Resistome profiling reveals transmission dynamics of antimicrobial resistance genes from poultry litter to soil and plant

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Abstract

Poultry farming is a major livelihood in South and Southeast Asian economies where it is undergoing rapid intensification to meet the growing human demand for dietary protein. Intensification of poultry production systems is commonly supported by increased antimicrobial drug use, risking greater selection and dissemination of antimicrobial resistance genes (ARGs). Transmission of ARGs within food chains is an emerging threat. Here, we investigated transmission of ARGs from chicken (broiler and layer) litter to soil and *Sorghum bicolor* (L.) Moench plant based on field and pot experiments. The results demonstrate ARGs transmission from poultry litter to plant systems under field as well as experimental pot conditions.

The most common ARGs detected were *cmx, ErmX, ErmF, lnuB, TEM-98* and *TEM-99,* while common microorganisms were represented by *Escherichia coli, Staphylococcus aureus, Enterococcus faecium*, *Pseudomonas aeruginosa,* and *Vibrio cholerae*. Further, we using next generation sequencing and digital PCR assay we also demonstrated transmission of ARGs from poultry litter to root and stem. Poultry litter is used as manure because of its high nitrogen content, risking of transmission of ARGs into human food chains. Evidence indicating ARG transmission illustrate the risk posed by antimicrobial treatment of poultry and could be useful in formulating appropriate strategies to limit ARGs transmission from one value chain to another, improving understanding of impacts on human and environmental health. The research outcome will help in further understanding on the transmission dynamics and threat evaluation of ARGs in poultry linked agriculture system to perpetuate environment and human/animal health.

Keywords: Antibiotic resistance gene, Plant microbiome, Poultry litter, Resistome, and Soil

1. Introduction

Globally, antibiotics are frequently used to cure illnesses and ensure the health and safety of humans as well as animals. They come in a variety of chemical classes that are targeted at diverse animal species and different regions around the world (Barra Caracciolo et al., 2022; de Mesquita Souza Saraiva et al., 2022). Such pervasive, unconstrained usage risks the common occurrence of high-level sub-inhibitory exposure in animal tissues and contamination of the environment, especially in areas associated with animal production (Khan et al., 2013; Lim et al., 2020; Zalewska et al., 2021). Due to numerous human activities, antibiotics have frequently been found in agroecosystems in recent years (Cheng et al., 2019; Kuppusamy et al., 2018). Through the direct release of faecal matter by medicated animals together with the application of contaminated manure, livestock farming has become one of the most frequent sources of antibiotic contamination in soil and water, and the anticipated expansion of global animal husbandry in the forthcoming years could make this scenario worse (Gurmessa et al., 2020). Excessive antimicrobial usage and environmental contamination can intensify selective pressure for development of antimicrobial resistance (AMR) in bacteria, increasing carriage of ARGs. (Gu et al., 2020; Qian et al., 2018; Zalewska et al., 2021).

Residual antibiotics discharged to the environment through animal manure, pharmaceutical and industrial wastewater or sewage, municipal solid waste reaching to the agriculture fields (Bombaywala et al., 2021; Khare, 2023; Le-Minh et al., 2010; Wang et al., 2020). Food safety concern due to residual antibiotics in agro-ecosystem possess a greater concern and challenge (Du and Liu, 2012; Geng et al., 2022; Pan and Chu, 2017) Researchers have reviewed fifty-one antibiotics in thirty-seven species of daily-consumed food crops such as potato, carrot, corn, tomato, lettuce, and wheat. Bioaccumulation of tetracyclines exhibited higher residue levels in plants than quinolones, sulfonamides, and macrolides, with median values ranging from 5.10 to 15.4 μg/kg dry weight while antibiotics likely to accumulate in plant root and their

concentrations in fruits observed to be low. Furthermore, authors speculated that compared with the plants grown in open field condition, accumulation of antibiotics was higher in plant grown under greenhouse condition, probably due to the higher residue levels of antibiotics in the greenhouse soil with intensive application of manure (Geng et al., 2022).

The World Health Organization (WHO) has suggested that we might be on the brink of a post-antibiotic age, where the advent of multidrug-resistant (MDR) microorganisms will reduce the efficacy of antibiotics for treating diseases (Alanis, 2005; Laxminarayan et al., 2013). The pathogens leading to fatality with antibiotic resistance are *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *P. aeruginosa*. (Murray et al., 2022) reported ~1 million deaths accountable to AMR and ~3·6 million fatal causalities related with AMR in 2019. It is now timely to address antimicrobial usage and issues related to AMR on a local to global scale.

Poultry farming has become an indispensable part of the animal husbandry sector especially in the low and middle-income growing economies (Gao et al., 2020; van Boeckel et al., 2017; Walia et al., 2019). Approximately 60 to 80 % of antibiotics used in human medicines today are also used in animal production (Boeckel et al., 2014; Mulchandani et al., 2023; van Boeckel et al., 2015), where misuse selects for antibiotic resistant bacteria in the gastrointestinal tracts of breeding and production animals. Release of waste (manure) will contribute greatly to non-point source contamination of antibiotic resistant bacteria and ARGs (Chee-Sanford et al., 2001). High proportions of residual antibiotics have also been found in animal manures (Khare, 2023; Martínez-Carballo et al., 2007; Mei et al., 2021; Qiao et al., 2012; Xu et al., 2020; Zhang et al., 2019; Zhao et al., 2010a, 2010b). Since farm manures are frequently used as fertilizers to enhance soil fertility, the presence of antibiotics, antibiotic resistant microorganisms and ARGs are priority global concerns regarding their possible impact on humans and other biota. The portfolio of ARGs contained within bacterial populations are collectively referred as the

"resistome" (Binh et al., 2008; Durso et al., 2011; Heuer and Smalla, 2007; Zhu et al., 2013). ARGs might infiltrate the environment through discharge of animal excreta, contaminating soil, water, and crops, influencing resistomes in each environment (Xu et al., 2020).

