18.75 ± 0.95	3.74 ± 0.05	1.35 ± 0.02	979.3 ± 2.3

NQ = Not quantifiable
* unit of measurement: g kg^{-1}

Whatman 42 filters, and the EC was measured in the clear samples.

TOC and TN values of the liquid samples were obtained using a CHN analyzer (Mod. Flash 2000, Thermo-Fisher, Massachusetts USA) after filtration through Whatman 42 filters and subsequent 500X dilution of the samples with MilliQ® water. The TOC and TN of the solid fractions were measured on aliquots of air-dried and sieved < 2 mm samples using a Shimadzu TOC-V CPN and a Shimadzu TNM-1 (Shimadzu Europe GmbH, Italy) for carbon and nitrogen analysis, respectively.

Moisture was evaluated using a gravimetric method (Reynolds, 1970). Weighed samples were placed in ceramic crucibles and left to dry in the oven at 105 °C until a constant weight was reached. Moisture was calculated as a difference in weight before and after the thermal drying of the samples.

2.3. Isolation of Escherichia coli strains

Twenty-seven *E. coli* strains were isolated from all the manure samples collected from weaning piglet pens (MW_A, MW_B, MW1_C, and MW2_C) (Table S1). The plate count agar method was used. Each sample was serially diluted 10-fold up to 10^{-9} in 0.9 % saline solution; 1 ml of all the dilutions was plated onto Chromocult Coliformen Agar (Merck). The plates were aerobically incubated at 37°C for 24 h when the colonies were distinctly visible for isolation. Blue/purple-coloured presumptive *E. coli* colonies were subcultured onto Luria Bertani (LB) agar to obtain pure cultures. Testing for oxidase activity and indole production confirmed the microorganism's identity. Each suspected oxidase-negative colony was cultured in sterile peptone water for 24 h in aerobiosis at 37°C. Five drops of Kovac's Indole reagent (Merck) were added to each tube following incubation. A positive result was shown by the presence of red or red-violet colour in the surface alcohol layer of the broth. Yellow colour indicated a negative result (Modesto et al., 2009).

2.4. Genomic DNA extraction, BOX PCR, and detection of antibiotic resistance susceptibility using the VITEK® 2

Genomic DNA from the 27 *E. coli* strains was extracted with the Wizard® Genomic DNA Purification Kit (Promega), following manufacturer instructions. Briefly, each strain was grown in LB broth at 37°C with shaking for 24 h at 140 rpm. Cells from 1 ml of each broth culture were then used for DNA extraction. For strains typing and differentiation, BOX-PCR (Versalovic et al., 1991) with the BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) was carried out according to Modesto et al., (2009). *E. coli* isolates, selected as representatives by different BOX-PCR profiles, were further characterised for their antibiotic susceptibility.

Strains were subcultured twice in LB broth (Merck) for 18–24 h at 37°C. A suspension of 0.5 McFarland was used to load the test cards for the VITEK2 according to the manufacturer's instructions (bioMérieux). The antibiotic susceptibility test cards with the standard European configurations analysed 20 antibiotics used in humans and animals and concentration ranges, in particular: ampicillin 2–32 μg/ml, amoxicillin-clavulanate (2:1 ratio) 2 and 1–32 and 16 μg/ml, cephalothin 2–64 μg/ml, cefoxitin 4–64 μg/ml, cefotaxime 1–64 μg/ml, ceftazidime 1–64 μg/ml, ticarcillin 8–128 μg/ml, ticarcillin-clavulanate (clavulanate at 2 μg/ml with ticarcillin a twofold dilution) 8 and 2–128 and 2 μg/ml, piperacillin-tazobactam (tazobactam at 4 μg/ml with piperacillin a twofold dilution) 4 and 4–128 and 4 μg/ml, imipenem 0.5–16 μg/ml, amikacin 2–64 μg/ml, gentamicin 1–16 μg/ml, netilmicin 1–32 μg/ml, tobramycin 1–16 μg/ml, nalidixic acid 2–32 μg/ml, ciprofloxacin 0.25–4 μg/ml, norfloxacin 0.5–16 μg/ml, ofloxacin 0.25–8 μg/ml, nitrofurantoin 16–512 μg/ml, and trimethoprim-sulfamethoxazole 20–320 μg/ml.

