Environmental Science Advances

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Environ. Sci.: Adv.*, 2025, **4**, 2127

Land application of beef cattle manure facilitates the transmission of antibiotic resistance genes from soil to lettuce

Yuepeng Sun, *[ab] Daniel Snow,[c] Harkamal Walia[d] and Xu Li[b]

The land application of livestock manure can have significant effects on the emergence and proliferation of antibiotic resistance genes (ARGs) and antibiotic residues in the soil–plant ecosystem. While previous studies have documented the effects of manure application on ARGs in either soil or plant compartments, research addressing its impact on ARGs concurrently in both soil and plants remains relatively limited. This study aims to assess the occurrence of ARGs in and on lettuce cultivated in soil with beef cattle manure application. Results showed that seven ARGs (bla_{TEM}, *erm*(B), *erm*(F), *tet*(M), *tet*(O), *tet*(Q), and *tet*(X)) and one class 1 integron–integrase gene (*intI*1) were quantified in both soil and lettuce compartments following manure application. The relative abundance of manure-borne *tet*(M), *tet*(Q), and *tet*(X) was significantly elevated in surface soils (Kruskal–Wallis tests, $p < 0.05$). Notably, *tet* genes increased by 1–3 orders of magnitude within the lettuce endosphere and roots, revealing a potential transmission from soil to lettuce. In contrast, the relative abundance of bla_{TEM}, *erm*(B), and *erm*(F) increased only in the episphere and root but not within the endosphere of lettuce. Additionally, concentrations of tylosin in surface soil showed positive correlations with *tet* genes, suggesting their potential roles in facilitating the ARG proliferation in the soil–plant ecosystem. In summary, this study demonstrates that manure application promoted the transmission of manure-borne ARGs from soil to lettuce, highlighting a potential pathway for human exposure to antimicrobial resistance through the food chain. This finding underscores the need for the development of manure management practices to mitigate ARG spread in agriculture.

Received 9th July 2025
Accepted 20th October 2025

DOI: 10.1039/d5va00204d

rsc.li/esadvances

Environmental significance

The land application of livestock manure facilitates the dissemination of antibiotic resistance genes (ARGs) into agricultural ecosystems, posing significant environmental and public health risks. This study demonstrates that manure application markedly increases the abundance of ARGs (*e.g.*, *tet*(M), *tet*(Q), *tet*(X)) in soil and lettuce, with certain genes enter into the edible endosphere of the plant. Since crops may serve as vectors for ARG transmission to humans, these findings underscore a critical food safety concern, highlighting the potential entry of antibiotic resistance into the food chain.

1 Introduction

While livestock manure can be used as a soil conditioner to provide nutrients and organics to soil, the antibiotic residues and antibiotic resistance genes (ARGs) in land applied manure could pose environmental concerns. Ranging from μg kg^{-1} to mg kg^{-1},[1] the antibiotic residues in livestock manure could pose selective pressure on soil microbiome, shape the resistome in soil,[2] and accumulate in plants.[3] For example, chlortetracycline, sulfamethazine, and tylosin, ranged from 0.3 to 9.3 μg kg^{-1}, were detected in the bulk soils and rhizosphere soils with the land application of beef cattle manure.[4]

Manure-borne ARGs can persist in soil as well as accumulate on and in crops.[5] Studies have shown that manure application increased the abundance and diversity of ARGs in different part of the receiving soil, *e.g.*, bulk soil,[4] surface soil,[5] and rhizosphere soil.[4] The manure-borne ARGs that persist in soil have been reported to originate from various livestock sources, including beef cattle manure,[5] swine manure[6] and poultry litter.[7] ARGs may persist in receiving soil, because manure-borne bacteria carrying ARGs can survive in soil.[8,9] ARGs originating from manure can transfer to soil bacteria *via* horizontal gene transfer (HGT),[10,11] and residual antibiotics in manure can pose selective pressures on soil bacteria.[12] In order to mitigate

[a]*School of Ecology and Environment, Inner Mongolia University, Hohhot 68588, China. E-mail: 111989064@imu.edu.cn*
[b]*Department of Civil and Environmental Engineering, University of Nebraska-Lincoln, Lincoln, Nebraska 68588, USA*
[c]*Nebraska Water Center, University of Nebraska-Lincoln, Lincoln, Nebraska 68588, USA*
[d]*Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, Nebraska 68583, USA*

the potential risk of ARGs, it is important to characterize the prevalence and persistence of ARGs in soil after manure application, as soil ARGs can transmit to crops that are eaten raw.[5]

The ARGs in soil can potentially reach the surface of the edible parts of commercial crops and may even enter the endosphere.[13] ARGs such as tetracycline (*tet*(A), *tet*(B) and *tet*(T)), macrolide (*erm*(B) and *erm*(F)), and sulfonamide (*sul*1) resistance genes have been detected on vegetables, *e.g.*, carrot, cucumber, lettuce, radish, pepper, and tomato planted in manured soil using polymerase chain reaction (PCR).[14] Tetracycline (*tet*(A), *tet*(C), *tet*(G), *tet*(M), *tet*(O) and *tet*(X)) and sulfonamide resistance genes (*sul*1) have been detected in the endophyte of lettuce and endive,[15] possibly due to the transmission of soil microbes carrying intrinsic ARGs to vegetable. Endophytic bacteria isolated from celery, pakchoi, and cucumber grown in soil fertilized with chicken litter exhibited cephalexin resistance.[16] Shotgun metagenomics have shown that manure application can significantly affect the resistome of soil or/and lettuce as well as the shared resistome between these two ecological niches.[17,18] Still, quantitative data on the abundance and distribution of ARGs across in different compartments of soil and vegetables is limited.

The objective of this study was to quantify the ARGs and class 1 integron–integrase gene (*intI*1) in different compartments of the soil (surface soil and rhizosphere soil) and lettuce (episphere, endosphere, and root) following the application of beef cattle manure. The *intI*1 was targeted in this study because it mediates the HGT by enabling the exchange of ARGs within gene cassettes among bacteria, and could serve as a potential marker of anthropogenic pollution.[19] In this greenhouse study, soil and plant samples collected at various growth phases of lettuce were quantified for ARGs using quantitative PCR (qPCR) and for residual antibiotics using liquid chromatography with tandem mass spectrometry. The correlations between ARGs and *intI*1 as well as those between ARGs and antibiotics were analyzed. With the results reported in the form of absolute abundance of ARGs and antibiotics, the findings of this study will contribute to the literature data that will support more accurate risk assessment associated with manure application for leaf-green vegetables at the pre-harvest stage.

2 Material and methods

2.1 Soil–lettuce ecosystems

Seeds of leaf lettuce (cultivar Green Salad Bowl, *Lactuca sativa*) were grown in rhizoboxes in a Biosafety Level 2 greenhouse at the University of Nebraska – Lincoln. Rhizoboxes (30 cm × 20 cm × 5 cm, $H \times L \times W$) were filled with sandy loam soil to a depth of 25 cm. Soils (22.5% silt, 15.0% clay, 62.5% sand) were prepared by mixing clean quartz sand into a silty clay soil from a farm near Lincoln, NE. The mixed sandy loam soil was defined as original soil in this study. The manure used in the greenhouse experiment was collected from the beef cattle feedlot at the Eastern Nebraska Research, Extension and Education Center in Mead, NE. The manure was broadcast to the surface of the original soil in greenhouse.