ARGs are widely distributed in soil and can be spread through horizontal gene transfer (HGT) and vertical gene transfer (VGT) through mating, transformation, and transduction within and across bacterial species (Aminov, 2011; Fan et al., 2019; Qiu et al., 2012). A small number of studies have explored the potential risk to human health from soil-hosted ARGs, with examples vegetables harvested after growth in soil that had been treated with manure (Han and Andrade, 2005; Jiang et al., 2011; Wang et al., 2015; Yang et al., 2016). Manure-borne ARGs might utilize soil microbiota as vehicles to migrate to the endospheric region of plants (Afzal et al., 2019; Xu et al., 2021), but integrated perspectives on dissemination of ARGs harbouring microbial communities in soil-plant systems have been limited (Ashbolt et al., 2013; Zhang et al., 2019).

The likelihood of antibiotic resistant bacteria (ARB) and multi-antibiotic resistant bacteria (MARB) transfer through manure to edible plant parts during soil fertilization is not clear. Therefore, an investigation into prevalence of ARGs containing microbial communities from its possible source to the plant system is logical to conceptualize a better understanding about pathways of ARG transmission in soil-plant environments. With the emergence of high throughput sequencing technologies with improved bioinformatics tools, it is now possible to detect and define entire microbial resistome profile in diverse habitats (Crofts et al., 2017; Lee et al., 2021). Here, we employ high throughput sequencing strategies to assess whether using poultry manure as an organic fertilizer can increase plant reservoirs of ARGs. The present investigation therefore aimed to 1) detect the transmission of ARGs from poultry manure to, soil and from soil to plant tissues, and, 2) to establish relationships, if any, among microbial

communities detected in different microhabitats (poultry litter, soil and plant) in pot and field-based studies.

2. Materials and methods

2.1.Experimental site and study design

The experiments were conducted during July-November, 2021. The sampling site was located at the farmer field at Babatpur (Varanasi, Uttar Pradesh) in India (25˚24' N, 82˚50' E, 83m height above mean sea level). The region has a seasonal tropical monsoonal climate with an average rainfall of 1110 mm annually and minimum to maximum temperature ranging between 8 - 22 ˚C (in January) and 28 - 42 ˚C (in June). The soil is well drained, inceptisol, silty loam (sand 31%; silt 63%, clay 2%) with 0.63% organic carbon (C), 0.14 % total nitrogen (N) and pH 7.7. Three distinct sub-sites were selected with known history of receiving poultry (broiler and layer) litter as organic manure from the past several years; together with a fourth, site plants were grown conventionally without poultry litter as a control.

The experimental plots were designed in randomized complete block design (RBD) including 3 blocks (5m × 3m) per treatment with 1m gaps. Each plot was thoroughly watered and *Sorghum bicolor* (L.) Moench seeds were surface sterilized before sowing as per described elsewhere with minor modifications (Mareque et al., 2015). Briefly, seeds were washed with tap water 2-3 times, incubated in 70% ethanol for 5 minutes, then surface disinfected by incubation in 4% perchloric acid ($HClO_4$) for 15 minutes, incubated again in 70% ethanol for 3 minutes, and eventually rinsed three times with sterile deionized water.

A separate pot experiment was set up in parallel to establish and validate the transmission dynamics of ARGs in different plant compartments (root and stem) for comparison the field experiment under more controlled conditions The pot experiment followed the same design the field study and was completed in triplicate.. Collected bulk soils from the same agricultural

142 plots were passed through a 2 mm mesh sieve to remove plant debris and used in the pot
143 experiment. Each clay pot (diameter: 30 cm; height: 25 cm) contained ~ 4.0 kg soil and were
144 watered and incubated overnight, followed by sowing of surface sterilized seeds of *Sorghum*
145 *bicolor* (L.). The pots and plots were watered on every third day depending upon its water-
146 holding capacity. Pesticides and fungicides were not applied during the experiment and weeds,
147 if any, were removed manually.

148 **2.2. Soil and poultry manure sampling**

149 Rhizospheric soil samples (0-20 cm depth) in triplicate were taken randomly just after
150 flowering stage of the plant (~55-60 days after sowing of crop) from each treatment. Normally
151 farmers harvest crops at this stage as a green fodder to animals. Plants were uprooted with
152 soils adhered to root systems. Extra soils were nipped out by squeezing the plant roots. By
153 tapping the plant root, rhizospheric soils were collected (Vishwakarma and Dubey, 2007). Soil
154 samples were mixed and homogenized, sieved (2mm mesh) to remove plant debris, transported
155 to the laboratory and stored at -20˚C until further processing. Soil sampling from pots was
156 conducted in the same manner. Poultry litter samples were collected from the broiler (a flock
157 of ~2,000 chickens) and layer (~10,000 chickens) chicken farms whose manures were used in
158 this study. Litters are generally dumped for a longer period as a stock. Due to storage, the upper
159 surface was dried, enabling the removal of upper crust and slightly moisture containing litters
160 were retained and used as manure as well as for DNA extraction. Litter samples were collected
161 from 10-25cm depth using sterilized spatula, stored in plastic bags aseptically, transported to
162 the laboratory and were stored at -20˚C prior to DNA isolation or application as manure in
163 experimental field plots and pots within 24h of collection.