The turbidity signal was automatically measured every 15 min for up to 18 h during the test. Growth curves were generated to identify the minimal inhibitory concentration MIC) values; the software used an algorithm specific to each antibiotic. *E. coli* ATCC 25922 was used as a control strain for the enterobacterial test card.

2.5. Detection of target ARGs and integron marker genes in E. coli strains

E. coli strains were analysed for the presence of ARGs encoding for β-lactamase (TEM), quinolone resistance gene (*qnr*S), and macrolide resistance genes, which operate through the production of rRNA methylase acting as a steric hindrance (*erm*B), and the RNA adenine *N*-6-methyltransferase gene (*erm*A) (Lim et al., 2012; Marchandin et al., 2001), the florfenicol export protein (*flo*R) and the chloramphenicol acetyltransferase gene (*cml*A) which confers chloramphenicol resistance (Bischoff et al., 2005), one of the tetracycline efflux pump gene (*tet*A), genes essential for the fluoride export mechanism (*fex*A and *fex*B) (Berbasova et al., 2017). The primers were obtained from previously published studies and are listed in Table S2. The amplification cycles were carried out in a volume of 25 μl consisting of 12.5 μl of Xpert Fast Hotstart Mastermix (GRiSP, Portugal), 9.5 μl of D_2O, 1 μl of 10 mM primer forward, 1 μl of 10 mM primer reverse and 1 μl of DNA 10 ng/μl. Touch-down PCRs were performed using an Applied BiosysTEMs Veriti Thermal Cycler with the following temperature profiles. After sample preincubation at 95 °C for 3 min, twelve PCR cycles were run under the following conditions: denaturation at 95 °C for 40 s, primer annealing at optimum temperature + 5 °C for 40 s and DNA extension at 72 °C for 1 min in each cycle. The 12 PCR cycles were then run under the following conditions: 95 °C for 40 s, primer annealing at optimum temperature for 40 s, and DNA extension at 72 °C for 1 min in each cycle.

Ten PCR cycles were run under the following conditions: denaturation at 95 °C for 40 s, primer annealing at optimum temperature – 5 °C for 40 s, and DNA extension at 72 °C for 1 min in each cycle. After the last cycle, the PCR tubes were incubated for 7 min at 72 °C and held at 4 °C. The annealing temperature was optimised for all primer sets. Detection and classification of class 1 integrons by means of PCR analysis was carried out as described in Waldron and Gillings (Waldron and Gillings, 2015). The integron classification assessed through the characterization of int1 variable region, allowed the identification of a clinical or environmental origin. Primers HS464 and HS463a allowed the identification of strains with class 1 integrons; intI1F165 and intI1R476 amplified only the strains with class 1 integrons of a clinical type.

The PCR products were purified using Nucleospin® gel and a PCR clean-up column kit (Macherey-Nagel) and then sequenced by Eurofins Genomics to verify the fairness of sequence genes.

The DNA sequence data were compared to data in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

2.6. Genomic DNA extraction and quantification of target ARGs in manure samples

Total DNA was extracted from 1 ml of the liquid manure samples listed in Table 1 by using a QIAamp Fast DNA Stool Mini Kit (Qiagen). DNA concentration (ng/μl) and purity (260/280 nm ratio) were measured using a spectrophotometer (Tecan Infinite 200 PRO, Tecan, Switzerland). Genes *erm*B, *cml*A, and *flo*R, listed in Table S2, were utilised to quantify ARGs in the manure samples. Furthermore, primers for *qnr*S, *tet*A, and TEM genes detection and quantification were designed in this study and adapted for Real-Time (Table S3). Each ARG was first amplified from *E. coli* strains. The amplicons were then purified using Nucleospin® gel and PCR clean-up (Macherey-Nagel) and were quantified using a NanoDrop™ 2000/2000c Spectrophotometer. Standard curves were generated with serially diluted amplicons up to 1×10^{-6} ng/μl.

qPCR was carried out using the Step One RealTime PCR sysTEM (Applied BiosysTEMs) in a volume of 10 μl, which consisted of 5 μl of Syber Green Master Mix (Thermofisher), 2 μl of D_2O, 1 μl of 10 mM primer forward, 1 μl of 10 mM primer reverse and 1 μl DNA 10 ng/μl. Cycling conditions were: preincubation at 95°C for 10 min, followed by forty cycles of cycling conditions: 15 s at 95 °C, 1 min at 60 °C and 1 min at 72 °C. The absolute number of target genes was derived after normalisation to standard curves. The limits of quantification ranged from 1.2 (for *qnr*S) to 1.77 (for *erm*B) x10^6 gene copies per μl in 100 ng of genomic DNA. All the q PCRs were carried out in triplicate for the standards and the microbial community DNA samples.

