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EDITED BY
Hua-Jun Huang,
Jiangxi Agricultural University, China

REVIEWED BY
Mianzhi Wang,
Yangzhou University, China
João Pedro Rueda Furlan,
School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil

*CORRESPONDENCE
Diana S. Aga,
dianaaga@buffalo.edu

†These authors share first authorship

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Co-occurrence of antimicrobials and metals as potential drivers of antimicrobial resistance in swine farms

Jena L. Congilosi[1†], Joshua S. Wallace[1,2†], Timothy P. Neher[3], Adina Howe[3], Michelle L. Soupir[3] and Diana S. Aga[1,2*]

[1]Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, NY, United States, [2]RENEW Institute, University at Buffalo, The State University of New York, Buffalo, NY, United States, [3]Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA, United States

The application of animal manures to cropland is an important nutrient recycling strategy in many parts of the world. Commonly, aggregated manure wastes contain chemical stressors including veterinary antimicrobials, heavy metals, and antimicrobial resistance genes (ARGs) that can stimulate the development and proliferation of antimicrobial resistance (AMR). While the presence of antimicrobials in manure is well-documented, the co-occurrence of other potentially impactful chemical stressors in swine manure remains underreported. This study quantifies and analyzes correlations between antimicrobials, metals, and certain ARGs present in manure samples from swine farms in Iowa, United States. Relationships between chemical stressors and different stages of swine production or feed composition are also investigated. Results revealed substantial levels of tetracyclines [up to 1,260 μg g^{-1} dry weight (d.w.) of manure for oxytetracycline] detected in all samples. Tiamulin, two ionophores (monensin and lasalocid), and one macrolide (tilmicosin) were detected at maximum class concentrations of 9.4, 0.547, and 0.472 μg g^{-1} d.w., respectively. The median relative abundances of *ermB* and *tetM* were 0.13 and 0.17 copies g^{-1} wet weight (w.w.) manure (normalized to 16S gene), respectively. Additionally, high levels of copper (Cu), iron (Fe), and zinc (Zn) were detected in all samples, with maximum concentrations of 887, 1,900, and 2,100 μg g^{-1} d.w., respectively. Notably, uranium (U) was detected in 11 samples, at concentrations up to 0.77 μg g^{-1}. A global analysis of AMR-stressor relationships using Spearman's rank correlation indicates Cu, and Ba are the most positively and significantly correlated with cytotoxic anhydrotetracycline (ATC) and/or anhydrochlortetracycline (ACTC) concentrations in all tested facilities (Cu-ATC: ρ = 0.67, p = 0.0093; Cu-ACTC: ρ = 0.75, p = 0.0022; Ba-ATC: ρ = 0.84, p = 0.0002). Interestingly, *ermB* and *tetM* genes were strongly, positively correlated to each other (ρ = 0.92, $p < 0.0001$), suggesting possible co-selection, despite the absence of correlation between ARGs and tetracycline concentrations. This study demonstrates the complexity of interactions between antimicrobials, metals, and ARGs in multiple manure storage pits prior to cropland application.

KEYWORDS

swine manure, ARGs, uranium, antimicrobials, metals, co-selection

1 Introduction

Antimicrobial resistance (AMR)-related deaths are increasing globally at an alarming rate, with an estimated 4.95 million AMR-linked deaths in 2019 alone (Antimicrobial Resistance Collaborators, 2022). As a result, it is critical to study the presence and mitigation of antimicrobial resistance gene (ARG) promoting agents released to the environment. Veterinary pharmaceuticals may be important ARG-promoting agents and are considered either medically-important or non-medically-important to humans (WHO, 2019). It has been suggested that both categories can have mutual impacts on human health, as current literature is inadequate to conclude otherwise (Wong, 2019). Although not used in humans, some non-medically-important antimicrobials, such as ionophores, may pose a threat to human health through cross-resistance or co-selection of AMR (Wong, 2019). Cross-resistance occurs when a single gene confers resistance to more than one agent due to structural similarities or common resistance mechanisms, while co-selection occurs where agents have genetically linked resistance genes and selection for one drug causes simultaneous selection for at least one other drug (Wong, 2019). Therefore, it is crucial to investigate the influences of both medically-important and non-medically important antimicrobials on AMR. Additionally, metals co-occurring with antimicrobials in agriculturally impacted soil and water have been linked to co-selection of ARGs (Wong, 2019). This can occur when metal concentrations exceed the minimum co-selective concentration (MCC), or the smallest metal concentration correlated with increased bacterial resistance to antimicrobials (Seiler and Berendonk, 2012).

Land application of manure is a major source of antimicrobial and metal release to agricultural environments (Chee-Sanford et al., 2009; Srivastava et al., 2017). Up to 90% of certain antimicrobials may be excreted unchanged, potentially retaining antimicrobial activity in soil receiving land-applied manure (Kumar et al., 2005). In 2020, more than 10.4 million kilograms of antimicrobials were sold in the United States (US) for agricultural use, including livestock disease control and growth promotion (USFDA, 2021). According to US Department of Agriculture (USDA) data on thirteen states, 93.5% of sites with more than 1,000 swine used antimicrobials in feed, 78.4% in water, and 92.4% administered antimicrobials by injection (USDA, 2019). Of the tested sites, 10.5% of nursery-age sites and 44.1% of grower/finisher sites administered antimicrobials in feed for growth promotion.

The introduction of antimicrobials to soil may stimulate the development and proliferation of AMR in bacteria exposed to these antimicrobials, which act as chemical stressors (Manyi-Loh et al., 2018). Similarly, certain metals necessary for cellular metabolic function are often added as mineral supplements to swine feed to promote growth and animal health (Hill et al., 2000; Medardus et al., 2014). Zinc (Zn) and copper (Cu) are often added to nursery swine diets to combat post-weaning diarrhea and stimulate growth (Hill et al., 2000; De Mille et al., 2022). However, an increased awareness of the negative impacts of excess metals on both the surrounding environment and AMR proliferation have raised concerns over the use of metals (ex. Cu, Zn) at high concentrations exceeding dietary requirements in livestock production (Hölzel et al., 2012; Dębski, 2016).

Our previous work revealed widespread occurrence of tetracycline antimicrobials and ARGs conferring resistance to tetracyclines in manure collected from dairy animals in three states in the United States (Hurst et al., 2019). However, no correlations were observed between the monitored tetracycline ARGs and tetracycline concentrations in manure, suggesting that other external pressures may induce expression of the observed tetracycline resistance. Importantly, a number of other studies report positive correlations between the concentrations of metals and/or antimicrobials, and ARG copies in swine manure (Zhang et al., 2015), swine lagoons and beef cattle storage ponds (Zhang et al., 2013), and manure-amended soil (Zhu et al., 2013). While the presence of antimicrobials in manure is well-documented (Congilosi and Aga, 2021), very little has been reported on the co-occurrence of other chemical stressors, such as potentially toxic metals, in swine manure that may stimulate proliferation and spread of AMR in exposed bacteria. The state of Iowa is currently the top swine producing state in the United States, with 23 million swine as of June 2022 (USDA, 2022). Therefore, this study seeks to: i) detect and quantify antimicrobials, potentially toxic metals, and select ARGs present in manure samples from swine farms in Iowa; and ii) explore possible relationships between antimicrobials, metals, and ARGs in swine manure from different stages of animal production and feed composition.

