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Complex interplay between composted manure application, metal contamination, and antibiotic resistance genes profile under tropical field conditions

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ABSTRACT

Manure applications in agricultural soils are a major driver of antibiotic resistance gene (ARG) dissemination, yet long-term effects of composted manure applications under tropical real field conditions remain unclear. This study assessed how successive composted manure applications influence soil physicochemical attributes, bacteriome and resistome profiles in the Brazilian Cerrado, including one site with naturally high heavy metal content. Across all sites, multidrug resistance genes were most abundant, followed by macrolide-lincosamide-streptogramin (MLS), tetracycline, β-lactam and glycopeptides resistance, aligning with predominance of Actinomycetota and Pseudomonadota as key ARG hosts. Manure increased soil pH and available phosphorus (P), with pH significantly shaping bacterial communities and pH and P the resistome in uncontaminated sites (2 and 3). However, in the metal-rich site (1), Cu was the dominant driver. Manure increased ARG richness and changed resistome structure but did not affect clinically relevant genes or resistome diversity. Metal resistance genes (MRGs), particularly for Cu and Zn, strongly influenced resistome dynamics, highlighting co-selection. Integrons integrase genes (*intI*) abundance increased in metal-depleted but not in metal-rich soils. While composting appears to mitigate ARG spread, particularly for clinically relevant genes, the high antibiotic use in livestock, large manure volumes, and potential for ARG persistence in tropical soils highlight the need for further research on manure treatment strategies and ARG fate in these environments.

Environmental Implication.

Our study highlights the environmental risks of antibiotic resistance gene (ARG) dissemination in tropical agricultural soils, emphasizing the role of manure application and heavy metal contamination in shaping soil resistome. While composted manure increased bacterial diversity and ARG richness, it did not significantly impact clinically relevant genes and resistome diversity, suggesting that composting may help mitigate ARG spread but does not eliminate it. Metals were the dominant drivers of ARG selection in the contaminated site, underscoring the role of co-selection mechanisms in maintaining resistance. However, manure applications increased integrons abundance, raising concerns about horizontal gene transfer and potential ARG proliferation into pathogens. These findings stress the urgent need for improved manure management policies in Brazil, where high antibiotic use in livestock and large manure volumes pose significant environmental and public health risks. Developing sustainable manure treatment strategies and monitoring ARG persistence are essential to limit antibiotic resistance proliferation in tropical agricultural ecosystems.

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1. Introduction

Antibiotics are widely used in veterinary medicine to prevent and treat diseases and promote animal growth (Wu et al., 2023). Animal husbandry consumes approximately 70 % of global antibiotics (Singh & Kim, 2025), significantly contributing to proliferation of antibiotic resistance genes (ARGs) and posing a major global health threat (Heuer et al., 2011; Wang et al., 2020a; Wu et al., 2023). Manure is commonly applied to agricultural soils due to its high nutrient and organic matter content (Rayne & Aula, 2020). However, following application, ARG abundance and diversity in soils often increase before gradually declining over time (Peng et al., 2017; Muhammad et al., 2020; Liu et al., 2021; Wang et al., 2021a). The type of manure also influences ARG levels, with swine manure generally leading to higher abundances than cattle, chicken, or mixed manure (Wu et al., 2023).

Successive manure applications can either increase (Peng et al., 2015; Liu et al., 2021) or have no effect (Ghosh & LaPara, 2007; Tang et al., 2015) on ARG abundance, depending on soil conditions. Manure amendments also alter soil resistome composition by modifying physicochemical properties (Xie et al., 2018; Guo et al., 2018; Pu et al., 2019; Deng et al., 2020; Wang et al., 2020b). A meta-analysis revealed that antibiotic-contaminated manure significantly increases total ARG and mobile genetic element (MGE) abundances by 591 % and 351 %, respectively, compared to unamended soils ($P < 0.05$). The greatest increase was observed for sulfonamide resistance genes (1121 %), followed by aminoglycoside (852 %) and tetracycline (763 %) resistance genes (Zhang et al., 2022). Long-term manure application can lead to linear or exponential ARG accumulation and shifts in associated bacterial communities. Although most ARG levels decline within 11 years after manure discontinuation, some, particularly tetracycline and macrolide ARGs, persist near their previous levels (Zhang et al., 2023a).