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2.3. Plant sampling and pre-treatment

Sorghum bicolor (*L*.) seeds were sown in July 2021 in experimental field plots and pots treated with different poultry manure as well as control plots/pots (devoid of poultry manure application). Crops were managed as per standard farmer practice (Roy and Barik, 2016). Root and stem samples were collected from plant at the time of maturation (November 2021), stored in plastic bags aseptically, and transported to the laboratory for downstream analyses. Plant samples were surface sterilized prior to extraction of total endophytic genomic DNA as described elsewhere with minor modification (Mendes et al., 2007). Briefly, roots and stems collected from *Sorghum* plants in each plot were washed in running tap water, then washed thrice with sterile deionised water to eliminate adhering soil particles. Subsequently, plant samples were washed sequentially with 70 % ethanol solution (10 minutes), 4 % (v/v) sodium perchlorate solution (5 minutes), 70% ethanol solution (2 minutes), then rinsed 5-7 times with sterile deionised water. An aliquot of 100µL from each final wash was plated on Nutrient Agar (NA) plates to confirm the absence of any residual surface bacteria. Plates were incubated at 28°C for 2-4 days. Sterilization of the samples (pieces of roots and stems) was found to be successful, as no growth was observed on the nutrient agar plates. Sterilized plant roots and stems were kept at 4 °C for further study.

2.4. Total genomic DNA isolation

Total genomic DNA was extracted from all the samples using DNeasy PowerSoil genomic DNA isolation kit (Qiagen, Germany) following the manufacturer's protocol **(Supp. Table 1)**. Genomic DNA quality and quantity was assessed using a Qubit 4 Fluorometer (Thermo Fisher Scientific, MA, USA) and confirmed by gel electrophoresis 0.8% (w/v) agarose gel (Brand/Source/Made) prepared in Tris-Acetate-EDTA (TAE) buffer and visualised in a Gel-

189 Doc system (BioRad, Germany). Extracted genomic DNA was preserved at -20 °C until further
190 processing.

191 **2.5. Library preparation for ARGs sequencing**

192 The Ion AmpliSeq AMR Research Panel (ThermoFisher Scientific, USA) (size: 748.82 kb)
193 comprised of two primer pools targeting 408 and 407 amplicons, respectively, in to evaluate
194 the presence of 478 ARGs (Soni et al., 2022). The AMR panel used in this study included
195 additional primers specific for 17 ARGs known to confer resistance against colistin (mobile
196 colistin resistance genes, *mcr*) and quinoline antibiotics. Panel characteristics included a
197 maximum 241bp, minimum size of 73bp, average size of 193bp, and median size of 205bp.

198 After quantification of the total genomic DNA, the template was diluted and for each
199 sample, 20 ng was used as an input for the library preparation. AmpliSeq libraries were
200 constructed using the AmpliSeq™ Library PLUS for Illumina kit (Ilumina, USA) following
201 the manufacturer's instructions. All libraries were quality checked using an Agilent
202 Bioanalyzer 2100 (Agilent Technologies, USA) and quantified using a Qubit™ dsDNA HS
203 Assay kit (Thermo Fisher Scientific, MA, USA) kit. An Illumina MiSeq platform was
204 employed for sequencing, using a 500 cycle chemistry availing MiSeq Reagent Kit v2 (2x250
205 bp read length).

206 **2.6.Data pre-processing and bioinformatics analysis**

207 In total of 59 samples (field stem control, FS2 was in duplicate) were sequenced on the
208 Illumina MiSeq sequencing platform, representing each experimental group in triplicate,
209 generating 4.09 GB data in total. The raw data was quality checked using FastQC (Andrews
210 and others, 2010) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and filtered
211 withan average quality score ≥25 using PrinSeq-Lite v0.20.4 (Schmieder and Edwards,

2011). Cleaned sequencing reads were analysed for the detection and identification of ARGs using the Resistance Gene Identifier (RGI) v5.2.1 on our local server and using the Comprehensive Antibiotic Resistance Database (CARD) v3.2.4 (Alcock et al., 2020). Open Reading Frame (ORF) prediction was performed using Prodigal (Hyatt et al., 2010) and homology search was performed using DIAMOND version 0.8.36 (Buchfink et al., 2015). We used DIAMOND version 0.8.36 (Buchfink et al., 2015) aligner with 95% identity with loose hit criteria using E-value < 5.234390e-02. Low sequence quality option was selected in Prodigal version 2.6.3 (Hyatt et al., 2010) anonymous mode for open reading frame prediction, supporting calls of partial AMR genes from short or low quality contigs (ignoring those less than 30 bp). The ARGs were obtained from the annotation files of RGI output in text file. Furthermore, for individual samples compiled to generate the cumulative ARGs abundance profile for all the samples. Then, the relative abundance profile and statistical analysis was carried out using STAMP software version 2.1.3 (Parks et al., 2014).

The obtained ARGs were analysed for the dominant antibiotic drug classes and involved resistance mechanisms along with the resistome and pathogen of origin analysis. The Venn diagram analysis using online server (https://bioinformatics.psb.ugent.be/webtools/Venn/) was performed for the identification of the unique ARGs present in the selected experimental groups and to know the genes detected in the poultry litter, plant root, and stem parts. The resistome profile of the observed pathogen of origin analysis was generated based on the hits obtained from the CARD database v3.2.4 (Alcock et al., 2020).

2.7. Statistical analysis

Statistical analysis was performed in the statistical analysis of taxonomic and functional profiles (STAMP) software version 2.1.3 (Parks et al., 2014) to identify significant ARGs (p-value $\leq$0.05, with Benjamini-Hochberg FDR) associationa in ARG occurrence in poultry litter

(broiler and layer), soil, and plant (root and stem) samples. Heatmap plots for the detected bacterial pathogens of origin were generated using ClustVis (Metsalu and Vilo, 2015). Venn diagrams were generated using the online server at https://bioinformatics.psb.ugent.be/webtools/Venn/.