Greenhouse experiments were established with three treatment scenarios involving varying amounts of manure: soil without manure as control (Con), soil with manure equivalent to 5 yr nitrogen need (T5, with 4 g manure per year × 5 year = 20 g, equivalent to 0.52 kg dry weight per m^2 based on manure application rates ranging between 0.34 and 2.2 kg dry weight per m^2 per year according to ref. 20), and soil with manure equivalent to 10 year nitrogen needs (T10, with 4 g manure per year × 10 year = 40 g). The entire experiment was replicated three rounds (*i.e.*, Sep–Dec 2019, Feb–Mar 2020, and Jun–Jul 2020). Within each round, 15 rhizoboxes were allocated to the three treatment scenarios, with 5 rhizoboxes per scenario. Thus, three rounds resulted in 15 biological replicate soil–plant model ecosystems (5 replicates per round × 3 rounds). For each round experiment, the greenhouse was maintained at air temperature of 15–18 °C with 16 h of photoperiod for lettuce. The lettuce was watered using an overhead irrigation system with deionized water containing supplemental nutrients. Details of the greenhouse experiments were descried in our previous work.[5]

2.2 Sample collection and DNA extraction

Samples were collected from the original soil and manure on week 0, from surface soil within the rhizoboxs in week 1, 3, and 6, and from rhizosphere soil as well as leaf and root of lettuce in week 6. Genomic DNA was extracted from 0.3–1.0 g of the samples using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). In total, 15 × 3 = 45 biological replicates were generated for both surface soil and rhizosphere soil. Microbes associated with lettuce were collected from the episphere and endosphere of lettuce leaves and from lettuce roots at the end of week 6. To collect microbes from the episphere, lettuce leaves were harvested by cutting below the cotyledonary node at the soil surface using ethanol-sterilized scissors. In total, 5 biological replicates were generated for both episphere and endosphere of lettuce.

Lettuce leaves were then transported into a 50 mL centrifugal tube and submerged in 45 mL autoclaved phosphate buffered saline (PBS) containing 0.02% Tween 20. The solutions were mixed on a vortex mixer for 1 min and then were shaken at 200 rpm at 30 °C for 2 h to recover bacteria from leaf surface. The washing solution was then centrifuged at 2800*g* for 30 min, and the resulting pellets were stored at −20 °C until DNA extraction.[21,22] To recover endospheric microbes, the leaf surfaces were pretreated with 30% hydrogen peroxide (H_2O_2) for 1 min and then rinsed three times using sterilized water. The leaf samples were further treated with 70% ethanol (Ricca Chemical, Arlington, VA) for 1 min and washed in sterilized water three times to eliminate the surface bacteria. The treated leaf samples were homogenized in a blender jar containing 100 mL of sterilized PBS (Sigma Aldrich, St. Louis, MO) for 30 s. The solution was then centrifuged at 2800 g for 30 min and the pellets collected were stored in −20 °C until DNA extraction.[22]

Rhizosphere soil was recovered from different locations of the root zone (top, medium, and bottom) at the end of week 6. The root segments from different rhizobox location were transferred to 50 mL tubes using ethanol-sterilized tweezers. After adding sterilized PBS to the tubes, samples were vortexed

for 1 min. The solutions with rhizosphere soil particles after removing roots were centrifuged for 5 min at 2800 g. The pellets were stored at −20 °C until DNA extraction.

Lettuce roots were recovered from the rhizoboxes. To eliminate surface bacteria from the roots, the roots were sterilized by immersion in 30% H_2O_2 for 30 min and then washed with sterilized water three times. Samples were then immersed in 70% ethanol for 1 min and washed in sterilized water 3 times. The washed roots were homogenized using a blender jar containing 100 mL of sterilized PBS for 30 s, and resulting solution was centrifuged at 2800 g for 30 min to collect the root particles for DNA extraction.[21,22]

2.3 ARG quantification

qPCR was performed to quantify the copy numbers of the 16S ribosomal RNA (rRNA) gene, the class 1 integron–integrase gene (*intI*1), and ARGs including β-lactamase (bla_{TEM}), macrolide (*erm*(B) and *erm*(F)), and tetracycline (*tet*(O), *tet*(Q), *tet*(M) and *tet*(X)) resistance genes on an Eppendorf Realplex2 thermocycler (Eppendorf, Hamburg, Germany). The selected ARGs were commonly found in soil with application of animal manure.[23,24] Each 20 μL qPCR reaction mixture contained 10 μL of 2× KiCqStart® Universal SYBR® Green ReadyMix™ (Sigma-Aldrich, St. Louis, MO), 1 μL each of forward and reverse primers (0.2 μM), 7 μL molecular-grade water (Sigma-Aldrich, St. Louis, MO), and 1 μL DNA template.[25] Primer sequence was listed in Table S1. The thermal cycling of qPCR amplification includes 2 min of initial denaturation at 95 °C, 40 cycles of the denaturation at 95 °C for 15 s, annealing at specific temperature for 15 s, extension at 72 °C for 20 s, melting curve for 45 s (95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s). The annealing temperature for the 16S rRNA, bla_{TEM}, *erm*, *intI*1, and *tet* genes were listed in Table S1. All qPCR runs had an amplification efficiency between 90% and 110% with an R^2 >0.95. Genes quantified in less than fifty percent of the technical replicates were considered false positives and were removed from the analysis. Each gene was quantified in duplicate with a standard curve and negative control. Results were reported as absolute abundance (copies per g dry weight (dw)) and relative abundance (copies per 16S rRNA gene copies), respectively.

2.4 Antibiotic analyses

Antibiotics in original soil, surface soil and manure were quantified using methods described in a previous study.[23] For each round, surface soils from five replicates were mixed to form a composite sample for each treatment scenario. Briefly, soil and manure samples (1 g) were weighed in 50 mL centrifuge tubes and mixed with 20 mL of acetonitrile and 15 mL of Mcllvain-EDTA. Surrogate was added to monitor extraction efficiency. Tubes were shaken on a Burrell Wrist-Action Shaker (Cole-Parmer, Vernon Hills, IL) and equilibrated for 30 min and centrifuged for 10 min at 2000*g*. The supernatant was decanted into a Labconco RapidVap™ evaporation tube (Labconco, Kansas City, MO). The process was repeated with 20 mL of acetonitrile and 15 mL of Mcllvain-EDTA. All extracts were combined, evaporated at 25 °C to approximately 20 mL on a RapidVap Vacuum Dry Evaporation Systems (Labconco, Kansas City, MO), mixed with deionized water to a final volume of 50 mL. Aqueous extracts were loaded onto a 200 mg Waters Oasis HLB solid phase extraction (SPE) cartridge (Waters, Milford, MA) aspirating at a rate of no more than 1 mL min^{-1}. SPE cartridges were eluted with 10 mL of acetonitrile. Purified extracts were evaporated under nitrogen to 50 μL, mixed with 100 μL of an internal standard solution, transferred to an autosampler vial, and stored at −20 °C.

Chlortetracycline, lincomycin, monensin, sulfamethazine, and tylosin in extracts were analyzed on a Waters Quattro Micro triple quadrupole mass spectrometer coupled with a Waters 2695 high pressure liquid chromatography (Waters, Milford, MA). Target compounds were chosen based on the antibiotic usage data from the cattle feedlot and expected persistence or chemical properties of the parent compounds. Demeclocycline and sulfachloropyridazine were used as surrogates, while sulfophenyl-$^{13}C_8$ and doxycycline were used as internal standards. Recoveries determined using sterile sand were 102 ± 39% for chlortetracycline, 18 ± 15% for lincomycin, 48 ± 30% for monensin, 108 ± 50% for sulfamethazine, and 162 ± 60% for tylosin.

2.5 Statistical analyses

Kruskal–Wallis rank sum tests were performed in *R* to determine statistically significant differences among different treatment scenarios. Multiple pairwise comparison using Dunn's post hoc test was used to identify which treatment scenarios are different, if there was a significant difference based on Kruskal–Wallis test result. Cliff's Delta values (δ) from Dunn's post hoc test were reported to indicate the effect sizes. *t* tests were used to determine if the mean difference between two treatment levels was statistically significant. Spearman's correlation analysis was performed in *R* to test the correlations between ARGs and *intI*1, and between ARGs and antibiotics.

3 Results and discussion

3.1 ARGs and *intI*1 in original soil and manure

The abundances of the total 16S rRNA gene were 7.02×10^6 and 1.36×10^9 copies per g dw in original soil and manure, respectively (Fig. S1a). The relative abundance of bla_{TEM}, *tet*(M), *tet*(O) and *intI*1 in the original soil ranged from 5.09×10^{-5} to 1.25×10^{-4} copies per 16S rRNA gene copy (Fig. S1b). The other ARGs were below the detection limits in soil. Seven targeted ARGs and *intI*1 were quantifiable in manure, with relative abundance ranging from 1.48×10^{-4} to 5.88×10^{-2} copies per 16S rRNA gene copy (Fig. S1c). *tet*(M), *tet*(O) and *tet*(Q) were the most abundant ARGs in manure samples.