ARG contamination in agricultural soils is a global concern, but its impact should be particularly relevant in Brazil, a major agricultural producer and third-largest consumer of veterinary antibiotics (9 % of global consumption), behind China and the USA. By 2030, Brazilian antibiotic use is projected to double compared to 2010 levels (Van Boeckel et al., 2015). The Cerrado biome, covering nearly 2 million km^2, is the world's most biodiverse savanna (Mizobe, 2019). Approximately 40 % of its land is used for agriculture, with 69 % allocated to pasture and 31 % to intensive cultivation, primarily soybean-corn rotation. Livestock production is expanding in this region, and manure is largely applied to grain-producing areas. Due to intense weathering, Cerrado soils are typically acidic, nutrient-poor, and phosphorus-deficient, requiring intensive chemical management, including limestone and phosphate applications (Brito et al., 2020). Despite Brazil's prominence in global agriculture, data on ARGs in its agricultural soils are limited (Bastos et al., 2018; Ferreira et al., 2024), with no studies specifically addressing the Cerrado biome.

Cerrado soils may naturally contain high heavy metal concentrations or accumulate them through anthropogenic activities, mainly from intensive agriculture and phosphate fertilizers (Cabral et al., 2023). Heavy metals are persistent in soils and act as long-term selective pressures on microbial community (Ji et al., 2012), often exerting a stronger influence on ARG dynamics than antibiotics themselves (Mazhar et al., 2021). Anthropogenic inputs, such as repeated manure application, increase heavy metal contents in agricultural soils (Peng et al., 2017; Guo et al., 2025), promoting ARG proliferation through co-selection with metal resistance genes (MRGs) (Maurya et al., 2020; Sun et al., 2021). ARGs and MRGs often co-occur on mobile genetic elements (MGEs), enabling metal-driven ARG enrichment (Guo et al., 2018). Co-selection also occurs via cross-resistance, in which a single resistance mechanism in a microorganism, like efflux pumps, provides protection against multiple types of toxic agents (Gillieatt & Coleman, 2024; Zhang et al., 2021a), or via co-regulation, in which different resistance genes, such as ARGs and MRGs, are controlled by the same regulatory elements; for example, a shared promoter or transcription factor (Vats et al., 2022).

Heavy metals' role in modulating ARGs has been extensively investigated in recent years. Microcosm studies have shown that metals such as Cd, Cu, and Zn influence ARG dynamics (Tongyi et al., 2020; Wang et al., 2021b; Li et al., 2022a; Fu et al., 2023), with effects observed across diverse terrestrial environments. Co-selection between ARGs and metals has been reported in anthropogenically impacted areas, including urban soils (Knapp et al., 2017; Zhao et al., 2019), mining sites (Sinegani and Younessi, 2017; Chen et al., 2019a; Zhong et al., 2021), and landfills (Li et al., 2024). Agricultural systems have also been studied, such as paddy fields (Zheng et al., 2023; Zhang et al., 2024) and soils amended with organic fertilizers like sewage sludge (Urra et al., 2019) and animal manure (Guo et al., 2018; Dong et al., 2022). However, no studies have assessed the effects of composted manure in agricultural soils already burdened with natural heavy metal contamination.

Composting is an effective strategy to mitigate the risks associated with fresh manure application, reducing ARG spread, promoting heavy metal complexation, and lowering pathogen loads (Peng et al., 2018; Awasthi et al., 2019; Manyi-Loh et al., 2016; Xie et al., 2016; Zheng et al., 2022). Its thermophilic phase can decrease ARG transfer to soil by 2–96 % and alter resistome composition (Deng et al., 2020; Xu et al., 2020a). Compared to mineral fertilizers or composts, fresh manure amendments generally increase ARG abundance across different soil types (Qian et al., 2018a; Yang et al., 2020; Li et al., 2022b; Chen et al., 2018). However, composting does not fully eliminate ARGs (Selvam et al., 2012; Ray et al., 2017), and manure-derived ARGs have a greater impact on soil resistome than those from sewage sludge (Wu et al., 2023). Some ARGs, including beta-lactam, MLSB, multidrug, and vancomycin, may even increase in response to composted manure applications (Zhang et al., 2022). Despite its benefits, composted manure remains a significant reservoir of ARGs (Wang et al., 2020a), and its long-term use in agriculture may still contribute to ARG persistence and propagation. Further research is needed to assess these risks under real field conditions, particularly in soils contaminated with heavy metals.