2.8 Validation of selected ARGs using digital PCR (dPCR)

The ARGs which were selected for the validations that were based on their presence in the poultry litter, treated soil and plant (root and stem) however must absent in the control samples [soil and plant (root and stem)]. Total five genes viz. *cmx, ErmF, ErmX, InuB,* and *ErmF.1* were selected for the validation purpose. The primers that we used for the validation are same which are there in the AMR panel, which we used for the resistome analysis **(Supp. Table S6)**. For digital PCR assay, we pooled the genomic DNA of each triplicate sample in one pool for each set (to reduce the cost) resulting in 20 pooled experimental samples representing one each from broiler and layer litter samples and nine each from field and pot experiment group (**Supp. Table S2**).

The primers were initially checked and optimised for the PCR conditions for gene amplification from the pooled genomic DNA samples. Later on, using the same PCR conditions dPCR assay was performed to validate the ARGs identified using the NGS. We used QIAcuity Digital PCR System (Qiagen, Germany) 96-well 8.5K nanoplate. The reaction mixture for the dPCR assay comprise of 4 µL EvaGreen master mix (Qiagen, Germany), 3 µL nuclease free water, 0.5 µL each forward and reverse primers (5pmol), 3 µL DNA template (total 9 ng) and 1µL EcoR1 (0.25 U/µL) high fidelity restriction enzyme (New England Biolabs, UK) making the total system of 12 µL. The dPCR cycling conditions were set as initial annealing at 95 °C for 5 minutes, 95 °C for 30 seconds, 58 °C for 20 seconds, 72 °C for 30 seconds for 30 cycles, and final denaturation 40 °C for 5 minutes. After completion of PCR run, the plates were

scanned using the green channel by the QIAcuity Software Suite (v.2.1.8) provided along with the instrument. The data were analysed and copy number per ng DNA was calculated as per the QIAcuity® User Manual Extension, 2021 (https://www.qiagen.com/us/resources/download.aspx?id=5d19083d-fa10-4ed2-88a0-2953d9947e0c&lang=en) as described in our previous study (Travadi et al., 2022). In dPCR, all the samples were run in duplicate and standard deviation error in the copy number of detected ARGs per ng DNA sample calculated in the MS Office 2016 Excel and plots were generated using GraphPad Prism version 5.0 (Motulsky, 2007).

3. Results and discussion

Present communication deciphers a relevant contemporary issue about spreading dynamics of ARGs in agro-ecosystem using updated molecular approaches *viz*. next generation sequencing and dPCR. Objectives were achieved through dual strategies i.e., pot and field-based studies, each having their own significance. Prior allows the microbial assessment in controlled regime without being interrupted to diverse environmental factors, whereas the later strategy facilitates conducting experiments under natural conditions where probability of ecological validity is more pronounced. Manure-borne ARGs might use soil bacteria as a vehicle enabling them to migrate into the plant endosphere and ARGs propagated into these soil bacteria could readily invade the plants (Mišić et al., 2022). It was surprising to understand their origin and transmission dynamics and mechanism of gene transfer such as plasmid, transposons, mobile genetic elements and horizontal gene transfer might be involved and play crucial role in the transmission of the detected ARGs (Huang et al., 2022). However, the majority of earlier research concentrated on specific plant microbiome regions and lacked an integrated perspective on how antibiotic resistance spreads in the soil-plant system (Yu-Jing Zhang, Hang-Wei Hu, a Qing-Lin Chen, Hui Yan, Jun-Tao Wang, Deli Chen, 2020).

The results obtained in the present study, further establish the hypothesis of extensive ARGs ingress in the agro-bio-ecosystem and therefore, immediate attention need to be focus through various measures in order to reduce the selective pressure on microbes against residual antibiotics in the environment. In this study, we performed AMR genes amplicon sequencing using illumina MiSeq system. We generated total 4.4 million reads corresponds to average 0.75 million reads per sample. **Supp. Table 3** comprise the output of sequencing run. ARGs were identified using by searching homology of the reads against the CARD database v3.2.4 (Alcock et al., 2020).

3.1. Resistome profile of the broiler and layer litter samples

In this research study, sequencing analysis of the ARGs in poultry broiler litter revealed dominance of *QnrS15* (12.66±0.13%), *APH(3')-IIIa* (7.07±3.26%), *TEM-99* (6.32±0.47%), *tet(Z)* (4.64±3.53%), *APH(6)-Id* (4.53±1.15%), *tetW* (4.41±4.20%), *TEM-98* (4.28±1.11%), *ErmX* (4.24±1.11%), *tet(A)* (4.15±3.28%). Among the total ARGs, top fifteen genes accounting for an overall 67.12% relative abundance while, the rest were less than 2.00% abundance. (**Supp. Table S4)**. The pathogen of origin associated with these ARGs are *E. coli* (31.76±1.04%), *Campylobacter coli* (8.43±2.30%), *P. aeruginosa* (6.49±2.51%), *Corynebacterium glutamicum* (5.84±4.50%), *Butyrivibrio fibrisolvens* (4.41±4.20%), Plasmid pNG2 (4.24±1.11%), *Shigella sonnei* (4.15±3.28%), *Bacteroides fragilis* (3.08±1.41%), *C. jejuni* (2.74±1.47%), and *Serratia marcescens* (2.61±1.89%) were in relative proportion >2.0%. Similarly, in the poultry layer litter *tetM* (33.71±0.88%), *ErmF* (9.84±1.39%), *APH(6)-Id* (6.12±0.73%), *aadS* (5.39±0.80%), *tetQ* (5.12±1.58%), and *vgaE* (4.17±1.00%), were found in higher proportion. The resistome profile for the layer litter was represented by *Erysipelothrix rhusiopathiae* (33.71±0.88%), *B. fragilis* (18.67±2.65%), *P. aeruginosa* (8.29±0.85%), *S. aureus* (8±1.65%), Transposon Tn4551 (5.39±0.80%), *C. glutamicum* (2.77±0.39%), *C. jejuni*

(2.66±0.34%), *Salmonella enterica* (2.3±0.24%), *Bacteroides coprosuis* (1.93±0.51%), *E. faecium* (1.79±0.34%), *S. sonnei* (1.66±0.13%), and *C. coli* (1.56±0.13%).