2.7. Data analysis

The estimation of the gene copy number for 100 ng/μl of total DNA for each sample was calculated according to the formula:

$$\frac{\text{ampliconng}^{*}6,0221^{*}10^{23}\text{molecules/mole}}{\text{length of dsDNAamplicon}^{*}660\text{g/mol}^{*}10^{9}\text{ng/g}}$$

where 660 g/mole = average mass of 1 bp dsDNA. amplicon= fragment resulted from the amplification via PCR dsDNA= double strand DNA

Statistical analysis was performed in the R environment (version 4.3.2.). R Packages *ggplot2*, *ggcorrplot*, *factoextra,* and package set *Tidyverse* was used for data handling and visualisation. Correlation matrix and principal component analysis PCA) were computed on a subset of data which included unprocessed manure only: MW_A, MG_A, MF_A, M_A, MW_B, MF_B, STOC_B, MW1_C, MW2_C, MF1_C, MF2_C, and STOC_C.

3. Results and discussion

3.1. Chemical and physical characterization of the manure samples

For assessing a chemical-physical profile of each analysed manure sample, pH, electrical conductivity, total organic carbon, and total nitrogen were measured at each different collection point (Table 2). Fresh, unprocessed manures (MW_A, MG_A, MF_A, MW_B, MF_B, MW1_C, MW2_C, MF1_C, and MF2_C) had pH value between 5.54 (MW_A, weaning stable of farm A) and 8.17 (MF1_C and MF2_C, fattening stables of farm C). Notably, pH of excreta may be influenced by animal feed, gut microbiome, health status, as well as environmental conditions, as some organic acids or amines influencing pH are volatile (Van Der Weerden et al., 2023). The highest pH values (11.45, DIG_B) was observed in Farm B digestate, coherently to similar findings in literature (Sun et al., 2023). Electrical conductivity of fresh unprocessed manures varied between 5.04 (MW_B) and 29.71 (MW1_C) mS cm^{-1}. The solid and digested samples showed elevated levels of TOC and TN, primarily attributed to the organic matter-rich composition of the samples. Unprocessed fresh manure samples had TOC content between 1.16 and 17.06 g L-1 (MF2_C and MW1_C, respectively), and TN content between 0.51 (MW_B) and 3.80 (MW1_C) g L^{-1}.

As expected, a high-water content (>90 %) was found in all the manure samples with the exception of those subjected to physical separation or centrifugation (SP_A, LS_B). Not surprisingly, higher TOC and TN content are found in thicker samples which.

3.2. Genetic characterization of E. coli isolates and their antimicrobial susceptibility profile

The genetic diversity of isolated *E. coli* strains was investigated using BOX-PCR. Out of 27 isolates, fourteen different clustering groups were identified. Strains STL 1/1, STL 1/2, STL 1/4, STL 1/5, STL 1/7, STL 1/8, STL 2/2, STL 1/9, STL 2/1, STL 2/5, STL 1/3, A1, AB1, and AB2 were chosen as representatives of each group and further characterised (Fig. S2). Antimicrobial resistance/susceptibility to both human and veterinary antibiotics of all *E. coli* isolated strains was analysed using Vitek™ assay.

Results of resistance/susceptibility strains towards each antibiotic tested are reported in Table S4. Data showed a 100 % sensitivity of *E. coli* strains against five antibiotics (Piperacillin/Tazobactam, Ertapenem, Imipenem, Meropenem, and Amikacine). All the other tested antibiotics showed a variable efficacy: sensitive strains ranged from 92 % (Ceftazimide/Tazobactam) to 20 % (Fosfomycin). In particular, the highest rate of *E. coli* resistance was found in Fosfomycin (80 %), followed by Amoxicillin/Clavulonic acid (74.3 %). Pig farmers commonly utilize these antibiotics to treat and prevent infections during the post-weaning period as well as to manage and control severe systemic infections in pigs (Burch and Sperling, 2018).