This study evaluated the impact of successive applications of composted animal manures on soil bacterial community and resistome profiles, focusing on diversity, resistome composition, and their correlations with soil attributes. It also assessed the effects of manure on clinically relevant ARGs, metal resistance genes, and integrons, which play a key role in horizontal gene transfer (HGT) (Li et al., 2017; Verraes et al., 2013; Ali et al., 2020). Integrons serve as markers of anthropogenic activity in natural environments and are directly associated with ARG transmission risks (Gillings, 2014; Hu et al., 2017; Li et al., 2017). To achieve these objectives, soil samples were collected from three agricultural sites in the Brazilian Cerrado biome, including one with naturally high metal content. The study aimed to address two key questions: a) How does successive composted manure application influence resistome and bacterial community of highly weathered tropical soils? and b) How do high heavy metal contents and other soil physicochemical properties affect antibiotic resistome and bacterial community?

2. Material and methods

2.1. Soil samples collection

Soil samples were collected at the municipalities of Rio Verde-Goiás State from two places (sites 1 and 2) and of Claraval-Minas Gerais State (site 3), in which distinct thermophilic composted manures have been applied for at least 4 years (Table 1). At site 2, the pig manure was just "stockpiled" for at least 70 days. All sites were in farmed rural properties and soil samples were taken from adjacent agricultural fields (called parallel soils), either with or without manure application. Compost is applied annually in all treatments. Sampling was performed in September-2021, prior compost application and crop seedings in sites 1

Table 1
Sample codes, sampling locations, compost compositions, and application periods.

Sample codes	Sampling locations	Compost compositions	Application periods
S1	Rio Verde	Control	–
S1-M_c	Rio Verde	Poultry litter + cattle manure compost	4 years
S2	Rio Verde	Control	–
S2-M	Rio Verde	Stockpiled pig manure	10 years
S3	Claraval	Control	–
S3-M_c	Claraval	Poultry litter compost	6 years

and 2. Site 3 was under a perennial coffee plantation. Each sample was composed of 10 subsamples randomly collected from top-soil layer (0–20 cm), in triplicates, which were homogenized, placed into plastic bags, and ice-transported to the laboratory. Within 48 h, soil aliquots were taken, placed into microtubes for DNA extraction, and freezer stored at −20 °C. The remaining soil samples were refrigerated (4 °C) until analysis of their physicochemical attributes.

2.2. Physicochemical attributes of the soils

Soil physicochemical attributes were determined according to Van Raij et al. (2001). In summary, soil-pH was measured in 0.01 mol L^{-1} $CaCl_2$ solution; available P and K were extracted by ionic exchange resin (K was quantified by flame photometry and P by colorimetry); soil texture was determined with Bouyoucos hydrometer using 1.0 mol L^{-1} NaOH as dispersant; and total-N was determined by the Kjeldahl method. OM was extracted by sodium dichromate and quantified by colorimetry. Total heavy metal (As, Cd, Cr, Cu, Hg, Ni, Pb, and Zn) contents were extracted following 3050-B method (USEPA, 1996) and quantified using inductively coupled plasma (Optical Emission Spectrometer iCAP 6300, Thermo Scientific).

2.3. DNA extraction

Total DNA was extracted using DNeasy Powerlyzer Power Soil Kit (Quiagen, Hilden, Germany), following manufacturer's specifications. Extracted-DNA quality was verified in a 1.0 % agarose gel and quantified with BioDrop uLite+ (Biochrom, United Kingdom). Metagenome sequencing was performed for 150 bp paired-end sequences on the Illumina NovaSeq 6000 platform by Novogene (Novogene Advancing Genomic, California, United States).