The dominant gene *QnrS15* is reported to be a plasmid-mediated and known to provide resistance against the quinolone antibiotics, while *APH(3')-IIIa* is also a plasmid-encoded aminoglycoside phosphotransferase providing resistance against aminoglycoside antibiotics (Trieu-Cuot and Courvalin, 1983). Similarly, *TetZ* is a tetracycline efflux protein found in Gram-positive bacteria and associated with plasmid DNA (Roberts, 2005). It would be interesting to understand residual load of antibiotics used in the poultry sector and we further speculate that the dominant ARGs classes detected in the poultry litter such as quinolones, tetracycline, beta-lactams and aminoglycoside. Recent studies reported the antimicrobial use (AMU) lead by tetracyclines were the most commonly used antimicrobial (overall 33,305 tonnes) and were predicted to increase by 9% by 2030. However, AMU intensity per antimicrobial class varied by country. Thailand had the highest proportion for penicillins, while Chile had maximum proportion for amphenicols (Mulchandani et al., 2023).

Previous studies carried out to understand the risk assessment and enrichment of the poultry manure induced ARGs in soil, food crops and agriculture environment reported that antimicrobial resistance against tetracycline, aminoglycosides, and sulphonamides as the most common antibiotics drug classes (Buta-Hubeny et al., 2022; Jadeja and Worrich, 2022). Research studies have revealed that agricultural soils fertilized with organic matter obtained from animals treated with antibiotics have enhanced naturally existing ARGs as well as introduced novel ARGs into the accompanying soil microbial population (Chen et al., 2016; Looft et al., 2012; Rieke et al., 2018; Udikovic-Kolic et al., 2014; Yu et al., 2017). *Tet(M)* is a ribosomal protection protein that provide resistance against tetracycline antibiotics. It is found on transposable DNA elements and which is known for horizontal transfer between bacterial species (Akhtar et al., 2009). Another ARG, *ErmF* known to confer the macrolides,

lincosamides and streptogramin B (MLSB) phenotype. Microorganisms with a constitutive MLSB phenotype express high-level of cross-resistance to MLSB (Kangaba et al., 2015). The comparative analysis between the broiler and layer litter resistome profiles revealed overall 58.80% ARGs shared between broiler and layer litter, while, 14.90% and 26.40% were exclusively present in the broiler and layer litter, respectively **(Supp. Fig. S1**).

Interestingly, the relative proportion of *tetM* (33.71±0.88%) and *ErmF* (9.84±1.39%) genes were found to be more in layer litter as compare to broiler litter, 2.32±0.86%) and 1.23±0.84%, respectively. In contrast to this, *QnrS15* (12.66±0.13%) and *APH(3')-IIIa* (7.07±3.26%) were found to be abundant in broiler litter as compared to the layer litter with relative proportion 0.03±0.01% and 1.07±0.08%, respectively. The difference in ARGs detected in the broiler and layer litter samples might be due to the differences in the dietary regimes. *tetW* (4.41±4.20%) in broiler litter, *tetQ* (5.12±1.58% in layer litter) were found to be either negligible or absent in control samples and not detected in the soil treated with the litter. Similarly, there were several ARGs showing such a phenomenon and it was puzzling to point, which genes are coming from which source, therefore we further selected the genes which were present in the poultry litter, absent in control and showing their further presence in the treatment groups. The probable reason for such observation is might be due to the dilution of the genes harbouring microbes in the soil treated with the litter.

3.2. Transmission of ARGs from broiler litter to plant under field experiment

The relative abundance of ARGs in soil treated with broiler litter is represented by *TEM-229* (34.07±15.70%), *TEM-98* (30.12±12.52%), *tetM* (5.03±6.99%), *tetX* (3.55±4.01%), *APH(6)-Id* (3.34±3.66%), *aadS* (2.96±4.14%), *APH(3')-IIa* (2.26±0.85%), and *ErmF* (2.01±2.77%). Similarly, ARGs in the root samples collected from the plants treated with broiler litter were dominated by *TEM-98* (33.27±24.20%), *TEM-229* (18.24±13%), *MIR-7*

(11.14±14.76%), *MIR-22* (8.00±11.31%), *TEM-99* (4.28±2.75%), and *NDM-31* (3.29±3.34%). Overall, ARGs with relative abundance ≥ 2.0 % accounted for the cumulative abundance of 80.30% in the field root samples treated with broiler litter. (Zhang et al., 2020) have analysed the microbial diversity and reported transmission of *aadE*, *tet(34)*, and *vanSB* as the shared ARGs along with the bacterial communities among leaves of the four vegetable crops.

Pathogens of origin associated with the resistome profile of the plant root treated with broiler litter under field condition were *E. coli* (40.74±23.26%), *Enterobacter cloacae* (21.36±27.35%), Bacteria (18.46±13.06%), *Citrobacter werkmanii* (3.29±3.34%), *Halobacterium salinarum* (2.56±2.92%), *P. aeruginosa* (2.40±2.94%), Plasmid pGT633 (1.19±1.08%), *S. aureus* (1.19±1.06%), *A. baumannii* (1.01±0.69%), and *Enterobacter asburiae* (1.00±1.42%) **(Supp. Fig. S2)**. *TEM* variants are resistant and reported for clinical resistance against beta-lactam-lactamase antibiotics while *MIR* beta-lactamases are plasmid-mediated beta-lactamases that confer resistance to oxyimino- and alpha-methoxy beta-lactams antibiotics in clinical isolates of *K. pneumoniae* (Papanicolaou et al., 1990). The relative abundance of the ARGs that were observed higher in proportion in the stem samples of the plants treated with broiler litter are *TEM-98* (59.20±5.08%), *TEM-229* (13.66±13.63%), *TEM-99* (5.67±3.62%), *NDM-31* (4.54±3.2%), and *OqxB* (2.24±1.93%). While, the microorganism associated were *E. coli* (69.06±6.78%), Bacteria (13.74±13.62%), *C. werkmanii* (4.54±3.2%), *P. aeruginosa* (2.66±0.96%), *Klebsiella variicola* (1.75±1.07%), *A. baumannii* (0.92±0.41%), *S. aureus* (0.92±0.78%), and Plasmid pNG2 (0.92±0.63%) **(Supp. Fig. S3)**. The *oqxAB* encode for a multidrug efflux pump reported from human clinical isolates of *Enterobacteriaceae* (Kim et al., 2009).