3.3. Occurrence of target antibiotic resistance and class 1 integron genes in E. coli strains

ARGs conferring resistance to β-lactams, tetracyclines, macrolides, and phenicols were selected, and their presence was studied in the 14 selected strains. Out of the nine investigated ARGs (Table S2), only six genes (*tet*A, TEM, *erm*B, *qnr*S, *flo*R, and *cml*A) were retrieved in the *E. coli* strains (Table 4); in particular, *tet*A, *cml*A, and TEM genes were found present in 10 strains, whereas *erm*A and *fex*A were not detected. Furthermore, *Int*1 gene was detected in the following 10 *E. coli* strains: STL 1/2, STL 1/3, STL 1/4, STL 1/5, STL 1/7, STL 2/2, STL 2/5, A1, AB1, and AB2 (Fig. S3).

All isolates resulted positive for clinical type class 1 integron, except STL 1/7, which showed the presence of the environmental type integron class 1 (Fig. S4).

All PCR-obtained amplicons for ARGs and *Int*1 were sequenced and

tested for a BLAST search against the NCBI database (Table S5). The occurrence of integron class 1 and its characterization as clinical and/or environmental through its variable region, indicated the extensive distribution of the ARGs mobilization system in the manure samples under study. Class 1 integron presence is considered an indicator of anthropogenic pollution (Waldron and Gillings, 2015; Corno et al., 2023), and our results showed that identifying the types of integrons could serve as a marker of the environmental or clinical origin of the strains (Martini et al., 2018). Finally, the finding of mainly clinically relevant *E. coli* strains confirmed that the use of antibiotics on farms led to the selection of resistant strains that could pose potential risks to both animal and human health (Maciel-Guerra et al., 2023; Wang et al., 2023).

3.4. Quantification of target ARG in manure samples

The ARGs and class 1 integron identified in *E. coli* isolates were also quantified by real-time PCR (qPCR) assays in all 18 samples (Table 1) collected in the three farms at different points along the waste management process (Fig. S1). Results, expressed as $\log_{10}$ of the number of copies per gram of dried sample, are shown in Fig. 2. The ARGs observed extensively in the manure samples underline the strong impact of antibiotic use and the subsequent dissemination of antibiotic resistance within animal husbandry. Additionally, the levels of detected ARGs showed variability across all samples from different farms (Fig. 2).

Specifically, within farm A, unprocessed manure samples obtained from all stables (MW_A, MG_A, and MF_A) displayed ARG counts, ranging from 10^{11} copies (*qnr*S count in fattening pig manure samples MF_A) to 10^{13} (*erm*B count in weaning pig manure MW_A).

Generally, MW_A showed the higher ARG counts. Samples from M_A were collected from the blending tank that receives manure from weaning, growing, and fattening stables. The ARG counts in this sample type likely reflect a weighted average of the three manure types, as expected.

On farm A, the dejections gathered and blended in M_A were later divided into solid and liquid phases, labelled SP_A and LP_A, respectively. Despite the completely different water content (approximately 98.8 % in LP_A and 71.8 % in SP_A), the ARG counts in SP_A consistently trended lower than LP_A. Specifically, TEM and *erm*B were consistently observed at lower levels in SP_A.

According to waste management protocol on farm A, the solid phase is discarded after separation, while the liquid phase undergoes treatment at the nitro-denitro treatment plant. The resulting waste (LP_A) exhibited the highest counts per gram dry mass of *cml*A, *tet*A, and *flo*R among all samples from farm A, while TEM counts highly decreased. Similarly, class 1 integron count increases during the treatment process, as the highest count is registered in STOC_Dep_A sample. Conversely, the *Int*1 count in solid fraction waste (SP_A) is the lowest observed in farm A.

Unprocessed manure samples from farm B, namely MW_B and MF_B (derived from weaning and fattening stables, respectively), showed a variable pattern in the counts of ARGs.