2.4. Metagenome analysis

Samples were filtered to discard sequences with Q-value < 20, using Trimmomatic v0.40. The paired-end sequences (R1 and R2) were joined by PEARv0.8. R1 sequences that did not merge were included along with merged sequences. Again, sequences with Q-value < 20 and less than 50 nucleotides were excluded using Trimmomatic v0.40. Quality control of the sequences was confirmed using FastQC v0.11.9. Then, 22 million randomized sequences per sample were selected to normalize the data. ARGs' survey was performed with DeepArg, SS mode, adopting default e-value ≤ 10^{-10} and probability ≥ 80 % (Arango-Argoty et al., 2018). ARG diversity was determined based on the number of identified ARG subtypes. Metal resistance genes (MRGs) were identified using the BacMet database via DIAMOND v2.0.14 (Buchfink and Xie, 2015), with an e-value < 10^{-5}, alignment identity ≥ 90 %, and a minimum alignment length of 25 amino acids. Integrons marker (class *Int*I genes) were identified using Integrall database (Moura et al., 2009), through DIAMOND v2.0.14, also applying an e-value < 10^{-5}, alignment identity ≥ 80 %, and a minimum of 25 amino acids. Taxonomic classification was performed using MG-RAST v.4 pipeline. Genus-level profiles were obtained using the RefSeq database, with an e-value < 10^{-5}, alignment identity ≥ 60 %, and minimum alignment length of 15 bp. Sequences were assembled using MEGAHIT v1.1.2, based on the de Bruijn graph approach. Contigs ≥ 500 bp were selected for the final assembly. To construct a non-redundant gene set, all gene sequences were clustered using CD-HIT v4.6.1 (90 % identity and 90 % coverage). ARG identification in contigs was performed using DeepARG (LS mode), applying an e-value ≤ 10^{-5} and a probability score ≥ 80 % (Arango-Argoty et al., 2018). Taxonomic classification of ARG-containing contigs was conducted using Kraken2 (Wood et al., 2019).

2.5. Statistical analysis

All data were tested for normality. Differences in soil physicochemical attributes were assessed using ANOVA followed by post-hoc Tukey-HSD tests. Venn diagrams were generated, considering genes present in at least two replicates. ARG abundance was evaluated using Welch's t-tests, with p-values corrected by the Bonferroni method. Shannon diversity indices for the resistome and bacterial communities in parallel soils (with and without composted manure) were calculated and compared via Welch's t-tests, also applying Bonferroni correction. Heatmaps of the 25 most abundant ARGs were generated using z-scores. The 25 most clinically relevant genes were selected based on literature (Martineau et al., 2000; Stoll et al., 2012; Devarajan et al., 2015; Qian et al., 2021). Taxaplots illustrating the main ARG-hosting phyla were created, while a dendrogram based on the mean abundance of ARG hosts at the class level, was constructed. Heatmaps of the 40 most abundant ARG-host genera within representative ARG classes were generated using z-scores. The effects of composted manure applications on those ARG abundances were analyzed using Welch's t-tests, with p-values corrected by the Benjamini-Hochberg method in STAMP (v2.1.3). ARG composition in parallel soils was compared using Chi-square tests.

Redundancy analysis (RDA) was used to assess clustering of resistome and bacterial community. Collinear soil physicochemical attributes were removed. Monte Carlo tests with 999 random permutations were conducted to determine the significance of soil attributes on the resistome and bacterial community. Procrustes analysis, using 999 permutations, evaluated relationships between the resistome profile and bacterial community structure. Mantel tests were used to correlate ARGs with MRGs. Statistical analyses, including ANOVA, Welch's t-tests, and Chi-square tests, were performed in R (v4.1.2). Shannon index, RDA, dendrogram, Mantel, and Procrustes analyses were conducted using the vegan package (v2.6–4). Heatmaps and Venn diagrams were generated using gplots (v3.2.0) and the taxaplot was generated with ggplot2 (3.5.1).

3. Results

3.1. Physicochemical attributes of the soils

Adjacent manured and unmanured soils from the same site exhibited similar textural class, reinforcing their parallelism, and all soils were acidic (pH ≤ 6.0) (Table 2). Among unmanured soils, S1 had higher pH, organic matter (OM), and Co, Cr, and Ni contents compared to the others (Table 2). At this site, Cr and Hg concentrations exceeded intervention values (IV) for potential human health risks, while Cu and Ni levels surpassed prevention values (PV) (CETESB, 2021). Manure applications did not consistently increase soils' organic matter or heavy metal contents but raised soil pH and available phosphorus (P) levels, regardless of manure type (Table 2).

3.2. Abundance and richness of ARGs in the soils

Metagenomic sequencing of the samples yielded 22135591–43907946 brute paired-end sequences; 8646296–21233087 merged sequences; and 78326–380642 contigs with at least 500 bp (Table S1). A total of 594 distinct ARGs were identified (Fig. 1A).

Table 2
Physicochemical attributes of parallel soils without (S1, S2, and S3) and with (S1-Mc, S2-M, and S3-Mc) successive composted manure applications.