3.2 ARGs and *intI*1 in surface soil after manure application

The relative abundance of targeted ARGs and *intI*1 in surface soil were quantified to be in the range of 3.18×10^{-7} to 2.44×10^1 copy per 16S rRNA gene copy (Fig. 1). T5 and T10 had comparable abundances of bla_{TEM}, *erm*(B), *erm*(F), *intI*1 and *tet*(O) to control. Manure application resulted in significantly

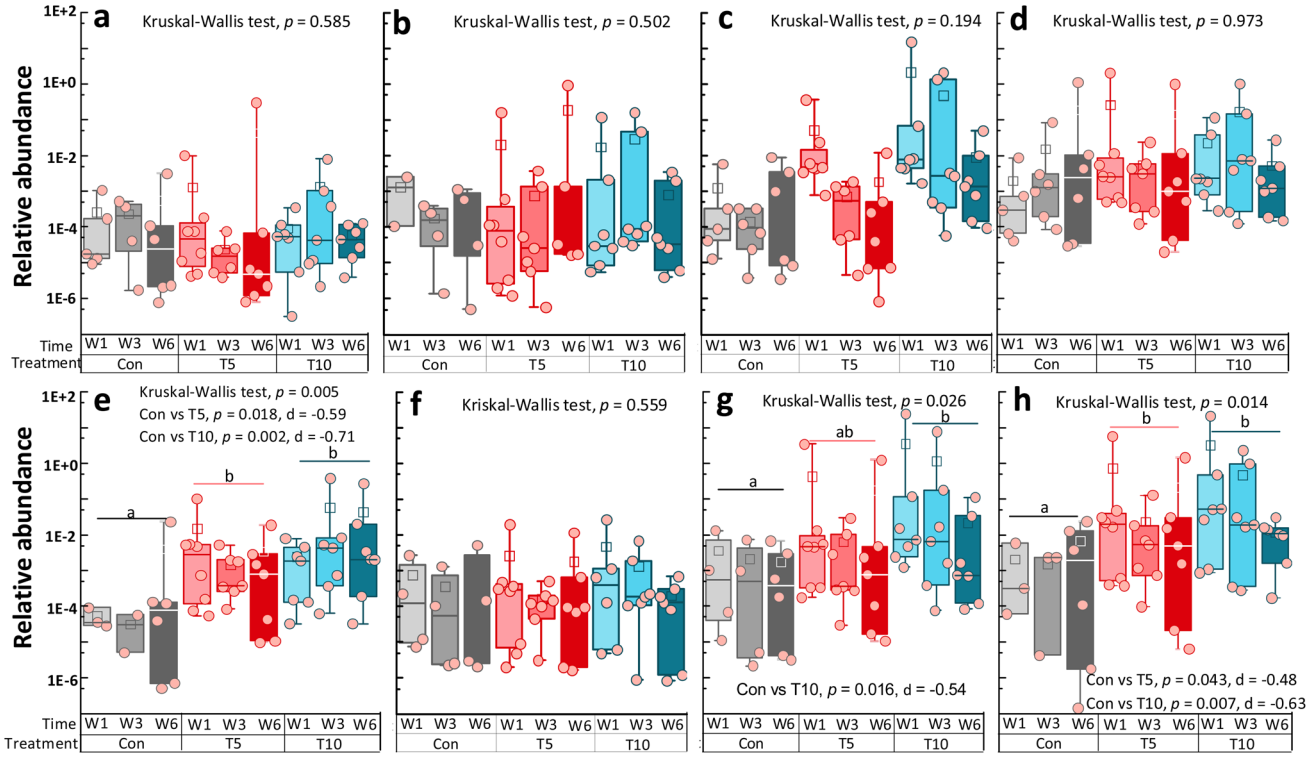

Fig. 1 Relative abundances of (a) bla_{TEM}, (b) *erm*(B), (c) *erm*(F), (d) *intI*1, (e) *tet*(M), (f) *tet*(O), (g) *tet*(Q), and (h) *tet*(X) in surface soil at different sampling time (W1: week 1, W3: week 3, W6: week 6). The upper and the lower whiskers represent the maximum and minimum values, the upper and the lower sides of the box represent 75% and 25% percentile of values, open square represents the mean value, and the line in the box represents the median value (Con: $n = 6$; T5 and T10: $n = 9$). Box group with different letter indicates significantly difference according to Kruskal–Wallis and multiple pairwise-comparison between control and treatment scenarios ($p < 0.05$).

higher relative abundances of *tet*(M), *tet*(Q), and *tet*(X) (Kruskal–Wallis test, $p < 0.05$). Sampling time had no statistically significant effects on the relative abundance of the tested ARGs and *intI*1 (Kruskal–Wallis test, $p > 0.05$). Thus, manure application resulted in enrichment of *tet* genes in surface soils, whereas no enrichment was observed for *bla* and *erm* genes.

Targeted ARGs and *intI*1 were all quantifiable in surface soil under the three treatment scenarios, with the absolute abundance from 4.09×10^2 to 9.86×10^6 copies per g dw (Fig. S2). Manure application had significant effects (Kruskal–Wallis test, $p < 0.05$) on the absolute abundance of *intI*1 and all targeted ARGs except for bla_{TEM}. Pair-wise comparisons between treatments show that the absolute abundances of ARGs and *intI*1 in T5 and in T10 were significantly higher than those in control (in Kruskal–Wallis test, $p < 0.05$). For example, the absolute abundance of *tet*(M) was 2–3 orders of magnitude higher in T10 than control. Throughout the growth period, the average absolute abundance of ARGs and *intI*1 in surface soil tended to decrease (Fig. S2). The average absolute abundance of *tet*(Q) in T10 decreased from 8.9×10^6 copies per g dw at week 1 to 9.8×10^5 copies per g dw at week 6.

The abundance of ARGs in surface soil tend to increase following the application of beef,[26] pig,[27] and chicken manure.[28] The sustained, elevated levels of *tet* and *intI*1 genes in manured soil were attributed to either the survival of manure-borne bacteria carrying ARGs in surface soil[29] or the enrichment of ARG-carrying bacteria in soil due to the selective pressure exerted by the antibiotic residues in manure.[30]

The two *erm* genes were not detectable in the original soil (Fig. S1a and b) but were detected at low absolute abundance in the surface soil of the control rhizoboxes after 1 week (Fig. S2b and c), suggesting that the *erm* abundance increased in soil during the course of the study. The increase in *erm* gene abundance observed during the study may be attributed to the activation of indigenous soil-borne microbes and/or potential minimal aerosol transfer[31] from manured to unmanured soils. Even though, the *erm* abundance were observed to significantly higher in T5/T10 compared to control. By week 6, the relative (Fig. 1) and absolute (Fig. S2) abundance of *bla* and *erm* genes in treated soil was lowered to the similar levels for the control soil (Fig. 1). That is, the relative abundance of manure-borne *erm* genes returned to the baseline level (the level in control experiments) within 6 weeks in this study. Previous studies have also demonstrated that the relative abundance of *erm*(F) genes returns to the baseline after 6 weeks in soil with dairy manure slurry,[32] while the relative abundance of *tet* and *erm* genes returned to the baseline after 2 to 6 weeks in soil with dairy and swine manure.[33] The reduction of *bla* and *erm* might be attributable to die-off of manure bacteria carrying these ARGs and the decay of extracellular DNA released from dead cells.[25,34]

The relative abundances of *tet*(M), *tet*(Q), and *tet*(X) remained approximately 1–2 orders of magnitude higher in

treated soil than those in control soils at Week 6 (Fig. 1). Together with the results that relative abundances of *tet* genes in manure were much higher than that in original soil (Fig. S1b and c), these results indicate that manure-borne *tet* genes could persist in soils. This may be due to the broad range of bacterial hosts of *tet* genes. For instance, *tet*(M) and *tet*(O) have been identified in *Enterococcus* spp.,[35] *Campylobacter jejuni*,[36] and *Clostridium septicum* from beef cattle manure.[37]

3.3 ARGs and *intI*1 in rhizosphere soil

The relative abundance of quantifiable ARGs and *intI*1 in rhizosphere soil ranged from 8.43×10^{-7} to 4.19×10^{-3} gene copies per 16S rRNA gene copy (Fig. 2). bla_{TEM}, *erm*(B), *tet*(M), *tet*(O) and *tet*(Q) were consistently detected in the rhizosphere soil samples of T5 and T10, but not in the control. Manure treatment caused significantly higher relative abundance of *tet*(M) (Fig. 2e) and *tet*(Q) (Kruskal–Wallis test, $p < 0.05$, Fig. 2g). Sampling location had no significant effects on the relative abundances of ARGs and *intI*1 (Kruskal–Wallis test, $p > 0.05$).