MW_B had a higher copy number of TEM and *qnr*S genes with respect to MF_B, while the latter had a higher count of *erm*B and *flo*R genes. In farm B, wastes from weaning and fattening stables undergo two different pathways: weaning swine dejections, MW_B are accumulated in a storage tank (STOC_B) and disposed of afterward, while fattening pig excreta (MF_B) are treated by anaerobic digestion in a biogas plant (DIG_B), hence physically separated into a liquid and a solid phase (LP_B and LS_B, respectively). *cml*A, *tet*A, *flo*R, and *erm*B counts in STOC_B highly increased with respect to MW_B, while TEM and *qnr*S counts showed the opposite trend. Anaerobic digestion (DIG_B) showed a reduction in TEM count (compared to original sample MF_B) and *qnr*S, *flo*R, and *erm*B, albeit to a lesser extent. Differently, the *cml*A gene count increased in digestate samples. Separated liquid and solid digestate phase LP_B and LS_B (94.7 and 68.4 % water content, respectively) in most cases contained similar amounts of ARGs, except for *erm*B (higher in LP_B) and *flo*R (higher in LS_B). The highest copy number of class 1 integron was found in MW_B and STOC_B samples. The *Int*1 count reached its minimum in the digestate (DIG_B), with a slight increase observed in the separated phases LP_B and LS_B derived from DIG_B.

In the waste management system of farm C, the process involves collecting waste from all stables and combining it in a single communal tank. Samples from the two weaning stables (MW1_C and MW2_C) showed different counts for each quantified ARG and *Int*1. This trend is also shown in MF1_C and MF2_C samples derived from fattening pigs and sows, respectively. As found in farm A and farm B, the collection tank STOC_C exhibited average counts of ARGs and *Int*1 across all individual sampling points.

Overall, due to the varied treatment processes employed by the three farms for pig manure, establishing a consistent trend in the count of ARGs is challenging. The only shared phase among the three farms is the storage phase, during which gene counts typically exceed those of the initial samples, except for TEM in farms A and C, *erm*B in farm A, and *Int*1 in farms B and C.

These findings hindered our ability to thoroughly elucidate the impact of these treatments on the concentration and dissemination of genetic determinants in livestock waste.

3.5. Correlations and principal components analysis of unprocessed manure samples

A correlation matrix of selected chemical and genomic variables is provided in Fig. 3. In unprocessed manure samples (MW_A, MG_A, MF_A, M_A, MW_B, MF_B, STOC_B, MW1_C, MW2_C, MF1_C, MF2_C, and STOC_C), *cml*A and *tet*A established the strongest correlation (correlation coefficient $\rho = 0.99$), followed by TOC-TN (0.80), *erm*B-*flo*R (0.74), *flo*R-*erm*B (0.74) and TEM-*Int1* (0.70). *qnr*S-TEM, *erm*B-*cml*A, and *erm*B-*tet*A also correlated to a lesser degree ($\rho = 0.58$, 0.49, and 0.48, respectively). Moreover, electric conductivity (EC) correlated with both *cml*A and *tet*A (0.58 and 0.54, respectively) while negatively correlated with *qnr*S (-0.69). The PCA individuals score (coloured by farm of origin) of unprocessed manure sample and genomic variables contribution to principal components is available in Figure 4. *cml*A, *tet*A, *Int*1, and *erm*B contribute to differentiating manure samples along the first principal component (20.79, 20.20, 19.23 and 18.20 %, respectively). Variables *qnr*S and *flo*R highly contribute (43.91 and 32.72 %, respectively) to the variance along component 2. Individuals' data points are scattered on both dimensions with very little clustering. Unprocessed manure samples from farm C are highly diverse and spread along both dimensions. Farm B samples are much more dispersed on Component 1 with respect to Component 2. The opposite is true for farm A samples (as they vary more along Component 2), and they also tend to be less dispersed.

Generally, observing the correlations among ARGs counts, the most interesting correlation can be evidenced among *Int*1 and TEM count (Fig. 3). This result allowed us to hypothesise that TEM can be preferentially transposed with this HGT system in these conditions as an advantageous gene for survival and adaptation in this environment. Vice versa, an anticorrelation was observed with the other ARGs, *cml*A, *erm*B, and *tet*A, as if the diffusion of TEM in the samples became preferential to the detriment of other genes.

Furthermore, a positive correlation in ARGs presence was observed preferentially among EC, which is one of the soil features most influenced by the salinity (Friedman., 2005), and *cml*A and *tet*A genes count. Of course, salinity levels and water saturation of soil can influence the survival of microorganisms in that environment in some way, impacting ARGs spread and stability. The fluctuation of ARGs among the different farms sampling points, or their tendency to increase in some of the collection points as of the storage tanks, cannot correlate with the number of bacteria surviving in the same samples, as described in (Checcucci et al., 2020; Bao et al., 2023).