Soil attributes	Soil treatments					
	Site 1		Site 2		Site 3	
	S1	S1-M_c	S2	S2-M	S3	S3-M_c
Clay/ g kg^{-1}	355 ± 8.5b	388.8 ± 8.8ab	292 ± 9.1c	311 ± 0.7c	421 ± 9.7a	305 ± 0.6c
Silt/ g kg^{-1}	158.3 ± 8.9b	192.7 ± 8.3a	58 ± 5.1c	55.3 ± 1.2c	170 ± 9ab	81.3 ± 3.8c
Sand/ g kg^{-1}	486.3 ± 0.9d	418.7 ± 1.9e	650 ± 4a	633.3 ± 1.3b	409 ± 1.2e	614 ± 3.2c
pH	5.7 ± 0.0003c	6.0 ± 0.01a	5.1 ± 0.01d	5.8 ± 0.02b	4.3 ± 0.02e	6.0 ± 0.01a
OM/ g kg^{-1}	42.3 ± 1.3b	50.9 ± 0.6a	28.5 ± 1.2c	27.6 ± 1.5c	33.8 ± 0.4d	35.0 ± 0.3d
P/ mg kg^{-1}	34.8 ± 0.6c	56.8 ± 1.1b	10.5 ± 1.9d	68.9 ± 2.8a	57.9 ± 0.1b	76.0 ± 2.3a
K/ mg kg^{-1}	1.3 ± 0.06c	1.4 ± 0.02c	1.4 ± 0.09c	1.6 ± 0.03bc	1.8 ± 0.01ab	1.9 ± 0.04a
N/ mg kg^{-1}	1684 ± 198a	2207 ± 327a	1057 ± 402a	889 ± 91a	1106 ± 315a	1677 ± 318a
Co/ mg kg^{-1}	22.5 ± 1b	29.9 ± 0.3a	3.5 ± 0.1e	4.9 ± 1d	10.6 ± 0.3c	6.2 ± 0.3d
Cr/ mg kg^{-1}	515.9 ± 4.8a	447.9 ± 3.8b	100.1 ± 2.6c	94 ± 4.1c	45.4 ± 2.5d	40.2 ± 1.6d
Cu/ mg kg^{-1}	72.3 ± 1.1b	99.6 ± 1.1a	15.6 ± 0.5f	24.1 ± 0.7e	70.3 ± 0.6b	46.4 ± 0.8d
Ni/ mg kg^{-1}	65.4 ± 1.6a	50.5 ± 0.3b	8.7 ± 0.2d	9.4 ± 0.5d	19.0 ± 0.7c	12.3 ± 0.5d
Pb/ mg kg^{-1}	7.9 ± 1.5bc	8.6 ± 1.3bc	11.4 ± 1.2ab	11.7 ± 0.6a	5.7 ± 0.6c	6.4 ± 0.9bc
Zn/ mg kg^{-1}	46.2 ± 0.6d	70.8 ± 0.6a	13.7 ± 0.4e	67 ± 0.5ab	62.1 ± 1.2b	51.9 ± 2.0c
As/ mg kg^{-1}	< 2	< 2	< 2	< 2	< 2	< 2
Cd/ mg kg^{-1}	< 2	< 2	< 2	< 2	< 2	< 2
Hg/ mg kg^{-1}	2.88 ± 0.36	2.17 ± 0.01	< 2	< 2	8.81 ± 1.93	< 2

Values are expressed as mean ± standard error (n = 3).
Different letters indicate contrasting mean values by the Tukey-HSD, $p \leq 0.05$.

Multidrug resistance genes were the most abundant (135 types), followed by β-lactam (101), macrolide-lincosamide-streptogramin (MLS) (80), aminoglycoside (70), tetracycline (48), glycopeptide (11), rifampicin (10), nucleoside (4), and bacitracin resistance genes (3) (Fig. 1A, Table S2).

The overall abundance of ARGs did not significantly differ between paired soils at each site (S1 vs. S1-Mc; S2 vs. S2-M; S3 vs. S3-Mc) (Tukey-HSD, $p > 0.05$). Multidrug resistance genes were the most abundant (49.5 %), followed by those conferring resistance to MLS (11.9 %), tetracyclines (6.8 %), glycopeptides (4.9 %), rifamycin (3.7 %), bacitracin (3.4 %), fluoroquinolones (2.4 %), β-lactams (2.3 %), aminoglycosides (1.9 %), nucleosides (1.8 %) and other categories (11.3 %) (Fig. 1B). No clear differences in ARG class abundances were observed among the different soils (Fig. S1).