The absolute abundance of ARGs and *intI*1 in rhizosphere soil ranged from 3.81×10^{2} to 1.37×10^{6} copies per g dw (Fig. S3). Manure application resulted in 1–3 orders of magnitude higher of *tet*(M) in T10 than that in control (Fig. S3). The abundances of the tested *tet* genes were elevated in the top rhizosphere soils. *erm*(B) was not quantifiable in control but was quantifiable in top rhizosphere soil of T5 and T10. Sampling location significantly affected the absolute abundances of bla_{TEM}, *erm*(F), *intI*1, *tet*(M) and *tet*(O), with significant higher abundance in top rhizosphere soil than that in bottom rhizosphere soil (Kruskal–Wallis test, $p < 0.05$).

Sampling locations had significant effects on the absolute abundance of ARGs (*e.g.*, bla_{TEM}, *intI*1, *tet*(M) and *tet*(O)) in rhizosphere soil in the present study (Fig. S3). This observation might indicate that vertical ARG transmission was limited. This vertical ARG transmission dependent on soil characteristics,[38,39] animal manure types,[18] and concentrations of antibiotics at different soil depths.[20] Irrigation water containing nutrients and the manure were both applied to the surface soil. There was likely a nutrient gradient in soil profile which lead to fewer ARG-carrying bacteria residing in the bottom layer of the rhizosphere.[38]

3.4 ARGs and *intI*1 in the episphere, endosphere, and root of lettuce

Manure application caused elevated relative abundance of ARGs associated with lettuce, more in episphere than in root and endosphere. When ARGs were normalized to the 16S rRNA gene, endosphere had lower relative abundances (9.99×10^{-8}–6.64×10^{-4} gene copies per 16S gene copy) of ARGs and *intI*1 than those in episphere (2.47×10^{-8}–1.31×10^{-2} gene copies per 16S rRNA gene copy) and root (1.01×10^{-7}–1.57×10^{-2} gene copies per 16S rRNA gene copy) (Fig. 3). In endosphere

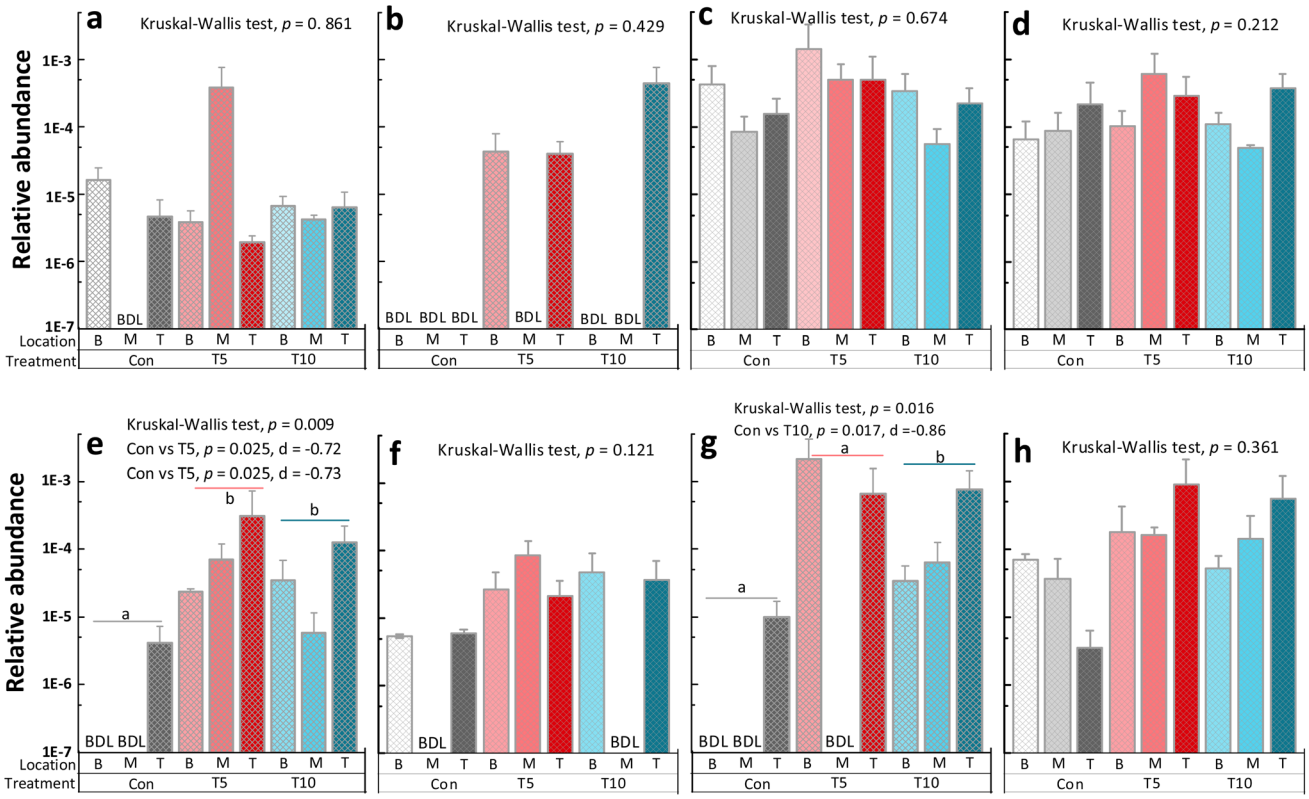

Fig. 2 Relative abundances of (a) bla_{TEM}, (b) *erm*(B), (c) *erm*(F), (d) *intI*1, (e) *tet*(M), (f) *tet*(O), (g) *tet*(Q) and (h) *tet*(X) in rhizosphere soil at different sampling locations (B: bottom, M: middle, T: top). Error bar indicates standard deviations ($n = 3$). Bar groups with different letters are significantly different from each other according to pairwise-comparisons ($p < 0.05$). BDL represents below detection limit.