2 Materials and methods

2.1 Chemicals and reagents

Primary analytical standards for lasalocid (LAS), monensin (MON), salinomycin (SAL), narasin (NAR), maduramycin (MAD), nonactin (NON, internal standard), nigericin (NIG, surrogate), sulfamethazine (SMZ), sulfadimethoxine (SDM), sulfameter (SMT), sulfamethiazole (SMI), sulfamerazine (SMR), sulfachloropyridazine (SCP), sulfadiazine (SPD), sulfamethoxazole (SMX), tetracycline (TC), oxytetracycline (OTC), demeclocycline (DMC, internal standard), minocycline (MIN, surrogate), azithromycin (AZI), clarithromycin (CLA),

erythromycin (ERY), roxithromycin (ROX), tilmicosin (TIL), and tylosin (TYL) were purchased from Sigma Aldrich (St. Louis, MO, United States). Spiramycin (SPI; as a mixture of spiramycin II, and III), and sulfathiazole (STZ) were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA, United States). Chlortetracycline (CTC), 4-epichlortetracycline (ECTC), anhydrochlortetracycline (ACTC), and anhydrotetracycline (ATC) were purchased from Acros Organics (now Fisher Scientific, Waltham, MA, United States). 4-Epitetracycline (ETC) was purchased from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ, United States) Tiamulin fumarate (TIA) was purchased from Sigma Aldrich (St. Louis, MO, United States). Standards of Phenyl–$^{13}C_6$–sulfamethazine ($^{13}C_6$-SMZ), D_4–sulfamethoxazole (D_4-SMX), $^{13}CD_3$-anhydroerythromycin ($^{13}CD_3$-AERY), and D_{10}–carbamazepine (D_{10}-CBZ) were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, United States). Surrogate standard D_{10}-tiamulin (D_{10}-TIA) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Liquid chromatography mass spectrometry (LC-MS) grade methanol (MeOH) and acetonitrile (ACN) were purchased from EMD Millipore (Darmstadt, Germany). High performance liquid chromatography (HPLC) grade ethyl acetate (EtOAc) was purchase from VWR Chemicals BDH® (Radnor, PA, United States). Disodium ethylenediamine tetraacetate (EDTA) and sodium hydroxide (NaOH) were purchased from Fisher Chemical (Fairlawn, NJ, United States). Citric acid monohydrate and anhydrous dibasic sodium phosphate (Na_2HPO_4) were purchased from Sigma Aldrich (St. Louis, MO, United States). Phosphoric acid, formic acid (88%), and Ammonium Acetate were of ACS grade and obtained through J.T. Baker (Phillipsburg, NJ, United States). The multi-element inductively-coupled plasma mass spectrometry (ICP-MS) certified reference standard (Model 82026-108) was purchased from VWR Chemicals BDH® ARISTAR® (Radnor, PA, United States). The rhodium (Rd) ICP-MS internal standard was purchased from Sigma Aldrich (St. Louis, MO, United States). TraceMetal™ Grade nitric acid (67%–70%) and hydrogen peroxide, 30% (Certified ACS grade), was purchased from Fisher Chemical (Waltham, MA, United States). Water (18.2 MΩ-cm) used throughout all experiments was purified using a Barnstead NANOpure™ Diamond system (Waltham, MA, United States).

2.2 Sampling approach

Swine manure samples were collected in summer 2020 from manure lagoons at swine farms in Iowa, United States. The locations of the farms where the manure samples were collected are not disclosed in this study to avoid privacy infringement. However, the farms were independently owned and geographically independent from each other. All manure samples collected were from swine farms representative of those typical in Iowa. 14 manure samples were collected from three stages of production: nine wean-finish (WF), four grow-finish (GF), and one gilt development (GD); and from farms using two types of feed compositions. The two feed rations were generally composed of corn, soybean, and distillers dried grains with solubles (DDGS), mixed in different proportions. Additionally, two facility ventilation practices, curtain sided and hybrid curtain/tunnel fan, were utilized at the studied farms. Ventilation practices varied with feed composition among farms. Data on stage of production and feed/facility practices were collected to provide further understanding for correlation statistics.

Manure samples were prepared using solid phase extraction (SPE) and analyzed for thirty antimicrobials including five ionophores, seven tetracyclines, nine sulfonamides, eight macrolides, and one pleuromutilin, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). One tetracycline (*tetM*) and one macrolide (*ermB*) gene were chosen to be analyzed based on their high occurrence in swine manure from previous literature (Whitehead and Cotta, 2013; Miller et al., 2020), and were quantified by quantitative polymerase chain reaction (qPCR) (Bio-Rad Laboratories, Inc.; Hercules, CA, United States). Genes conferring resistance to aminoglycosides (*AMG: str*, *aadD*, and *aadA2*), beta-lactams (*BLA: bla*$_{PSE}$, and *bla*$_{OXA}$-10), chloramphenicol (*CAP: cmlA5*, and *cmlA1*), fluroquinolones (*FQ: floR*), lincosamides (*LIN: lnuC*, and *lnuA*), sulfonamides (*SUL: sul2*, and *sul1*), tetracyclines (*TET: tetX, tetW, tetT, tetO, tetM, tetL, tetA*, and *tet36*), macrolides (*MAC: ermT, ermQ, ermF, ermC, ermB, erm36*, and *erm35*), and four mobile genetic elements (*MGE: intl3, intl2, intl1F165-clinical*, and *intl1-a-marko*) were monitored for presence by high-throughput qPCR (Biomark, Inc.; Boise, ID, United States). Finally, manure samples were digested and analyzed by ICP-MS for ten metals including barium (Ba), cadmium (Cd), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), lead (Pb), strontium (Sr), uranium (U), and zinc (Zn).

2.3 Extraction methods

2.3.1 Ionophore extraction

Approximately 100 mg of lyophilized, and homogenized manure were added to a 15-ml polypropylene centrifuge tube, spiked with 200 µl of NIG surrogate (200 ng ml^{-1}), allowed to equilibrate for 30 min, and extracted using the method described in Hurst et al. (2018) without modification. Prior to LC-MS/MS analysis, samples were spiked with 50 ng ml^{-1} internal standard (NON) and split into 2× 200 µl aliquots for quantification by single-point standard addition; one aliquot was spiked with 20 µl of 500 ng ml^{-1} ionophore native mix in mobile phase (50 ng ml^{-1} in vial), and the other aliquot was volume adjusted with 20 µl mobile phase diluent.

2.3.2 Sulfonamide, tetracycline, macrolide, and tiamulin extraction

Approximately 100 mg of lyophilized, homogenized manure were added to a polypropylene centrifuge tube, spiked with 200 µl of surrogate standard mix (200 ng ml^{-1} final concentration for each of

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TABLE 1 Optimized parameters for LC-MS/MS analysis of sulfonamides, tetracyclines, macrolides, and tiamulin (m/z values for precursor and fragment ions, fragmentor voltages, collision energies, and quantifying: qualifying ion ratios).