Furthermore, ARGs which might have been transmitted from poultry litter to plant were identified. In the field experiment, we found total four ARGs, which were exclusively present in the broiler litter, stem and/or root of the test samples and they may or may not be present in

the soil treated with the broiler litter. However, they were absent in all the controls i.e. the control soil and stem and or root without application of broiler litter. We found that *cmx*, *QnrVC3*, *ErmX*, and *dfrD* and *cmx*, *SAT-4*, *lnuB* and *ErmF* which might have been travelled from litter to root and stem, respectively. Moreover, *cmx* was common in both root and stem **(Fig. 1a, 1b, and 1c)**. The presence of the ARGs in the litter sample but their absence in the litter treated soil can be explained by the fact that the litter sample gets dilutes in the soil. Further, their reappearance in the stem and/or root can be explained by the facts that these genes or genes carrying bacteria further accumulated and enriched in the plant system.

3.3 Transmission of ARGs from broiler litter to plant in pot experiment

The above experiment was also performed under the controlled condition. The pot experiment revealed the dominance of *TEM-229* (11.80±9.18%), *ErmX* (7.31±2.90%), *TEM-98* (7.08±4.05%), *APH(6)-Id* (5.85±2.63%), *dfrA15b* (4.95±3.66%), *tetX* (4.65±3.41%), *tet(Z)* (4.60±4.00%), and *aadS* (4.19±4.99%) ARGs in the soil treated with broiler litter. Similarly, *E. coli* (18.73±5.12%), Bacteria (11.8±9.18%), *P. aeruginosa* (8.41±4.90%), Plasmid pNG2 (7.31±2.90%), *C. glutamicum* (5.71±4.27%), *B. fragilis* (5.21±3.84%), *S. enterica* (5.08±3.66%), Transposon Tn4551 (4.19±4.99%), and *S. aureus* (3.00±1.69%) were observed as the dominant pathogen of origin in the soil samples treated with broiler litter in the pot experiment. Similarly, in the root samples treated with the broiler litter, *TEM-98* (25.94±11.75%), *TEM-229* (20.17±10.43%), *TEM-99* (9.38±8.09%), *LEN-28* (3.63±3.10%), *OXA-935* (3.58±2.26%), *ACT-68* (2.99±1.67%), *NDM-31* (2.97±2.37%), and *MIR-7* (2.89±4.08%) were abundant. The observed pathogen of origin in the root samples treated with broiler litter is represented by *E. coli* (38.54±11.72%), Bacteria (20.27±10.44%), *P. aeruginosa* (7.15±2.03%), *E. cloacae* (7.07±8.36%), *K. variicola* (3.67±3.06%), *E. asburiae* (2.99±1.67%), and *C. werkmanii* (2.97±2.37%) **(Supp. Fig. S4)**. ARGs transmission in plants

has been shown to be mediated from manure as source of bacteria harbouring mobile genetic elements (MGEs), including plasmids, transposons, and integrons (Heuer and Smalla, 2007; Zhu et al., 2013). Therefore, there may be potential threats to living biota and the surrounding environment assuming ARGs grow exponentially or remain pervasive in livestock ecosystems (Negreanu et al., 2012; Wang et al., 2015).

The broiler litter treated plant stem samples revealed the dominant ARGs enriched by *TEM-98* (58.52±2.18%), *TEM-229* (36.19±3.40%), *TEM-99* (1.70±0.55%), *Halobacterium halobium* 23S rRNA mutation conferring resistance to chloramphenicol (0.46±0.20%), *E. coli mdfA* (0.19±0.03%), *APH(6)-Id* (0.18±0.20%), *smeD* (0.18±0.20%), *TEM-70* (0.15±0.12%), *TEM-182* (0.11±0.06%), and *P. aeruginosa catB7* (0.10±0.07%). Similarly, detected pathogen of origin in the plant stem samples were *E. coli* (61.08±2.41%), Bacteria (36.19±3.40%), *P. aeruginosa* (0.66±0.32%), *H. salinarum* (0.46±0.20%), *Stenotrophomonas maltophilia* (0.18±0.20%), *A. baumannii* (0.12±0.11%), *K. variicola* (0.12±0.05%), *S. marcescens* (0.11±0.08%), and *Haemophilus parainfluenzae* (0.11±0.06%). The ARGs *aadS, APH(4)-Ia,* and *Erm(36), ErmQ, SAT-4, Lactobacillus reuteri cat-TC, Thermus thermophilus* 23s rRNA conferring resistance to pleuromutilin antibiotics, and *APH(3')-Via* were found to be transmitted from broiler litter to plant parts **(Fig 1d, 1e and 1f)**. If, we compare the field and pot experiment, only *SAT-4* was found to be common in both the experimental setup.