Gene richness did not correlate with abundance across ARG classes. For instance, β-lactam resistance genes were highly diverse (101 types, Fig. 1A) but accounted for only 2.3 % of total ARG abundance in soils (Fig. 1B). Similarly, aminoglycoside resistance genes (70 types) represented just 1.9 % of total abundance.

3.3. Resistome composition modulation

Soils receiving composted manure exhibited a greater diversity of ARGs, particularly at site 3 (Fig. S2). At site 1, 370 genes were shared between parallel soils, with 36 unique to S1 and 39 to S1-Mc (Chi-square test, $p = 0.73$) (Fig. S2A). At site 2, 382 genes were shared, while 28 and 42 were unique to S2 and S2-M, respectively (Chi-square test, $p = 0.09$) (Fig. S2B). At site 3, 373 genes were shared, but S3-Mc exhibited significantly more unique ARGs (56) compared to S3 (26) (Chi-square test, $p < 0.001$) (Fig. S2C).

Composted manure application did not affect the most frequent genes in resistome at site 1 but altered the abundance of nine genes at site 2 and 16 genes at site 3 (Fig. 2A). However, no clear pattern emerged, as some genes increased (e.g., *acrB* and *bacA*), while others decreased (e.g., *muxB*, *Pur Res Prot*, and *vanR*). At site 3, the clinically relevant genes *aac3* and *msrA* increased following manure applications (Fig. 2B).

3.4. Bacterial community, resistome diversities, and soil physicochemical attributes

Soil bacterial diversity varied significantly across all sites (Welch's *t*-test, $p < 0.05$), with higher diversity in manured soils, particularly at sites 2 and 3 (Fig. S3A). The smaller difference at site 1 suggests greater convergence in bacterial community' diversity. However, resistome diversity did not significantly differ after composted manure applications at any site (Welch's *t*-test, $p > 0.05$) (Fig. S3B).

Redundancy analysis (RDA) showed that samples clustered based on soil attributes, with the first two axes explaining ~96 % of bacterial community' variation (adjusted $R^2 = 0.68$) (Fig. 3A). Despite differences in location, compost sources, and application periods, manured soils from sites 2 and 3 (S2-M and S3-Mc) had similar bacterial community compared to their unmanured counterparts (S2 and S3), likely due to increased soil pH. In contrast, bacterial community at site 1 were largely unaffected by manure applications and were influenced by high heavy metal concentrations, particularly Cu and Ni (Fig. 3A). For the resistome, the first two axes explained ~79 % of the variation (adjusted $R^2 = 0.37$) (Fig. 3B). Again, pH and available P were key factors shaping the resistome at sites 2 and 3, while Cu was the primary driver at site 1.

3.5. Correlations between MRGs and ARGs

Metal resistance genes (MRGs) for Cr, Cu, Ni, and Zn were positively correlated with the most abundant ARG classes in the soils (Table 3). Cu resistance genes showed the highest associations, correlating with seven ARG classes, followed by Zn (six), Cr (four), and Ni (two). In contrast, Co and Pb resistance genes exhibited no correlation with ARG classes.

3.6. Interactions between bacterial community and resistome

A significant positive correlation was observed between bacterial community structure and resistome within each site (Procrustes, $M^2 = 0.45$, $r = 0.68$, $p < 0.0001$), as well as between resistome and bacterial community structure (Mantel, $r = 0.77$, $p < 0.0001$) (Fig. 4). Unamended soil resistome from sites 2 and 3 (S2 and S3) showed lower correlations with bacterial community structure (Fig. 4). In contrast, their respective composted manured soils (S2-M and S3-Mc) exhibited stronger correlations between their resistome and bacterial communities. At site 1, both unamended and manured soils (S1 and S1-Mc) showed strong correlations between bacterial community structure and resistome, likely due to the high heavy metal contents that diminished the impact of manure application.