Antimicrobial	Precursor ion	Fragmentor energy (V)	Quantifying product ion (CE[a] eV)	Qualifying product ion (CE[a] eV)	Quant: Qual ratio
Ionophores[b]					
Lasalocid (LAS)	629	230	393 (30)	593 (30)	3.69
Monensin (MON)	693	260	461 (55)	501 (57)	4.72
Maduramicin (MAD)	939	220	877 (31)	719 (75)	24.1
Salinomycin (SAL)	773	260	431 (55)	531 (45)	3.57
Narasin (NAR)	787	270	431 (60)	531 (50)	4.06
Sulfonamides					
Sulfachloropyridazine (SCP)	285	108	156 (9)	92 (29)	0.88
Sulfadimethoxine (SDM)	311	136	156 (17)	108 (29)	1.80
Sulfamethizole (SMI)	271	108	156 (9)	92 (25)	1.12
Sulfamerazine (SMR)	265	108	92 (29)	108 (25)	1.07
Sulfameter (SMT)	281	108	92 (29)	108 (29)	1.22
Sulfamethoxazole (SMX)	254	108	92 (29)	108 (25)	1.15
Sulfamethazine (SMZ)	279	136	92 (33)	124 (25)	1.10
Sulfadiazine (SPD)	251	108	92 (13)	156 (25)	4.79
Sulfathiazole (STZ)	256	108	92 (25)	156 (9)	1.10
Tetracyclines					
Anhydrochlortetracycline (ACTC)	461	136	444 (17)	154 (29)	1.60
Anhydrotetracycline (ATC)	427	136	410 (13)	154 (25)	2.26
Chlortetracycline (CTC)	479	136	444 (21)	154 (29)	2.56
4-Epichlortetracycline (ECTC)	479	136	444 (21)	98 (41)	1.36
4-Epitetracycline (ETC)	445	136	410 (17)	427 (9)	9.31
Oxytetracycline (OTC)	461	136	426 (21)	443 (21)	6.53
Tetracycline (TC)	445	136	410 (17)	154 (25)	4.08
Macrolides					
Azithromycin (AZI)	375	121	591 (20)	158 (10)	1.35
Clarithromycin (CLA)	749	196	158 (25)	591 (14)	6.90
Erythromycin (ERY)	717	164	158 (29)	559 (9)	3.33
Roxithromycin (ROX)	838	215	158 (34)	679 (19)	3.30
Spiramycin II (SPI2)	443	125	174 (18)	582 (9)	5.88
Spiramycin III (SPI3)	450	125	174 (19)	597 (10)	7.69
Tilmicosin (TIL)	435	114	174 (22)	695 (13)	1.08
Tylosin (TYL)	917	295	174 (39)	772 (29)	8.67
Pleuromutilin					
Tiamulin (TIA)	495	60	192 (15)	119 (45)	1.32
Surrogate STDs					
Nigericin (NIG)	742	190	657 (26)	461 (30)	1.99
$^{13}C_6$-Sulfamethazine ($^{13}C_6$-SMZ)	285	136	98 (33)	162 (25)	7.59
D_{10}-Tiamulin (D_{10}-TIA)	505	60	119 (15)	202 (40)	1.65
$^{13}CD_3$-anhydroerythromycin ($^{13}CD_3$-AERY)	721	170	162 (25)	562 (5)	8.20
Minocycline (MIN)	458	136	441 (14)	154 (30)	17.1
Internal STDs					
Nonactin (NON)	754	220	185 (35)	369 (31)	4.11

(Continued on following page)

TABLE 1 (*Continued*) Optimized parameters for LC-MS/MS analysis of sulfonamides, tetracyclines, macrolides, and tiamulin (m/z values for precursor and fragment ions, fragmentor voltages, collision energies, and quantifying: qualifying ion ratios).

Antimicrobial	Precursor ion	Fragmentor energy (V)	Quantifying product ion (CE[a] eV)	Qualifying product ion (CE[a] eV)	Quant: Qual ratio
D_4-Sulfamethoxazole (D_4-SMX)	258	104	96 (25)	112 (25)	1.19
D_{10}-Carbamazepine (D_{10}-CBZ)	247	110	204 (35)	202 (20)	5.66
Democlocycline (DMC)	465	136	154 (33)	289 (29)	1.46

[a]CE, Collision energy.
[b]Ionophore ions and fragmentor energy from Hurst et al. (2018).

DMC, $^{13}C_6$-SMZ, D_{10}-TIA, $^{13}CD_3$-AERY), and allowed to equilibrate in the dark for 30 min. Samples were then extracted using the method described in Wallace and Aga (2016) with slight modification. Following SPE elution, MeOH eluates were reduced to 200 µl under a stream of N_2 gas and spiked with 25 µl internal standard mix (50 ng ml^{-1} of each D_{10}-CBZ, D_4-SMX, DMC in the final concentration), and reconstituted to 1 ml with 0.1% formic acid in water. Aliquots of each manure extract were taken to make 2x, 10x, and 50x dilutions to quantify analytes based on standard addition. This procedure is explained in more detail in Section 2.5 below. Undiluted and diluted extracts were split into 2× 200 µl aliquots for quantification by single point standard addition. One aliquot was spiked with 20 µl of 500 ng ml^{-1}, 1 µg ml^{-1}, or 2 µg ml^{-1} analyte mixtures in 0.1% formic acid in water, resulting in final concentrations of 50 ng ml^{-1}, 100 ng ml^{-1}, or 200 ng ml^{-1} in vial, respectively. Unspiked samples were volume normalized with 20 µl of 0.1% formic acid in water only. Samples were vortexed and then centrifuged for 5 min at 7,000 *g* prior to LC-MS/MS analysis.

2.3.3 DNA extraction

The DNA from 0.25 g wet weight (w.w.) of manure samples were extracted with the MagAttract® PowerSoil® DNA EP Kit (Qiagen; Germantown, MD, United States) and an epMotion® 5075 automated robot (Eppendorf; Framingham, MA, United States). The eluted DNA was further cleaned with a Clean and Concentrator kit (Zymo Research; Irvine, CA, United States). The concentrations of DNA were measured with the Quant-iT™ dsDNA Assay Kit, high sensitivity (ThermoFisher Scientific Inc.; Waltham, MA, United States) and DNA samples were stored at −80°C until further analysis.