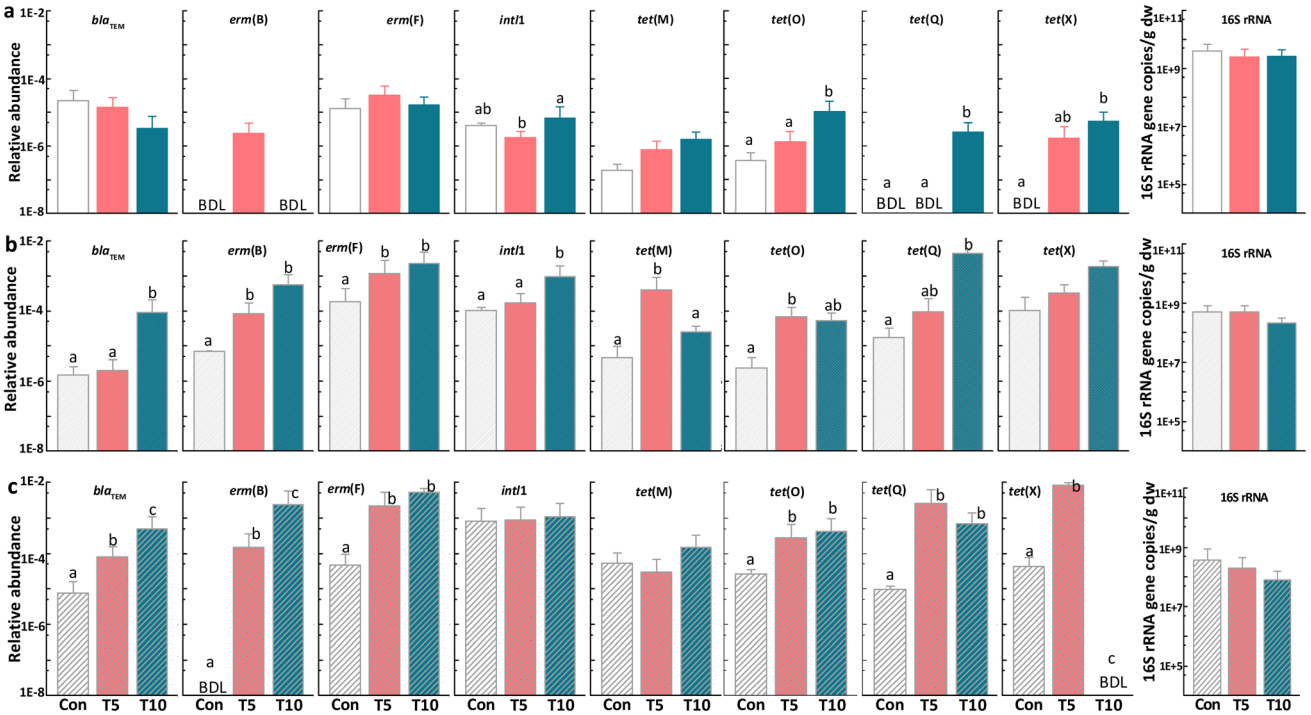

Fig. 3 16S rRNA gene copies per gram dry weight and relative abundances of target ARGs and *intI*1 in the (a) endosphere, (b) episphere, and (c) root of lettuce. Error bars represent standard deviations (n = 3). Bars labeled with different letters are significantly difference according to pairwise-comparisons (p < 0.05). Half of the detection limit values were used for statistical analyses. BDL represents below detection limit.

(Fig. 3a), the relative abundances of *tet*(O), *tet*(Q) and *tet*(X) were significantly (t-test, p < 0.05) higher in T10 compared to control. In episphere (Fig. 3b), the relative abundances of most targeted ARGs and *intI*1 were significantly (t-test, p < 0.05) higher in T5 or T10 than in control. In root (Fig. 3c), T10 and T5 had significant higher (t-test, p < 0.05) abundance of ARGs except for *tet*(M), compared to control. The data in Fig. 3 show that manure application led to the transmission of *tet* genes from soil to lettuce, resulting in elevated abundances of *tet* genes in episphere and root of lettuce.

The absolute abundance of targeted ARGs ranged from 3.05 $\times 10^2$ to 2.05 $\times 10^4$ copies per g dw in endosphere, 3.30 $\times 10^2$ to 2.81 $\times 10^5$ copies per g dw in episphere, and 3.10 $\times 10^2$ to 2.09 $\times 10^4$ copies per g dw in root (Fig. S4 and 3). Comparable 16S rRNA gene copies (10^8–10^9 copies per g dw) were found in three treatment scenarios (Fig. 3). The absolute abundance of detected ARGs in different compartments of T5 and T10 was 1–3 orders of magnitude higher than the corresponding compartments in control, *i.e.*, *tet*(O), *tet*(Q) and *tet*(X) genes in endosphere, *erm*(F), *tet*(M), *tet*(O) and *tet*(X) genes in episphere, bla_{TEM}, *erm*(B) and *erm*(F) genes in root (Fig. S4).

The present study analyzed ARGs in different compartments of a soil–plant ecosystem, enabling an opportunity to reveal the transmission of ARGs. The ARGs on and in plants generally originated from surface soils,[40,41] *e.g.*, *tet*(M), *tet*(Q) and *tet*(X) in endosphere and episphere (Fig. 3a and b), suggesting that manure-borne ARGs that survived in surface soil might be the source of ARGs on/in lettuce. *tet*(M), *tet*(Q) and *tet*(X) were also detected in lettuce endosphere and soil (~10^4–10^7) amended with poultry litter[7,41] and swine manure.[7] While previous studies revealed the presence of *tet* genes in soil or lettuce, our study provided the first comprehensive analysis of *tet* genes across multiple compartments of the soil–plant continuum. The nutrients in manure contribute to the production of capsular polysaccharides and exopolysaccharides, which play important roles in the interactions between episphere *Escherichia coli* and plant surface.[42] Bacteria surviving in the episphere may have a chance to enter the endosphere.[5] Human pathogens such as *Salmonella* can internalize into plants through injured stems, leaves, and flowers.[43] Together, the *tet* genes detected in endosphere and episphere of lettuce (Fig. 1) as well as in surface soil suggested that manure-borne *tet* transferred from surface soil to the lettuce episphere. The transfer of ARGs from surface soil to the episphere is likely to occur through splash and physical contact.[38] Subsequently, the episphere ARGs may enter the lettuce endosphere through openings such as stomata or hydathodes. These transmission pathways are supported by source tracker and metagenomic analysis in previous study, which revealed that ARGs in surface soil was sources of ARGs in episphere and endosphere compartments of lettuce.[5]

Similarly, *tet* genes can transmit *via* the under-ground route: manure–rhizosphere soil–root. This was suggested by significantly elevated abundances of *tet* genes in rhizosphere soil and root following manure application. Internalization of microbes into root often involves the recruitment of microbes to the vicinity of the root followed by the entry of microbes into the root.[44] This mechanism cannot be ruled out in the current study. The proposed transmission mechanism or pathways