3.4 Transmission of ARGs from layer litter to plant in field experiment

The relative abundance of ARGs found to be enriched in the soil samples which were treated with layer litter indicated that *TEM-98* (22.54±14.01%), *TEM-229* (16.04±8.43%), *tet(C)* (6.33±7.37%), *APH(6)-Id* (5.49±4.45%), *tetX* (5.25±7.43%), *ErmT* (3.91±5.10%), *oleB* (3.70±5.24%), *aadS* (3.43±4.86%), and *APH(3')-IIa* (3.10±4.39%). Furthermore, ARGs *TEM-98* (39.81±6.18%), *TEM-229* (22.02±16.36%), *MIR-7* (7.81±10.47%), *TEM-99* (6.47±6.09%),

NDM-31 (2.22±1.37%), and *ADC-151* (2.05±2.90%) were enriched in the plant root samples treated with layer litter in the field experiment. Layer chicken litter treated field setup revealed top five ARGs in the plant root samples, accounting for 79.94% cumulative abundance. While in the pot experiment, plant root samples treated with layer litter indicated the enrichment of accounting for more than eighty-five percent cumulative abundance. The dominant pathogens were *E. coli* (49.45±9.30%), Bacteria (22.12±16.35%), *E. cloacae* (8.83±11.43%), *P. aeruginosa* (5.72±4.67%), *Acinetobacter pittii* (2.47±3.50%), *C. werkmanii* (2.22±1.37%), *A. baumannii* (1.21±0.59%), and *E. asburiae* (1.06±1.50%) in the plant root samples. The majority of antibiotics administered are not completely absorbed in the chicken intestine, and up to 90% of the amount injected can be expelled in the faeces (Kumar et al., 2005). This could have further implications for bio-augmentation of the ARGs in the food chain through the poultry sector. In recent study major bacterial pathogens for the broiler poultry were *E. coli*, *Salmonella* spp., *Clostridium* spp., and *Campylobacter* spp. (Fathima et al., 2022; Kim et al., 2022) which is supports with this study.

Similarly, the ARGs *ErmT* (25.40±17.42%), *TEM-99* (21.43±19.73%), *TEM-98* (17.46±14.59%), *TEM-229* (6.35±5.94%), *ADC-207* (5.56±7.86%), *tetM* (4.76±6.73%), *CTX-M-244* (4.76±6.73%), *tetX* (4.76±6.73%), and *Erm(36)* (4.76±6.73%) were observed in the stem samples treated with layer litter. *E. coli* (43.65±29.12%), Plasmid pGT633 (25.40±17.42%), *A. pittii* (5.56±7.86%), *B. fragilis* (4.76±6.73%), *E. rhusiopathiae* (4.76±6.73%), *Lactobacillus reuteri* (4.76±6.73%), and *Micrococcus luteus* (4.76±6.73%) were observed dominant pathogen of origin in stem samples treated with layer litter in field condition **(Supp. Fig. S5)**. Here, *cmx, oqxA, LEN-27,* and *ErmX* were found to be transmitted from layer litter to the roots of the plant. However, none of the genes were found in the stem **(Fig. 2a, 2b, and 2c)**.

3.5 Transmission of ARGs from layer litter to plant in pot experiment

In the pot experiment, *tetM* (16.70±2.80%), *vgaE* (11.23±2.22%), *APH(6)-Id* (7.41±2.82%), *tet(L)* (6.70±2.22%), *dfrG* (6.06±1.59%), *aadS* (5.95±2.99%), and *APH(3')-IIIa* (4.53±0.85%) were found to be dominant in the pot soils treated with layer litter. The associated microorganisms were characterized by *S. aureus* (18.72±3.66%), *E. rhusiopathiae* (16.70±2.80%), *P. aeruginosa* (9.19±2.90%), *C. coli* (6.74±0.82%), *Geobacillus stearothermophilus* (6.7±2.22%), Transposon Tn4551 (5.95±2.99%), *C. glutamicum* (4.97±1.26%), *B. fragilis* (3.35±0.91%), and *Listeria monocytogenes* (3.32±0.86%) in the pot soil treated with layer litter. The root samples treated with layer litter were dominanted by *ErmT* (21.94±17.20%), *TEM-98* (21.42±18.67%), *Halobacterium halobium* 23S rRNA mutation conferring resistance to chloramphenicol (14.70±13.70%), *TEM-229* (12.88±14.18%), *Chlamydomonas reinhardtii* 16S rRNA (rrnS) mutation conferring resistance to streptomycin (3.24±4.44%), *TEM-99* (3.20±2.35%), and *Mycoplasma hominis* 23S rRNA with mutation conferring resistance to macrolide antibiotics (3.17±4.49%). The manure-borne ARB that infiltrated plant tissues may pass the carrying ARGs to the endophytic bacteria of the plant via horizontal gene transfer (HGT) (Sørensen et al., 2005; Xu et al., 2021). Therefore, prevalence and cryptic transmission of the ARGs in the different soil-plant compartments from manure-mediated source is necessary to understand the probable molecular mechanism in agro-ecosystem.

Similarly, pathogen of origin in the plant root samples from the layer litter treated samples were represented by *E. coli* (28.92±21.01%), Plasmid pGT633 (21.94±17.20%), *H. salinarum* (14.70±13.70%), Bacteria (12.94±14.27%), *C. reinhardtii* (6.22±5.89%), *M. hominis* (3.17±4.49%), and *Thermus thermophiles* (2.25±1.95%).The ARGs which were detected in the layer litter treated plant stem samples were represented by *TEM-98* (64.16±7.40%), *TEM-229* (27.74±10.98%), *TEM-99* (1.98±0.67%), *smeD* (1.53±1.73%), *H. halobium* 23S rRNA mutation conferring resistance to chloramphenicol (0.52±0.32%), *E. coli*

mdfA (0.47±0.14%), *OXA-937* (0.31±0.32%), *TEM-237* (0.21±0.20%), *P. aeruginosa catB7* (0.21±0.26%), and *APH(6)-Id* (0.14±0.07%). The detected pathogen of origin was represented by *E. coli* (67.38±7.79%), Bacteria (27.78±10.95%), *S. maltophilia* (1.53±1.73%), *P. aeruginosa* (1.00±0.78%), *H. salinarum* (0.52±0.32%), *S. aureus* (0.34±0.23%), *C. reinhardtii* (0.16±0.10%), and *A. baumannii* (0.13±0.15%). The ARGs *AAC(3)-IV, tet(G), dfrA15b, tetO, AAC(6')-Ie-APH(2'')-Ia,* and *NDM-30* were found to be possibly transmitted from layer litter to plant **(Fig 2d, 2e, and 2f)**. They were present in the layer litter, soil treated with layer litter and plant root samples.