2.4 Liquid chromatography-tandem mass spectrometry methods

Ionophores were analyzed according to Hurst et al. (2018), but with runtime increased from 20 to 20.5 min to allow for complete elution of ionophores (Hurst et al., 2018). Sulfonamide, tetracycline, macrolide, and tiamulin antimicrobials were analyzed using an Agilent 6410 triple quadrupole mass spectrometer equipped with an Agilent 1100 HPLC system and electrospray ionization (ESI) source operated in positive mode (Palo Alto, CA, United States). Analytical separation of antimicrobials was achieved using a 10-µl injection volume on a Raptor™ C_{18} column (100 mm, 2.1 mm I.D, 2.7 µm particle size) (Restek Corp.; Belefonte, PA, United States), and flow rate of 0.4 ml min^{-1}. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in ACN. The gradient profile consisted of 10% (B) ramped to 35% (B) over 4.5 min and 55% (B) over 2.5 min. This condition was held for 3 min before returning to the starting mobile phase of 10% (B). The total run time for each injection was 17 min. The analytical figures of merit for each analyte, including method limits of detection (LOD), can be found in the Supplementary Section S2.1 and Supplementary Table S1.

2.4.1 Metal analysis

Lyophilized manure was digested for metal analysis according to US Environmental Protection Agency (USEPA) Method 3050B with minor modifications to ensure complete effervescence. Briefly, 0.2 g of lyophilized manure was added to a 50-ml digestion tube with reflux cap, and refluxed in 2.5 ml (1:1, v:v) water: Fuming nitric acid in a heating block for 15 min at 95°C. The tube was then removed from the heating block and cooled to ambient temperature. An additional 1.25-ml of nitric acid was then added into a tube and refluxed again for 30 min at 95°C. Samples were again cooled and a third 1.25 ml volume of nitric acid was added into a tube and refluxed at 95°C for 30 min. Next, samples were carefully digested in 0.5 ml of water and 0.75 ml of 30% hydrogen peroxide solution at 95°C until the peroxide reaction dissipated (effervescence ends). Tubes were removed from the heating block, cooled, and an additional 0.5 ml of 30% hydrogen peroxide solution was added before refluxing again at 95°C until effervescence ceased. This step was performed three times prior to a final 30-min reflux. Finally, acid digestates were diluted to 25 ml with water, spiked with 10 ng ml^{-1} internal standard ^{103}Rd and filtered using a 0.45 µm PTFE (Environmental Express®; Charleston, SC, United States) filter before analysis by ICP-MS. Metals were quantified using a Thermo X-Series 2 ICP-

MS (ThermoFisher Scientific Inc., Waltham, MA, United States) using Collision Cell Technology (CCT) mode for Fe, Co, Sr, Cd, and U to reduce polyatomic interferences), and standard mode for Mn, Cu, Zn, and Ba. Metals were quantified based on their respective external standard curves using the most abundant isotope, normalized to the internal standard ^{103}Rd. Samples were analyzed undiluted, 10x diluted, and 50x diluted due to the wide range of metal concentrations present.

2.5 Quality assurance and standard addition spiking concentration for liquid chromatography-tandem mass spectrometry

The following quality control/assurance criteria were established following the suggested guide in a publication by Angeles and Aga (2018) to ensure positive detections. The criteria requires: 1) an ion intensity ≥ 10^3 for all monitored m/z signals; 2) peaks for monitored fragment ions must have area ratios for quantitative:qualitative (Q:q) within 20%, 35%, 60%, and 40% of the expected ratios (Table 1) for ionophores, sulfonamides, tetracyclines, and macrolides, respectively, 3) peaks must elute within a ±0.5-min retention time (RT) window around the corresponding standard peak; and 4) the calculated concentration must be above the established LOD for the particular analyte (Angeles and Aga, 2018). Tolerance percentages for criteria 2 were determined by the range at which 95% of the spiked sample ratios were considered accepted ratios for each analyte class. To consider an analyte as positive detection, three out of the four criteria needs to be met.

Here, we applied standard addition to quantify analytes due to the absence of commercially-available, stable isotope-labelled tetracycline standards, and to maintain a consistent quantification for all antimicrobial analytes. Standard addition quantification requires that the spiked concentrations be 50%–100% of the expected concentration in the sample (ThermoFisher Scientific, 2016). Preliminary analyses revealed variable, analyte-dependent concentrations. For example, the detected analytes of all antimicrobial classes analyzed within the same LC-MS/MS method, exhibited a concentration range of up to about 9,000 times the lowest detection within a single sample. Therefore, it was necessary to analyze both undiluted and diluted samples at three different standard addition levels to quantify analytes present at high and low concentrations. In brief, for each sample or diluted sample, a corresponding 200-µl aliquot was spiked with native analytes and analyzed in series with the unspiked aliquot for quantification. The undiluted and 2x diluted samples utilized 50 µg L^{-1} and 100 µg L^{-1}, respectively, of standard-added concentrations, and 10x and 50x diluted samples utilized 200 µg L^{-1} standard-added concentrations to quantify for all analytes.

2.6 Antimicrobial resistance gene quantification

The *ermB*, *tetM*, and 16S rRNA gene copies were quantified in DNA samples by qPCR using standard curves specific to each gene ranging from 10^1 to 10^7 gene copies. The sample DNA was diluted 1:10 prior to quantification. There were three technical replicates included for each sample. We removed any outliers above 1.5 x the standard deviation of the three sample replicates. Absolute gene copies were based on the standard curve, and copies $gram^{-1}$ of manure were back-calculated using the following equation:

$$Absolute\ abundance\left(\frac{copies}{gram}\right) = \frac{copies}{reaction} \times 10\ dilution\ factor \times \frac{100\ \mu L\ extracted\ volume}{2\ \mu L\ reaction\ vol} \times \frac{1}{0.25\ g\ manure}$$

2.7 Statistical methods

Statistical tests to determine AMR-stressor relationships for the manure was performed using Spearman's rank correlation in R. Feed ration compositions were coded -1 and 1 such that negative ρ values indicate correlation to diet 1 and positive ρ values indicate correlation to diet 2. Samples with non-detects were treated as 0 µg g^{-1} for correlation calculations to avoid data gaps during statistical analysis that could lead to spurious correlations.

3 Results and discussion

3.1 Antimicrobial detections

Out of 14 swine manure samples analyzed, 12 contained ionophores. However, only LAS and MON were detected, with concentrations ranging from 0.002 µg g^{-1} to 0.547 µg g^{-1} d.w. These results are notable because narasin is currently the only ionophore approved in the US for use in swine to promote growth (Rovira and Sturos, 2016). The presence of LAS and MON in manure may be attributed to their use as manure additives during storage to reduce foaming, rather than as animal medication (Berg, 2012). This is highly likely given that tiamulin combined with ionophores could be toxic to swine, and therefore should not be used together in feed (Rovira and Sturos, 2016).