PCA (Figure 4) did not always show a clusterization tendency for all samples from the same farm. Indeed, the distribution of ARGs revealed a high diversity among manure samples from farm C. This farm employs

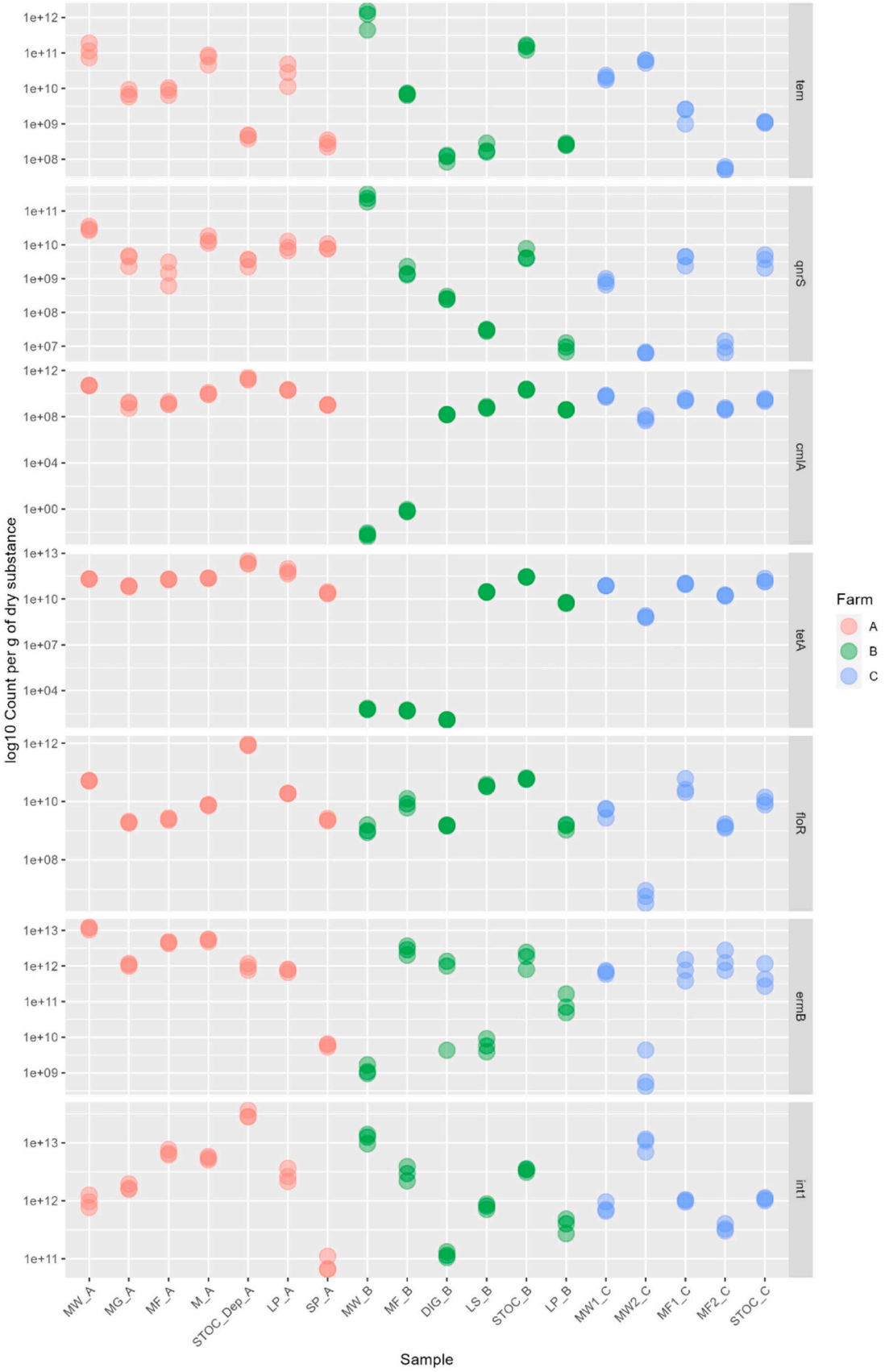

Fig. 1. ARGs and Integron count in all three farms' samples. Counts have been normalised on gram (g) of dry samples.