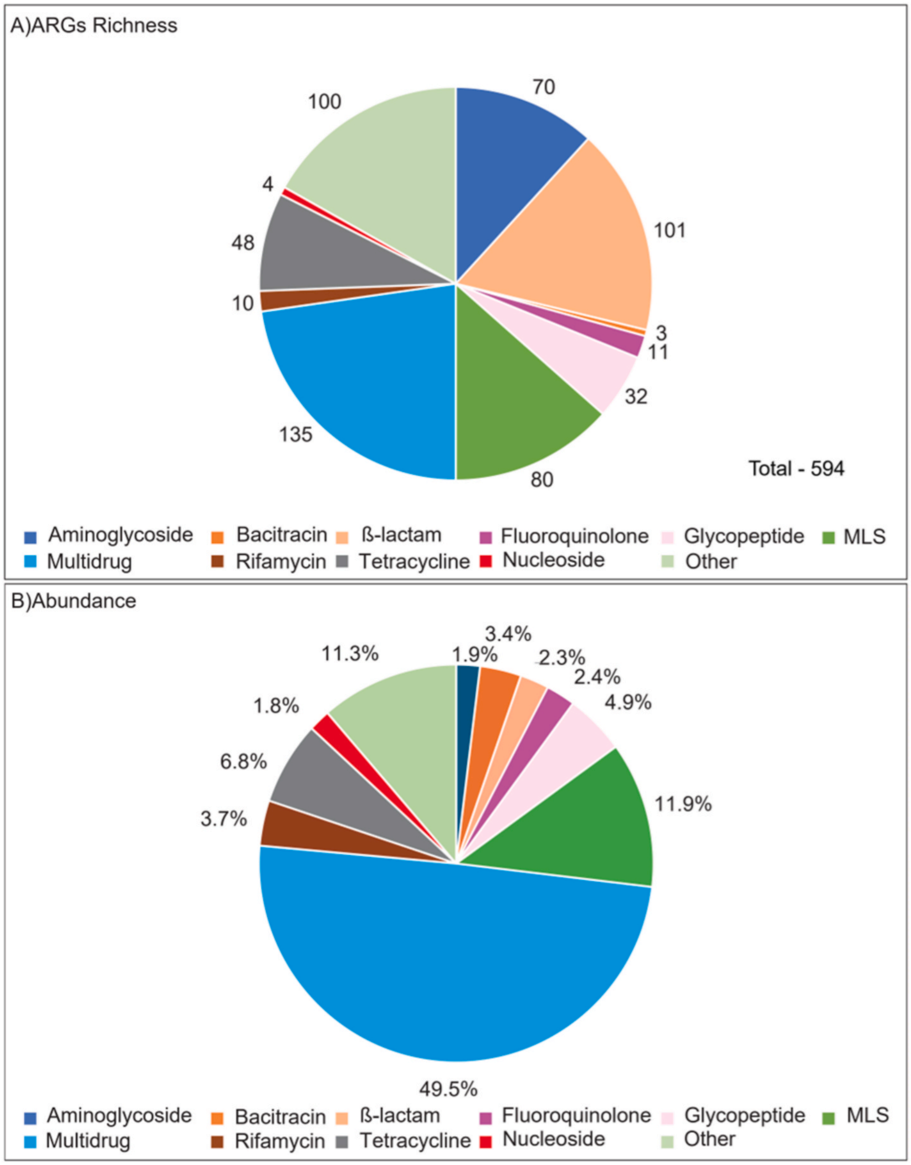

Fig. 1. Richness (A, refers to the number of genes present within each of the most abundant classes) and abundance (B, refers to percentage of ARGs for a specific class of antibiotic in relation to their total).

3.7. Co-occurrence for microbial taxa and ARGs

At phylum level, ARG hosts were predominantly Actinomycetota (54.9–75.4 %) and Pseudomonadota (22.8–43.3 %), jointly accounting for over 83 % of ARG host abundance across all treatments. At class level, Actinomycetes and Alphaproteobacteria were the most dominant groups (Fig. 5A). ARG host profiles clustered according to soil physicochemical attributes, as shown in the dendrogram (Fig. 5B). Similar clustering was observed in parallel soils from site 1 (S1 and S1-Mc), characterized by high heavy metal contents, and in manured soils from sites 2 and 3 (S2-M and S3-Mc), which exhibited higher pH. The multidrug resistance class encompassed the highest number of host genera (28), followed by aminoglycoside and glycopeptide resistance genes (23 each) (Fig. 5C), whereas beta-lactam and fluoroquinolone resistance genes were hosted by only four genera. Notably, *Streptomyces* and *Bradyrhizobium* were the most diverse ARG hosts, carrying 36 and 28 distinct ARGs, respectively (Table S3).

3.8. Integrons abundance

Integrons were quantified by identifying genes from integrase genes (*intI*). In heavy metal-rich soils (site 1), integrase abundance did not significantly differ between soils with or without compost application (Fig. 6). In contrast, at sites 2 and 3, compost applications led to an increase in *intI* abundance.