Tetracyclines dominated the antimicrobial profiles of each sample, though each contained a unique combination of tetracycline analytes (Figure 1). In this study, OTC was detected in all samples with concentrations ranging from 2.39 to 1,260 µg g^{-1} d.w. Oxytetracycline is generally used in veterinary medicine as a

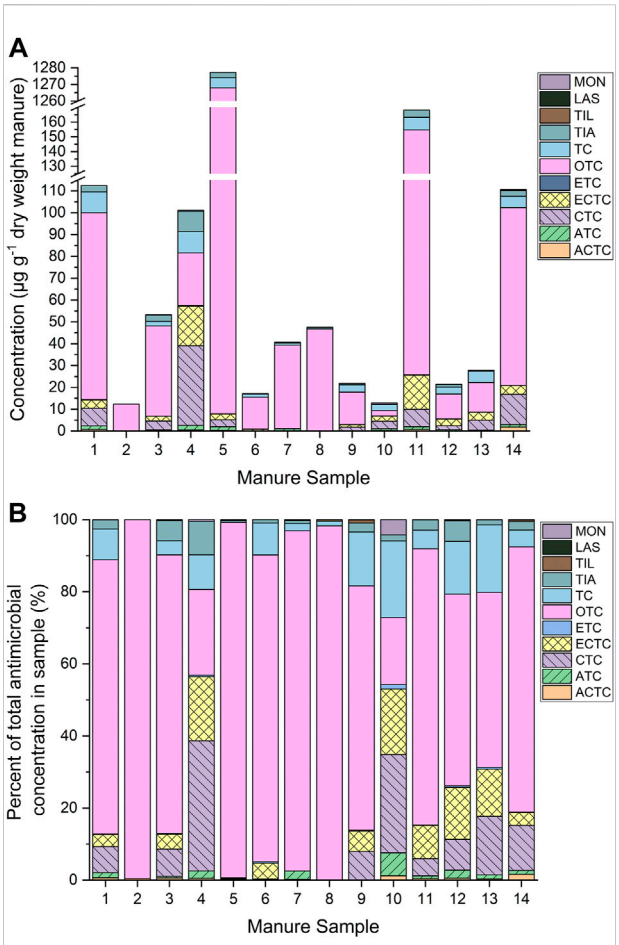

FIGURE 1
(A) Antimicrobial and transformation product concentrations and **(B)** antimicrobial and transformation product profiles of 14 swine manure samples from Iowa farms, analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Antimicrobials: Anhydrochlortetracycline (ACTC), anhydrotetracycline (ATC), chlortetracycline (CTC), epichlortetracycline (ECTC), epitetracycline (ETC), oxytetracycline (OTC), tetracycline (TC), tiamulin (TIA), tilmicosin (TIL), lasalocid (LAS), and monensin (MON).

broad spectrum antimicrobial for the treatment of gastrointestinal and respiratory diseases (Aktas and Yarsan, 2017). Oxytetracycline is typically administered *via* intravenous or intramuscular route, however, oral administration is more ideal when administering to large populations of food-producing animals (Martin-Jimenez et al., 1997). The supplementation of feed with sub-therapeutic concentrations of OTC has also been reported to promote swine growth (Soler et al., 2016). Most of the detections in this study fell within literature values of OTC in swine manure, which were reported from 0.21 to 354 µg g^{-1} d.w. (Martínez-Carballo et al., 2007; Chen et al., 2012). Additionally, the US Food and Drug Administration (FDA) approved dosage for swine is between 6.61 and 19.8 mg kg^{-1} body weight depending on treatment purpose (USFDA, 2004). Sample 5 is a notable exception with 1,260 µg g^{-1} d.w., which may be attributable to recent, wide-spread therapeutic OTC use in response to systemic infection.

Chlortetracycline and ECTC were detected in about 75% of the samples at concentrations ranging from 0.75 to 37 µg g^{-1} d.w., while TC and ATC detections ranged from 0.25 to 9.7 µg g^{-1} d.w in 71%–93% of samples. ACTC and ETC were present in the lowest concentrations, ranging from 0.0204 to 1.79 µg g^{-1} d.w., but were present at concentrations above the LOD in 86%–93% of samples. Oxytetracycline was the most detected tetracycline with detections frequency decreasing according to the series: OTC > CTC > ECTC > TC > ATC > ACTC > ETC. This trend is in general agreement with the values reported by Qiao et al. (2012), who reported swine manure detections of CTC (9 µg g^{-1} d.w.) and OTC (2.5 µg g^{-1} d.w.) well above those of other tetracyclines (Qiao et al., 2012). The relatively high detection frequency and concentrations of tetracyclines were anticipated because tetracyclines represent the largest class of antimicrobials sold for use in swine by mass, with a total of 3,948,745 kg sold in the US for agricultural use in 2020 (USFDA, 2021).

Of the macrolide antimicrobials monitored, only TIL was detected, and it was present in only 4 manure samples. Concentrations did not exceed 0.472 µg g^{-1} d.w. for TIL. Despite the use of ERY in swine, ERY residues were not detected in any of the manure samples. In contrast, TIA was present in all samples at concentrations ranging from <LOD to 9.4 µg g^{-1} d.w. According to literature, the recommended dosage is 10 mg TIL and 12 mg TIA per kg of body weight per day, administered *via* animal feed (Perruchon et al., 2022).

Recent work by Hughes and Andersson (2012) indicates that sub-MIC levels of antimicrobials can lead to rapidly enriched resistance mutations and *de novo* ARG selection in impacted pathogens. The low-levels of antimicrobials promote selection of variants with reduced susceptibility to antimicrobial activity by allowing for continued microbial growth in the presence of the chemical stressor and opportunity for horizontal transfer. Although only a few significant statistical relationships were observed during this study, it is possible that the low antimicrobial levels are contributing to the proliferation of AMR in a complex, synergistic fashion that is not readily apparent by traditional statistical analyses. As a result, it is imperative to examine selection pressure holistically and not discount the potential impact of low-level antimicrobials on AMR development in swine manure and in soil that receives manure application.

3.2 Metal detections

Metals can be classified as essential, non-essential, less toxic, and highly toxic according to their importance to metabolic function (Kochare and Tamir, 2015). However,

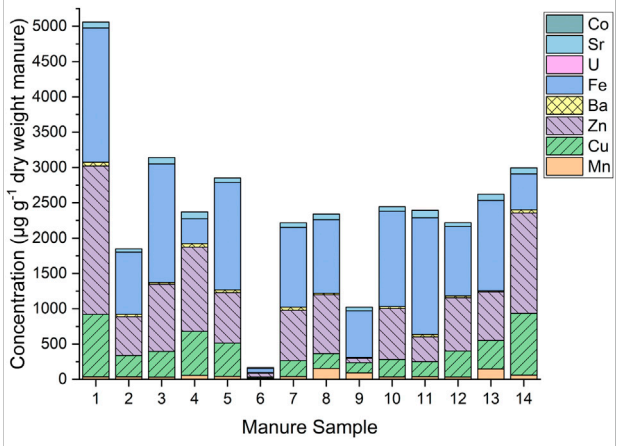

FIGURE 2
Metal concentrations in swine manure samples from Iowa farms, measured by inductively-coupled plasma mass spectrometry (ICP-MS). Cobolt (^{59}Co), strontium (^{88}Sr), uranium (^{238}U), iron (^{56}Fe), barium (^{137}Ba), zinc (^{66}Zn), copper (^{65}Cu), and manganese (^{55}Mn).