We speculate that the selected genes may further get biomagnified in the different experimental compartments and provide interesting insights in the dynamic nature of AMR transmission from litter to the plant. Therefore, a thorough investigation of plant resistomes is of essence to develop a clearer understanding of the pathways of ARG transmission and will give significant scientific support for the incorporation of natural resistomes in management methods and framework for evaluating the risks to human health (Achard et al., 2005; Xu et al., 2021). According to (Chen et al., 2019) naturally occurring concentrated soil ARGs can migrate into plants via the rhizosphere microbiota. (Zhang et al., 2019) have reported that treatment of cattle manure enhanced the proportion of ARGs in plant roots, whilst the application of poultry manure augmented ARGs into the different soil and plant microbiome compartments.

3.6. Digital polymerase chain reaction (dPCR) for validation of selected ARGs

The antimicrobial resistance genes (*ermF, cmx, InuB*, and *ermX*, and *ermF.1*) selected for the validation were found to be enriched in plant root and stem compartments of the plant where the litter was applied however their copies in the control samples were almost negligible **(Fig. 3 and Supp. Table 5)**. The ARGs represented by *InuB* (n=3040.70±9.50), *ErmF*

(n=1469.26±23.93), and *ErmF.1* (n=1428.76±62.42) in the layer litter while *cmx* (n=152.82±17.27) and *ErmX* (n=13.15±1.17) were found to be in low copy number (**Fig. 4 and Supp. Table 5**). Further, *cmx* (n=3546.43±75.90), *ErmX* (n=1179.05±12.65), *ErmF* (n=474.85±31.16), *InuB* (n=472.98±23.25), and *ErmF.1* (n=423.91±17.85) were found to be dominant in broiler litter samples. Here 'n' represents gene copy number per ng DNA sample along with ±standard deviation. The load of *InuB* and *ErmX* is higher in layer litter while *cmx* was found to be higher in broiler litter samples. The layer litter treated field samples indicate a similar load of these ARGs in the root and stem samples. While these ARGs in the field samples for the broiler litter were detected in relatively lower copy numbers.

The dPCR assay is highly sensitive and provides accurate quantification of the copy number of genes based on Poisson distribution of the statistical compositional analysis (Travadi et al., 2022). The results were compared for each group and revealed the copy number of genes identified for the selected group. The maximum copy genes in the broiler litter were represented by *cmx*, *ErmX*, *ErmF*, *InuB* and *ErmF.1*. Furthermore, these ARGs were not detected in the different control groups while observed in the treatment samples in the field experiment samples for broiler, and layer litter samples. Similarly, the copy number of these ARG detected in the layer litter is represented by *InuB*, *ErmF*, *ErmF.1*, *cmx*, and *ErmX*. The experiment with layer litter samples revealed the higher copies in the stem compartment as compared to the broiler litter group in both the field as well as the pot experimental setup. Therefore, we speculate that the ARGs transmission from the layer litter seems to be highly efficient compared to the broiler litter as demonstrated in the experimental results followed by validation through dPCR.

Conclusions

This study concludes that poultry litter can acts as source of ARGs that can spread into field soils and plants. ARGs like *cmx*, *ErmX*, *ErmF*, *lnuB*, *TEM-98* and *TEM-99* with microorganisms such as *E. coli*, *S. aureus*, *E. faecium*, *P. aeruginosa*, and *V. cholerae* were found to be shared in poultry litter-treated soil and plants. Furthermore, ARG profile of the different experimental samples in this research study, indicate the role plasmid mediated horizontal gene transfer, transposon elements and other biological routes in the microbial communities in response to the broiler and layer litter application. However, it requires further investigation and future deliberations to understand the ingress of ARGs in the agriculture ecosystem. The research study provides considerable indication of the transmission of ARGs from poultry litter into an agro-ecosystem, emphasizing the importance of responsible antimicrobial use in poultry production. Understanding the risk of ARG transmission should support formulation of strategies to incorporate natural resistomes into agro-ecosystems and develop frameworks to evaluate environmental risks due to the spread of AMR.

Data availability: The raw sequencing reads produced in this study have been deposited with the National Centre for Biotechnology Information (NCBI) in the Sequence Read Archive (SRA) with the bio-project accession number PRJNA839566.

Conflicts of interest: The authors declare no competing interests.

Author contribution statement: Animesh Tripathi: Experimentation, Sample Collection, Analysis and Manuscript Writing; **Dinesh Kumar**: Data Analysis, Methodology, and Manuscript Writing; **Priyank Chavda**: Experiments (NGS library preparation, and sequencing); **Dalip Singh Rathore:** Digital PCR assay and Methodology; **Ramesh Pandit**: Data Analysis and Manuscript correction; **Damer Blake:** Review and Editing; **Fiona Tomley:**

Review and Editing; **Madhvi Joshi**: Project Administration, Review & Editing; **Chaitanya G Joshi**: Conceptualization, Funding Acquisition; and **Suresh Kumar Dubey:** Conceptualization, Supervision, Writing, Review & Editing.

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