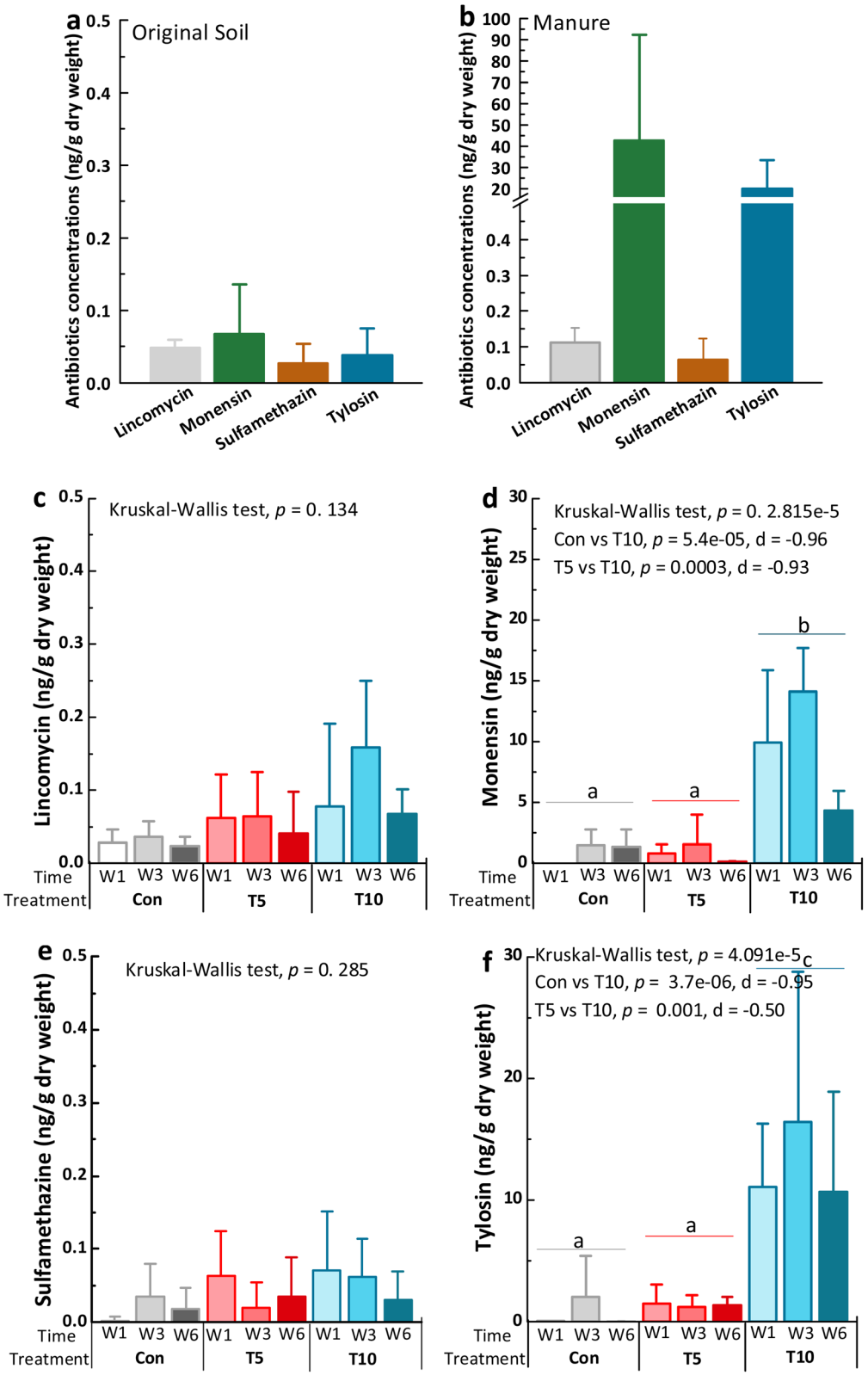

Fig. 4 Lincomycin, monensin, sulfamethazine, and tylosin concentrations in (a) original soil and (b) manure, and in surface soil at week 1, 3, and 6 (c–f). Bar groups with different letters are significantly different according to pairwise comparison ($p < 0.05$) where sampling times are treated as replicates. Error bars represent standard deviations ($n = 4$).

warrant further investigation by employing techniques such as stable isotope probing or metagenomics.

The *bla* and *erm* genes were significantly enhanced in episphere following manure application (Fig. 3b), while no significant difference was observed between the control and T5 or T10 in the endosphere (Fig. 3a). This observation indicates that *erm* and *bla* genes in endosphere were likely derived from soil-borne microbes. However, *erm* was not detected in original soils (Fig. S1). Plant seeds are a possible source of ARGs in endosphere here, as demonstrated in the soil–rice ecosystem.[45] Interestingly, *bla* and *erm* abundances increased significantly in roots, compared to control, after manure application (Fig. 3c). Roots can recruit ARG-carrying bacteria from rhizosphere soil, allowing the transmission of ARGs from soil to lettuce.[41,46] This is consistent with the elevated *bla* and *erm* levels in the rhizosphere soil (Fig. 2b and c). bla_{TEM}, *erm*(B) and *erm*(F) have seldom been quantified in the produce roots. One study provided qPCR-based evidence showing that the presence of bla_{TEM} in lettuce roots that may originated from layer chicken manure.[47] How *erm* and *bla* genes transmit from soil to root of lettuce remain to be investigated in the future.

3.5 Antibiotic residual in surface soil

The antibiotic concentrations in original soil, manure, and manured soil were determined (Fig. 4). Four antibiotics, *i.e.*, lincomycin, monensin, sulfamethazine, and tylosin, were detected in manure (Fig. 4a and b). The mean concentrations in manure were 13.45 ± 0.03 ng g^{-1} dw for monensin, 49.67 ± 0.07 ng g^{-1} dw for tylosin, 0.11 ± 0.04 ng g^{-1} dw for lincomycin, and 0.06 ± 0.05 ng g^{-1} dw for sulfamethazine. The concentrations of all four antibiotics in original soil were lower than 0.10 ng g^{-1} dw.

In surface soil, monensin and tylosin were the most abundant antibiotics, with concentrations ranging at 0.13–14.50 ng g^{-1} dw (Fig. 4d) and at 0.05–16.42 ng g^{-1} dw, respectively (Fig. 4f). The concentrations of lincomycin and sulfamethazine were mostly below 0.10 ng g^{-1} dw except for lincomycin of T10 at Week 3 (0.16 ng g^{-1} dw). Manure application resulted in significantly higher concentrations of monensin in T10 (4.34 ± 0.03 to 14.15 ± 0.06 ng g^{-1} dw) (Kruskal–Wallis test, $p = 5.4 \times 10^{-5}$, $\delta = 0.96$) than that in control (0.00 to 1.47 ± 0.03 ng g^{-1} dw), and significant higher concentrations of tylosin in T10 (10.69 ± 0.03 to 16.42 ± 0.09 ng g^{-1} dw) (Kruskal–Wallis test, $p = 3.7 \times 10^{-6}$, $\delta = 0.95$) than that in control (0.05 ± 0.04 to 2.03 ± 0.02 ng g^{-1} dw). The antibiotic concentrations of manured soil in the present study were consistent with values from previous study showing that chlortetracycline, sulfamethazine and tylosin was ranged from 0.3–9.3 ng g^{-1} dw in the bulk and rhizosphere soils with application of beef manure.[4]

Spearman correlation analysis revealed positive correlations between antibiotic concentration and the relative abundance of ARGs (Table 1). For example, lincomycin showed significant correlations with *tet*(Q) and *tet*(X), and tylosin exhibited significant correlations with *erm*(F), *tet*(Q) and *tet*(X). These correlations suggest that these antibiotics could exert selective pressure for the emergence and proliferation of ARGs in soil, directly or indirectly. Tylosin, in particular, exerts selective pressure on tylosin-resistant populations and thus lead to the increased prevalence of *erm*(F).[48] Positive correlations were also observed among relative abundance of ARGs, *e.g.*, *tet*(X) correlated with *erm*(B), *erm*(F), *tet*(O) and *tet*(Q). The significant correlation between *erm*(F) and both *tet*(Q) and *tet*(X) suggests that tylosin might co-select *erm*(F) as well as *tet*(Q) and *tet*(X).[34] An illustrative example is the co-location of *erm*(F) and *tet*(X) on the same transposon in the obligate anaerobe *Bacteroides fragilis*.[49] Other factors, such as undetected antibiotics in the present study and manure-induced changes in soil properties,[9] could influence ARG survival in surface soil. Further research is needed to investigate their relationships with ARG abundance.

3.6 Environmental significance

The land application of beef cattle manure could introduce antibiotics and ARGs to cropland. The findings of the present study revealed the increased levels of *tet* in both soil and lettuce as a consequence of beef cattle manure application. Notably, manure-borne *tet* genes that persist in surface soil may contribute to the presence of *tet* genes in endosphere and root of lettuce, posing potential health risks to consumers through the food chain. Our results further highlight the increased likelihood of *tet* transmission from soil to crop through both above- and under-ground routes. Further research is needed particularly on the mitigation of ARGs in soils and crops under

Table 1 Spearman's correlation coefficient (ρ, 0.05 *, 0.01**, 0.001***) between relative abundances of ARGs and antibiotic concentrations

	Lincomycin	Monensin	Sulfamethazine	Tylosin	bla_{TEM}	*erm*(B)	*erm*(F)	*intI*1	*tet*(M)	*tet*(O)	*tet*(Q)
Lincomycin											
Monensin	**0.82****										
Sulfamethazine	0.65	0.43									
Tylosin	**0.73***	0.48	**0.87****								
bla_{TEM}	−0.17	−0.35	0.27	0.17							
erm(B)	0.45	−0.03	0.57	0.50	0.65						
erm(F)	0.63	0.50	**0.67***	**0.65***	0.23	0.57					
*intI*1	0.21	0.46	0.28	0.05	0.43	0.15	0.38				
tet(M)	0.50	0.40	0.33	0.50	0.37	0.48	**0.72***	0.41			
tet(O)	0.18	−0.03	**0.73***	0.48	0.60	0.67	0.70	0.30	0.27		
tet(Q)	**0.83****	0.47	**0.85****	**0.80***	0.20	**0.78****	**0.78***	0.16	0.47	0.65	
tet(X)	**0.70***	0.38	**0.82****	**0.68****	0.22	**0.72****	**0.80****	0.20	0.50	**0.72***	**0.92*****

various environmental conditions and manure types, and the quantitative assessment of ARG risks (particular in the edible parts of lettuce) on the food safety. Correlating abundance of ARGs in edible parts of lettuce and food safety is essential for evaluating potential consumer risks and informing the development of agriculture practices and food safety policy.