some of the essential metals such as Cu, Zn, Co, Cr, Mn, and Fe can be toxic if administered in excessive doses. For example, the recommended levels of supplementation for growing swine are 6 mg kg^{-1} body weight for Cu and 100 mg kg^{-1} body weight for Fe and Zn (NRC, 2012). Copper, Fe, and Zn were detected in all samples, with maximum concentrations of 887, 1,900, and 2,100 µg g^{-1} d.w., respectively. The concentrations of Cu and Zn observed in this study exceeded the MCCs of 11.8 µg g^{-1} and 22.8 µg g^{-1} in manure, respectively; these levels are consistent with other studies (Hölzel et al., 2012; Seiler and Berendonk, 2012). For example, Zhang et al. (2012) reported Cu concentrations between 77.62 and 1,521.43 mg kg^{-1} (d.w.), and Zn concentrations between 63.37 and 1,622.81 mg kg^{-1} (d.w.) in swine manures (Zhang et al., 2012). Additionally, Moral et al. (2008) reported values of 11–80 g Cu m^{-1}, 87–168 g Fe m^{-1}, and 75–533 g Zn m^{-1} in swine manure slurries (Moral et al., 2008). These elevated levels of Cu and Zn could be due to typical feed-mineral supplementation practices, driven by poor animal absorption (Ji et al., 2012).

Cobalt was present in one sample <0.55 µg kg^{-1} while Cd was not detected. Notably, the sample containing the highest total metal concentrations (sample 1, Figure 2), does not correspond with the sample containing the highest total antimicrobial concentrations (sample 5, Figure 1A). However, there is a moderate positive correlation between total metal concentrations and total antimicrobial concentrations (correlation coefficient, ρ = 0.64, p = 0.015). Iron concentrations were generally highest in WF swine, and Fe, Ba, and U concentrations were generally higher in swine fed with feed composition 1 (composed of corn, soybean, and DDGS, mixed in specific proportion).

Uranium (U) was included as a targeted metal in the ICP-MS analysis as a result of the detection of ^{238}U during an initial survey analysis of digested manure. Observed U was present in all but two samples at concentrations ranging from 0.0048 to 0.77 µg g^{-1} (d.w.). U concentrations exceeding the USEPA drinking water maximum contaminant level (MCL) of 0.030 mg L^{-1} prompted further research into possible U contamination in the areas surrounding the sample locations (USEPA, 2004).

A number of sites in Iowa reportedly handled and disposed of U and other radioactive materials in the 1950s–1960s. One Iowa disposal site was categorized as a class "b- significant threat to the environment—action required" site in 1991 by the Iowa Department of Natural Resources (IDNR) (IDNR, 2021a). The IDNR reports the site was used in the early 1950s for uncontrolled disposal of unknown quantities and types of radioactive materials. Additionally, purification and manufacture of high purity U in support of the US Department of Energy (USDOE) occurred on the site (Shirley, 1996). U shavings were similarly burned on the ground surface near the waste burial sites, requiring remediation in the 1980s. Groundwater and soil sampling conducted between the 1990s and 2008 report U levels as high as 7,500 µg L^{-1} (in groundwater) at this disposal site post-remediation; however, off-site migration has not been reported. Additionally, the army and Atomic Energy Commission (AEC) performed nuclear weapon assembly, storage, and disassembly at an ammunition plant located in Iowa between 1947 and 1975 (Fuortes, 2001; IDNR, 2021b). These activities lead to site contamination with depleted U. In 1990, the site was placed on the National Priorities List for Uncontrolled Hazardous Waste Sites by the USEPA (IDNR, 2021b).

As of 2021, two public drinking water systems in Iowa, serving a population of 2,037 reported U detections ranging from 0.015 to 0.030 mg L^{-1} (USEPA, 2004; USEPA, 2021). Despite the historic use and disposal of U near the sampling locations, it remains unclear whether the U detected in swine manure originated from ingestion of feed and water or leaching into the manure pit. Our results do not suggest enrichment, however, a significant, positive correlation between U and diet composition 1 suggest possible feed contamination related to crop production or grain formulation. Further work should be performed to determine whether swine farming enriches the U, or if it is merely an artifact of former regional use. Additionally, it is possible that positive U detection is a result of direct manure contact with soils in swine lagoons. Nevertheless, the detection of U presents a potential concern for animal and human health, and has the potential to stimulate the proliferation of AMR (USEPA, 2004; Zhou et al., 2022).

TABLE 2 Levels of antimicrobial resistance genes, antimicrobials, and metals detected in swine manure samples from manure lagoons at 14 swine farms in Iowa.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Diet	1	1	2	2	1	2	1	2	2	2	1	2	2	2
Stage	WF	GD	WF	GF	WF	GF	WF	GF	WF	WF	WF	GF	WF	WF
Gene presence (# genes)														
TET	1	3	5	2	2	4	2	5	3	3	2	6	6	5
SUL	ND	ND	1	ND	ND	1	ND	ND	ND	ND	ND	ND	ND	2
MGE	ND	ND	2	1	1	2	ND	0	2	ND	1	ND	ND	3
MAC	1	1	6	2	ND	5	ND	5	5	3	1	4	6	5
AMG	ND	ND	2	1	0	3	ND	2	1	1	ND	2	2	3
Gene abundance (copies g^{-1} wet weight, normalized to 16S gene)														
ermB	0.09	0.05	0.20	0.17	0.02	0.05	0.03	0.43	0.18	0.63	0.03	1.03	1.44	0.08
tetM	0.30	0.11	0.16	0.13	0.05	0.08	0.10	0.18	0.20	0.29	0.34	3.15	5.36	0.11
Antimicrobial concentration (μg g^{-1} dry weight)														
ACTC	0.74	0.053	0.310	0.52	0.327	0.056	0.089	ND	ND	0.157	0.77	0.113	0.094	1.79
ATC	1.61	<LOD	0.25	2.07	1.65	<LOD	0.93	<LOD	<LOD	0.82	1.22	0.48	0.32	1.19
CTC	8.1	<LOD	4.0	37	3.2	<LOD	<LOD	<LOD	1.73	3.5	8.0	1.83	4.5	13.8
ECTC	3.78	<LOD	2.20	18.0	2.63	0.75	<LOD	<LOD	1.24	2.34	15.6	3.08	3.66	4.0
ETC	0.198	<LOD	0.0868	0.353	0.142	0.0643	0.0331	0.0204	0.0550	0.161	0.197	0.0987	0.1247	0.145
OTC	86	12.3	41	24.1	1,260	14.6	38	47	14.8	2.39	129	11.4	13.5	81
TC	9.6	<LOD	2.07	9.7	6.2	1.53	0.82	0.59	3.27	2.74	8.7	3.14	5.2	5.1
ERY	<LOD	<LOD	ND	ND	<LOD	ND	<LOD	ND	ND	ND	ND	<LOD	ND	ND
TIA	2.90	<LOD	2.99	9.4	3.11	0.154	0.333	<LOD	0.55	0.209	4.9	1.21	0.39	2.63
TIL	ND	ND	0.0965	ND	ND	ND	ND	0.187	0.190	ND	ND	ND	ND	0.472
LAS	0.007	ND	0.004	ND	0.002	ND	0.059	0.004	ND	ND	ND	0.059	ND	0.075
MON	0.016	ND	0.028	0.444	0.003	ND	0.028	0.023	0.011	0.547	0.036	0.010	0.006	0.021
Metals (μg g^{-1} dry weight)														
Mn	35	35	31	57	42	16	40	154	92	35	39	32	149	60
Cu	887	303	365	624	473	16.22	226	211.1	144.4	247	212.9	368	402	877
Zn	2,100	549	952	1,193	713	57.5	713	835	61.9	722	350	755	689	1,420
Ba	53.2	34.64	22.37	49.1	40.49	6.73	45.4	20.81	12.06	27.53	35.85	26.76	15.21	44.6
Fe	1,900	880	1,680	352	1,520	57.9	1,130	1,040	660	1,350	1,650	980	1,280	508
U	0.77	0.096	0.0324	0.0253	0.2	ND	0.146	0.0294	ND	0.0048	0.338	0.0326	ND	0.149
Sr	84.8	47.0	89.6	95.5	63.0	11.56	62.7	79.6	52.9	62.0	106.1	55.0	85.8	85.4
Co	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	<LOD	<LOD	0.000479