Fig. 2. Correlation matrix of ARG counts, integron counts, and selected physical or chemical variables. All variables were normalised on sample dry matter content. Data was subset considering only unprocessed manure samples. Statistically significant ($p<0.05$) correlation coefficients (Pearson's) are indicated only.

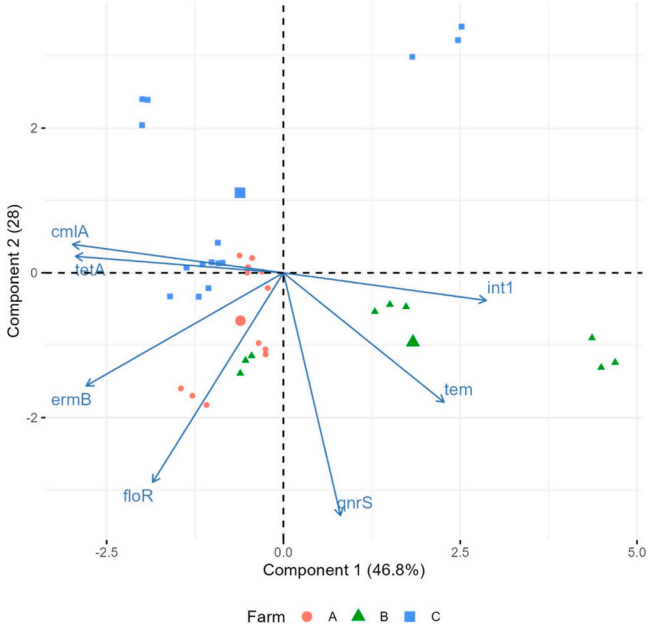

Fig. 3. Principal component analysis on selected manure samples and variables. Individual scores of the two principal components are plotted as points. Individuals have been coloured according to the farm of origin. Each group's average score is represented as an upsized point. Variable contribution to each principal component is represented as a vector.

the most simplified waste management system, resulting in fewer non-uniform samples.

Some ARGs seem to be characteristic of some samples, as *erm*B and *flo*R genes, typical of farm A samples, and *Int*1 TEM, and *qnr*S in manure samples from farm B. However, based on our data, the occurrence of specific ARGs does not appear to be associated with the impact of distinct environments or farming practices. Instead, it seems to be more randomly distributed, as if the variability in HGT of ARGs in this environment, susceptible to frequent fluctuations in conditions, remains unpredictable.

4. Conclusion

Our results highlight the challenge of formulating a clear prediction regarding the spread of ARGs in this environment and, consequently, in the food chain, where it becomes a severe health risk to humans. A clear pattern of ARG presence in the three different farm did not emerge, as each farm's manures had their unique composition. Additionally, no clear relation between ARG abundance and farm managerial practices could be established. Nevertheless, we found some correlation between ARGs dissemination and one of the most prevalent MGE systems in swine livestock environment, namely *integron class 1* gene. Among considered ARG, it has been established a positive correlation between TEM gene and *Int*1, suggesting the hypothesis that TEM may be present in class 1 integrons cassettes. On the contrary, *cml*A and *tet*A correlated negatively with *Int*1, leaving to hypothesize the presence of other genetic mechanisms for ARGs dissemination in this environment.

Future research will be essential to explore both ARGs and bacterial load decrease in manure after applying chemical-physical treatment to abate ARBs viability. Therefore, highlighting the importance of environmental conditions in the diffusion of antibiotic resistance, additional and more in-depth studies will allow the tracking, prediction, and ultimately prevention of environmental damage resulting from the use of wastewater or manure as a fertiliser.

Ethics approval

This article does not contain any studies with human or animal participants performed by any of the authors.

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CRediT authorship contribution statement

Diana Luise: Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Donatella Scarafile:** Writing – review & editing, Writing – original draft, Formal analysis. **Alice Checcucci:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Enrico Buscaroli:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Paola Mattarelli:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Monica Modesto:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Paolo Trevisi:** Writing – review & editing, Funding acquisition, Conceptualization. **Ilaria Braschi:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Francesca Bugli:** Writing – review & editing. **Sonia Blasioli:** Writing – review & editing, Formal analysis, Data curation. **Maura Di Vito:** Writing – review & editing, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Consent for publication

The authors agreed to publish in Environmental Research.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116774.

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