Author contributions

Yuepeng Sun: methodology, writing-original draft preparation, investigation. Daniel Snow: writing-reviewing & editing, supervision. Harkamal Walia: writing-reviewing & editing, supervision. Xu Li: funding acquisition, supervision, conceptualization, writing-reviewing & editing.

Conflicts of interest

The authors declare that there are no known competing financial interests.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary materials. No additional datasets were created or used beyond those presented here. Supplementary information: the abundance of the 16S rRNA gene and ARGs in original soil and manure; absolute abundances of ARGs in surface soil at different sampling time; absolute abundances of ARGs in rhizosphere soil of different sampling locations; absolute abundances of ARGs in endosphere, episphere and root of lettuce; primers used for ARG quantification. See DOI: **https://doi.org/10.1039/d5va00204d**.

Acknowledgements

Support for this project was provided by the National Science Foundation (1805990) and the Robert B. Daugherty Water for Food Institute, and "High-level talents under the Steed Plan" that hosted by Inner Mongolia University (10000-23112101/155). Authors want to thank Dr Zach Staley for helping on soil sample collection.

References

1 W. Y. Xie, Q. Shen and F. Zhao, Antibiotics and antibiotic resistance from animal manures to soil: a review, *Eur. J. Soil Sci.*, 2018, **69**, 181–195.
2 H. Heuer, A. Focks, M. Lamshöft, K. Smalla, M. Matthies and M. Spiteller, Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil, *Soil Biol. Biochem.*, 2008, **40**, 1892–1900.
3 K. Kumar, S. C. Gupta, S. Baidoo, Y. Chander and C. J. Rosen, Antibiotic uptake by plants from soil fertilized with animal manure, *J. Environ. Qual.*, 2005, **34**, 2082–2085.
4 C. Chen, G. K. Guron, A. Pruden, M. Ponder, P. Du and K. Xia, Antibiotics and antibiotic resistance genes in bulk and rhizosphere soils subject to manure amendment and vegetable cultivation, *J. Environ. Qual.*, 2018, **47**, 1318–1326.
5 Y. Sun, D. Snow, H. Walia and X. Li, Transmission Routes of the Microbiome and Resistome from Manure to Soil and Lettuce, *Environ. Sci. Technol.*, 2021, **55**, 11102–11112.
6 Z. Chen, W. Zhang, L. Yang, R. D. Stedtfeld, A. Peng, C. Gu, S. A. Boyd and H. Li, Antibiotic resistance genes and bacterial communities in cornfield and pasture soils receiving swine and dairy manures, *Environ. Pollut.*, 2019, **248**, 947–957.
7 J. Huang, J. Mi, Q. Yan, X. Wen, S. Zhou, Y. Wang, B. Ma, Y. Zou, X. Liao and Y. Wu, Animal manures application increases the abundances of antibiotic resistance genes in soil–lettuce system associated with shared bacterial distributions, *Sci. Total Environ.*, 2021, **787**, 147667.
8 M. Kyselková, L. Kotrbová, G. Bhumibhamon, A. Chroňáková, J. Jirout, N. Vrchotová, H. Schmitt and D. Elhottová, Tetracycline resistance genes persist in soil amended with cattle feces independently from chlortetracycline selection pressure, *Soil Biol. Biochem.*, 2015, **81**, 259–265.
9 W.-Y. Xie, S.-T. Yuan, M.-G. Xu, X.-P. Yang, Q.-R. Shen, W.-W. Zhang, J.-Q. Su and F.-J. Zhao, Long-term effects of manure and chemical fertilizers on soil antibiotic resistome, *Soil Biol. Biochem.*, 2018, **122**, 111–119.
10 H. Heuer, H. Schmitt and K. Smalla, Antibiotic resistance gene spread due to manure application on agricultural fields, *Curr. Opin. Microbiol.*, 2011, **14**, 236–243.
11 T. Lima, S. Domingues and G. J. Da Silva, Manure as a potential hotspot for antibiotic resistance dissemination by horizontal gene transfer events, *Vet. Sci.*, 2020, **7**, 110.
12 H. Heuer, Q. Solehati, U. Zimmerling, K. Kleineidam, M. Schloter, T. Müller, A. Focks, S. r. Thiele-Bruhn and K. Smalla, Accumulation of sulfonamide resistance genes in arable soils due to repeated application of manure containing sulfadiazine, *Appl. Environ. Microbiol.*, 2011, **77**, 2527–2530.
13 Y.-J. Zhang, H.-W. Hu, Q.-L. Chen, H. Yan, J.-T. Wang, D. Chen and J.-Z. He, Manure application did not enrich antibiotic resistance genes in root endophytic bacterial microbiota of cherry radish plants, *Environ. Int.*, 2020, **86**, e02106–02119.
14 R. Marti, A. Scott, Y.-C. Tien, R. Murray, L. Sabourin, Y. Zhang and E. Topp, Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest, *Appl. Environ. Microbiol.*, 2013, **79**, 5701–5709.
15 F.-H. Wang, M. Qiao, Z. Chen, J.-Q. Su and Y.-G. Zhu, Antibiotic resistance genes in manure-amended soil and vegetables at harvest, *J. Hazard. Mater.*, 2015, **299**, 215–221.
16 Q. Yang, S. Ren, T. Niu, Y. Guo, S. Qi, X. Han, D. Liu and F. Pan, Distribution of antibiotic-resistant bacteria in chicken manure and manure-fertilized vegetables, *Environ. Sci. Pollut. Res.*, 2014, **21**, 1231–1241.
17 K. Fogler, G. K. Guron, L. L. Wind, I. M. Keenum, W. C. Hession, L.-A. Krometis, L. K. Strawn, A. Pruden and M. A. Ponder, Microbiota and antibiotic resistome of

lettuce leaves and radishes grown in soils receiving manure-based amendments derived from antibiotic-treated cows, *Front. Sustain. Food Syst.*, 2019, **3**, 22.

18 G. K. Guron, G. Arango-Argoty, L. Zhang, A. Pruden and M. A. Ponder, Effects of dairy manure-based amendments and soil texture on lettuce-and radish-associated microbiota and resistomes, *Msphere*, 2019, **4**, e00239.

19 M. R. Gillings, W. H. Gaze, A. Pruden, K. Smalla, J. M. Tiedje and Y.-G. Zhu, Using the class 1 integron–integrase gene as a proxy for anthropogenic pollution, *ISME J.*, 2015, **9**, 1269–1279.

20 X. Tang, C. Lou, S. Wang, Y. Lu, M. Liu, M. Z. Hashmi, X. Liang, Z. Li, Y. Liao and W. Qin, Effects of long-term manure applications on the occurrence of antibiotics and antibiotic resistance genes (ARGs) in paddy soils: evidence from four field experiments in south of China, *Soil Biol. Biochem.*, 2015, **90**, 179–187.

21 D. S. Lundberg, S. L. Lebeis, S. H. Paredes, S. Yourstone, J. Gehring, S. Malfatti, J. Tremblay, A. Engelbrektson, V. Kunin and T. G. d. Rio, Defining the core *Arabidopsis thaliana* root microbiome, *Nature*, 2012, **488**, 86–90.

22 Y. Zhang, C. Jewett, J. Gilley, S. L. Bartelt-Hunt, D. D. Snow, L. Hodges and X. Li, Microbial communities in the rhizosphere and the root of lettuce as affected by Salmonella-contaminated irrigation water, *FEMS Microbiol. Ecol.*, 2018, **94**, fiy135.