Diet: 1 or 2 composed of corn, soybean, and distillers dried grains with solubles (DDGS), mixed in different proportions. Two facility ventilation practices: 1) curtain sided and 2) hybrid curtain/tunnel fan. (Ventilation practices varied with feed composition). Stage of swine production: WF-Wean-Finish, GF-Grow Finish, GD-Gilt Development. Gene classes: Tetracycline (*TET*), sulfonamide (*SUL*), mobile genetic elements (*MGE*), macrolide (*MAC*), aminoglycosides (*AMG*). Genes: macrolide (*ermB*) and tetracycline (*tetM*). Antimicrobials: anhydrochlortetracycline (ACTC), anhydrotetracycline (ATC), chlortetracycline (CTC), epichlortetracycline (ECTC), epitetracycline (ETC), oxytetracycline (OTC), tetracycline (TC), erythromycin (ERY), tiamulin (TIA), tilmicosin (TIL), lasalocid (LAS), and monensin (MON). Metals: manganese (Mn), copper (Cu), zinc (Zn), barium (Ba), iron (Fe), uranium (U), strontium (Sr), cobalt (Co), ND: Not detected.

3.3 Antimicrobial resistance gene detections

The *ermB* and *tetM* genes have been quantified in swine manure from storage pits in previous studies (Whitehead and Cotta, 2013; Miller et al., 2020). In this study, manure samples contained between 0.2 and 1.44 copies g^{-1} manure (w.w.) of the macrolide-encoding ARG *ermB* (normalized to the 16S rRNA copies). This range of relative abundance was higher than previously reported. For instance, in Nebraska stored manure the reported *ermB* relative abundance was 0.017 per 16S rRNA gene w.w., and an Iowa study measured 0.010 per 16S rRNA gene

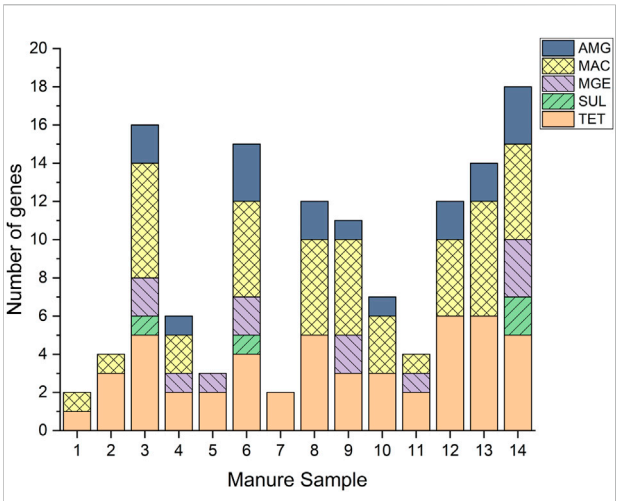

FIGURE 3
Antimicrobial resistance gene presence in 14 swine manure samples from Iowa farms, measured by quantitative polymerase chain reaction (qPCR). Quantified genes: Tetracycline gene (*tetM*), and macrolide gene (*ermB*). Gene classes: macrolide (*MAC*), aminoglycosides (*AMG*), sulfonamide (*SUL*), tetracycline (*TET*), and mobile genetic element (*MGE*).

w.w. (Lopatto et al., 2019; Miller et al., 2020). A much wider range of *tetM* genes were present in manure, with the number of copies ranging between 0.05 and 5.36 copies g^{-1} w.w. relative to the 16S rRNA gene (Table 2). The *tetM* gene has the widest bacteria host range of all tetracycline genes and is considered highly transferable, being located on transposons and integrases (Roberts, 2005). A previous study reported *tetM* gene copies as three times more abundant than 16S genes in a swine manure storage pit in Iowa (Alt et al., 2021).

Notably, *TET* genes were present in all manure samples, with the highest number of different genes detections in samples 12 and 13 (Figure 3). Interestingly, samples 12 and 13 have the fourth and sixth lowest total tetracycline concentrations of the 14 samples, respectively. These results comport with our previous work, reporting no observable correlation between tetracyclines and the corresponding *TET* ARGs (Hurst et al., 2019). *MAC* genes were present in all but two samples, samples 5 and 7, neither of which had macrolide detections. The presence of macrolides alone is insufficient to account for the presence of the MAC resistance observed in the study, considering non-detection of macrolides in 67% of samples that contained MAC resistant genes. These results suggest that co-selection of *TET* and *MAC* genes by other antimicrobials and chemical stressors could be an important ARG proliferation mechanism in the studied swine systems. Only three samples contained *SUL* genes (3, 6, and 14), however, none of the manure samples showed any positive sulfonamide detections. The ionophore, tetracycline, macrolide, and tiamulin antimicrobials detected in these samples, and the overall high levels of resistance genes further suggest that co-selection or cross-resistance is an important mechanism of AMR spread in manure. The greatest number of different *MGE*s was detected in sample 14 (3 of the 4 *MGE*s included in the method), which also exhibited the second highest total metal content, while all three *AMG*s detected during this study were present in samples 6 and 14. Interestingly, the sample containing the lowest number of total ARGs had the highest total metal content and third highest total antimicrobial concentration (sample 1, Table 2), indicating the need for correlational analyses to help elucidate the AMR mechanisms and selection pressures more fully. The absence of positive relationship between potential selection agents and total ARG content suggests complex relationships between existing selection agents and potentially susceptible microbiota, or that additional, unmonitored agents are present in manure and contributing to ARG development and proliferation; support for these hypotheses require further investigation.

3.4 Correlation statistics

There has been a notable historical absence of relationships between ARGs and antimicrobials in literature to date (Ji et al., 2012; Hurst et al., 2019; Komijani et al., 2021). However, several studies have reported strong correlations between ARGs and heavy metals in water (Komijani et al., 2021; Zou et al., 2021), animal manures (Ji et al., 2012; Liu et al., 2022), and soils (Ji et al., 2012; Knapp et al., 2017). Therefore, a global analysis of AMR-stressor relationships was performed for the manure sample set using Spearman's rank correlation (Figure 4; Supplementary Section S4; Supplementary Tables S4, S5).