23 R. E. Barrios, H. K. Khuntia, S. L. Bartelt-Hunt, J. E. Gilley, A. M. Schmidt, D. D. Snow and X. Li, Fate and transport of antibiotics and antibiotic resistance genes in runoff and soil as affected by the timing of swine manure slurry application, *Sci. Total Environ.*, 2020, **712**, 136505.

24 M. C. Hall, N. A. Mware, J. E. Gilley, S. L. Bartelt-Hunt, D. D. Snow, A. M. Schmidt, K. M. Eskridge and X. Li, Influence of setback distance on antibiotics and antibiotic resistance genes in runoff and soil following the land application of swine manure slurry, *Environ. Sci. Technol.*, 2020, **54**, 4800–4809.

25 Y. Zhang, C. Zhang, D. B. Parker, D. D. Snow, Z. Zhou and X. Li, Occurrence of antimicrobials and antimicrobial resistance genes in beef cattle storage ponds and swine treatment lagoons, *Sci. Total Environ.*, 2013, **463**, 631–638.

26 E. Miller, M. Spiehs, T. M. Arthur, B. Woodbury, E. Cortus, A. Chatterjee, S. Rahman and J. W. Schmidt, Cropland amendment with beef cattle manure minimally affects antimicrobial resistance, *J. Environ. Qual.*, 2019, **48**, 1683–1693.

27 T. Guo, C. Lou, W. Zhai, X. Tang, M. Z. Hashmi, R. Murtaza, Y. Li, X. Liu and J. Xu, Increased occurrence of heavy metals, antibiotics and resistance genes in surface soil after long-term application of manure, *Sci. Total Environ.*, 2018, **635**, 995–1003.

28 T. He, R.-C. Wei, L. Zhang, L. Gong, L. Zhu, J. Gu, Y.-L. Fu, Y. Wang, D.-J. Liu and R. Wang, Dissemination of the *tet*(X)-variant genes from layer farms to manure-receiving soil and corresponding lettuce, *Environ. Sci. Technol.*, 2021, **55**, 1604–1614.

29 Q.-L. Chen, X.-L. An, H. Li, Y.-G. Zhu, J.-Q. Su and L. Cui, Do manure-borne or indigenous soil microorganisms influence the spread of antibiotic resistance genes in manured soil?, *Soil Biol. Biochem.*, 2017, **114**, 229–237.

30 L. G. Marutescu, M. Jaga, C. Postolache, F. Barbuceanu, N. M. Milita, L. M. Romascu, H. Schmitt, A. M. de Roda Husman, P. Sefeedpari and S. Glaeser, Insights into the impact of manure on the environmental antibiotic residues and resistance pool, *Front. Microbiol.*, 2022, **13**, 965132.

31 L. Song, C. Wang, G. Jiang, J. Ma, Y. Li, H. Chen and J. Guo, Bioaerosol is an important transmission route of antibiotic resistance genes in pig farms, *Environ. Int.*, 2021, **154**, 106559.

32 N. Fahrenfeld, K. Knowlton, L. A. Krometis, W. C. Hession, K. Xia, E. Lipscomb, K. Libuit, B. L. Green and A. Pruden, Effect of manure application on abundance of antibiotic resistance genes and their attenuation rates in soil: field-scale mass balance approach, *Environ. Sci. Technol.*, 2014, **48**, 2643–2650.

33 J. Muurinen, R. Stedtfeld, A. Karkman, K. Parnanen, J. Tiedje and M. Virta, Influence of manure application on the environmental resistome under Finnish agricultural practice with restricted antibiotic use, *Environ. Sci. Technol.*, 2017, **51**, 5989–5999.

34 Y. Zhang, D. D. Snow, D. Parker, Z. Zhou and X. Li, Intracellular and extracellular antimicrobial resistance genes in the sludge of livestock waste management structures, *Environ. Sci. Technol.*, 2013, **47**, 10206–10213.

35 E. O. Ngbede, M. A. Raji, C. N. Kwanashie and J. K. P. Kwaga, Antimicrobial resistance and virulence profile of enterococci isolated from poultry and cattle sources in Nigeria, *Trop. Anim. Health Prod.*, 2017, **49**, 451–458.

36 G. D. Inglis, J. F. Gusse, K. E. House, T. G. Shelton and E. N. Taboada, Tetracycline resistant *Campylobacter jejuni* subtypes emanating from beef cattle administered non-therapeutic chlortetracycline are longitudinally transmitted within the production continuum but are not detected in ground beef, *Microorganisms*, 2019, **8**, 23.

37 Z. Yu, F. C. Michel Jr, G. Hansen, T. Wittum and M. Morrison, Development and application of real-time PCR assays for quantification of genes encoding tetracycline resistance, *Appl. Environ. Microbiol.*, 2005, **71**, 6926–6933.

38 S. Jechalke, S. Schreiter, B. Wolters, S. Dealtry, H. Heuer and K. Smalla, Widespread dissemination of class 1 integron components in soils and related ecosystems as revealed by cultivation-independent analysis, *Front. Microbiol.*, 2014, **4**, 420.

39 P. Yang and J. D. van Elsas, Mechanisms and ecological implications of the movement of bacteria in soil, *Appl. Soil Ecol.*, 2018, **129**, 112–120.

40 L. Jauregi, L. Epelde, I. Alkorta and C. Garbisu, Antibiotic resistance in agricultural soil and crops associated to the application of cow manure-derived amendments from conventional and organic livestock farms, *Front. Vet. Sci.*, 2021, **8**, 633858.

41 Y.-J. Zhang, H.-W. Hu, Q.-L. Chen, B. K. Singh, H. Yan, D. Chen and J.-Z. He, Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes, *Environ. Int.*, 2019, **130**, 104912.

42 S. Seo and K. R. Matthews, Exposure of *Escherichia coli* O157: H7 to soil, manure, or water influences its survival on plants and initiation of plant defense response, *Food Microbiol.*, 2014, **38**, 87–92.

43 X. Guo, J. Chen, R. E. Brackett and L. R. Beuchat, Survival of Salmonellae on and in tomato plants from the time of inoculation at flowering and early stages of fruit development through fruit ripening, *Appl. Environ. Microbiol.*, 2001, **67**, 4760–4764.

44 J. Edwards, C. Johnson, C. Santos-Medellín, E. Lurie, N. K. Podishetty, S. Bhatnagar, J. A. Eisen and V. Sundaresan, Structure, variation, and assembly of the root-associated microbiomes of rice, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, E911–E920.

45 X.-y. Li, W.-f. Wu, C.-y. Wu, Y. Hu, Q. Xiang, G. Li, X.-Y. Lin and Y.-G. Zhu, Seeds Act as Vectors for Antibiotic Resistance Gene Dissemination in a Soil–Plant Continuum, *Environ. Sci. Technol.*, 2023, **57**, 21358–21369.

46 C. Wang, R. Hu, P. Strong, W. Zhuang, W. Huang, Z. Luo, Q. Yan, Z. He and L. Shu, Prevalence of antibiotic resistance genes and bacterial pathogens along the soil–mangrove root continuum, *J. Hazard. Mater.*, 2021, **408**, 124985.

47 J.-Y. Chen, L.-J. Qin, T. Long, R.-T. Wu, S.-H. Niu, S. Liu, W.-K. Deng, X.-D. Liao and S.-C. Xing, Effortless rule: effects of oversized microplastic management on lettuce growth and the dynamics of antibiotic resistance genes from fertilization to harvest, *J. Hazard. Mater.*, 2025, **492**, 138046.

48 D. B. Holman and M. R. Chénier, Impact of subtherapeutic administration of tylosin and chlortetracycline on antimicrobial resistance in farrow-to-finish swine, *FEMS Microbiol. Ecol.*, 2013, **85**, 1–13.

49 B. Speer, L. Bedzyk and S. A, Evidence that a novel tetracycline resistance gene found on two Bacteroides transposons encodes an NADP-requiring oxidoreductase, *J. Bacteriol.*, 1991, **173**, 176–183.