The following metals exhibited the most significant overall positive correlation with ATC and/or ACTC across the sample set: Cu (ATC: $\rho = 0.67$, $p = 0.0093$; ACTC: $\rho = 0.75$, $p = 0.0022$), and Ba (ATC: $\rho = 0.84$, $p = 0.0002$). *TetM* was not significantly correlated to any tetracycline antimicrobial concentrations. Interestingly, *ermB* and *tetM* genes are strongly, positively correlated to each other, which could suggest co-selection, despite the lack of individual ARG and tetracycline correlations. The number of different *TET* genes detected had strong negative correlations with ATC ($\rho = -0.60$, $p = 0.024$), which could be due high cytotoxicity of ATC relative to the native compound or a bactericidal mode of action different from the native compound (Halling-Sørensen et al., 2002), which likely inhibited bacterial growth and variant selection when present (Hughes and Andersson, 2012). The number of different *SUL* genes detected exhibited strong, positive correlation with TIL ($\rho = 0.71$, $p = 0.0041$), indicating possible co-selection between the macrolide and sulfonamide resistance genes. Moreover, the presence of *TET* and *MAC* were negatively correlated with Ba concentrations (TET: $\rho = -0.60$, $p = 0.0242$) (*MAC*: $\rho = -0.73$, $p = 0.0033$), suggesting that Ba exhibits limited selection pressure in the studied systems. The limited number of significant correlations observed in this study suggest the relationship between chemical stressors and

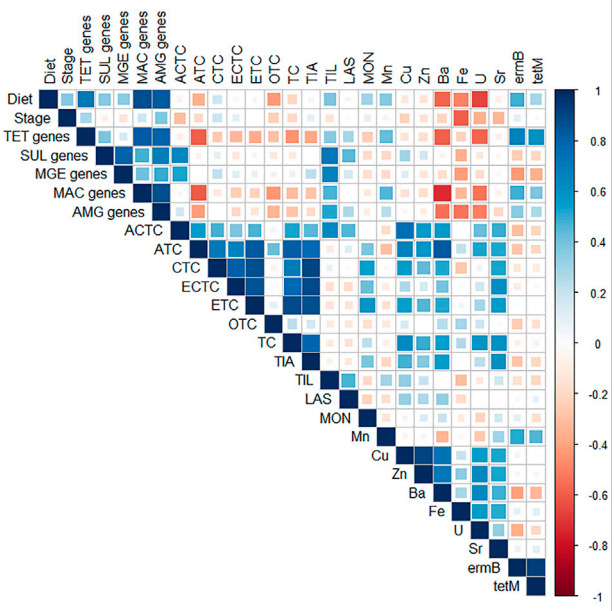

FIGURE 4
Spearman correlation matrix between antimicrobial [anhydrochlortetracycline (ACTC), anhydrotetracycline (ATC), chlortetracycline (CTC), epichlortetracycline (ECTC), epitetracycline (ETC), oxytetracycline (OTC), tetracycline (TC), tiamulin (TIA), lasalocid (LAS), and monensin (MON)] antimicrobial resistance genes (ARGs) [macrolide (*ermB*) and tetracycline (*tetM*)], ARG classes [macrolide (*MAC*), aminoglycosides (*AMG*), sulfonamide (*SUL*), tetracycline (*TET*), and mobile genetic element (*MGE*)], metals [manganese (Mn), copper (Cu), zinc (Zn), barium (Ba), iron (Fe), uranium (U), strontium (Sr), cobalt (Co)], diet, and stage of swine production (Stage). Correlation coefficients colored according to the value as depicted by scale, where a strong positive correlation is indicated by a dark blue box and a strong negative correlation is depicted by a dark red box. Size of squares corresponds to *p*-value, where correlations with *p*-value > 0.05 are considered insignificant and therefore left blank. Sulfonamides, spiramycin II and III (SPI2, SPI3), erythromycin (ERY), tylosin (TYL), and cobalt (Co) not included due to lack of detections in >1 sample, or concentrations < LOD.

ARG proliferation is extremely complex and may require studies under controlled conditions to fully understand the role of chemical stressors and the mechanisms of ARG development and spread in manure. Additionally, Lundström et al. (2016) reported low-level (1–10 μg L^{-1}) tetracycline exposure in aquatic environments resulted in increased expression of both *TET* resistance genes and genes conferring resistance to different classes of antimicrobials, supporting the conclusion of co-selection of *TET* and *MAC* genes observed in this study (Lundström et al., 2016).

4 Conclusion

This study analyzed and compared levels of antimicrobials and metals to ARGs in swine manure, to determine any correlations between the levels of ARGs and the types of chemical stressors present in manure. No strong positive correlation between ARGs and the metals analyzed were observed. Instead, a weak correlation between antimicrobial concentrations and the presence of corresponding class of resistance genes was observed. For example, *tetM* gene concentrations were not correlated with tetracycline antimicrobials. Genes conferring resistance to specific classes of antimicrobials occurred at sites that did not contain those compounds, such as *SUL* gene presence in samples with only ionophore, tetracycline, macrolide, and pleuromutilin detections. Furthermore, results indicated *tetM* and *ermB* copies correlated significantly with each other. These findings suggest possible co-selection or cross-resistance, as indicated by (Lundström et al., 2016). It should be noted that some metal concentrations (Fe, Ba, and U) were positively correlated to diet 1, while Fe concentrations were positively correlated to an earlier stage of life (ex. WF). Future studies exploring correlations between AMR and other potential chemical stressors in manure are necessary to reveal other possible AMR-promoting agents. Additionally, the presence of U in 85% swine manure samples at levels up to 0.77 μg g^{-1} was unexpected and merits additional research to determine to what extent do chemical stressors other than antimicrobials contribute to AMR spread in agroecosystems.

Animal manure is an important source of nutrients and organic matter that can be used to improve soil health. However, untreated manure may be a continuous source of chemical stressors that increase selection pressure promoting AMR development and proliferation in receiving soils. The potential for AMR transmission from edible crops grown in soils fertilized with untreated manure, and the lack of understanding of the degree of human exposure to AMR generated through agriculture is a critical issue within the One Heath Framework. Results from this research provide new information that can be used to encourage better management practices that will lead to the reduction of AMR spread in agroecosystems.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://datadryad.org/stash/dataset/doi:10.5061/dryad.v6wwpzh03.

Author contributions

JC: Conceptualization, methodology, investigation, writing—original draft. TN: Conceptualization, methodology, investigation, writing—original draft. AH: Conceptualization, funding acquisition, reviewing of manuscript. MS: Conceptualization, funding acquisition, reviewing of manuscript.

JW: Conceptualization, methodology, writing—original draft. DA: Conceptualization, writing—original draft, funding acquisition.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.1018739/full#supplementary-material

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