4. Discussion

Successive manure applications influence soil microbial community, often enhancing bacterial diversity through physicochemical modifications, particularly in pH and nutrient availability (Liu et al., 2012; Sun et al., 2015; Zhang et al., 2018; Neher et al., 2020; Xu et al., 2020b; Li et al., 2021a; Schlatter et al., 2022; Zhang et al., 2023a). In this study, manured soils exhibited higher pH and available P. The pH is a crucial factor shaping microbial community, especially in highly weathered Cerrado soils with high acidity (Liu et al., 2012; Tan et al., 2013; Alovisi et al., 2020).

At site 1, where heavy metal contamination was high, Cu and Ni

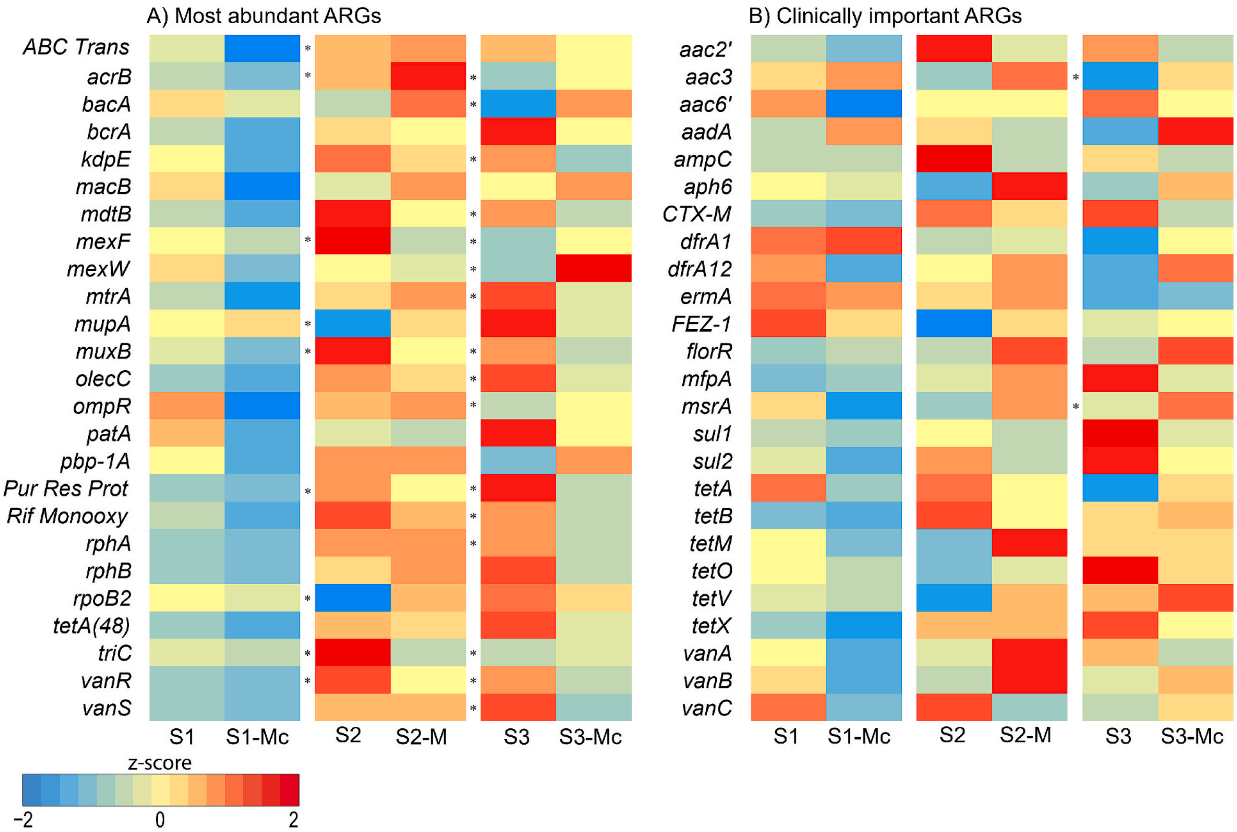

Fig. 2. Heatmaps displaying the 25 most abundant ARGs in soils (A) and the 25 most clinically relevant ARGs (B). Asterisks next to treatment pairs denote significant differences between parallel fields, as determined by the Welch's *t*-test ($p \leq 0.05$).

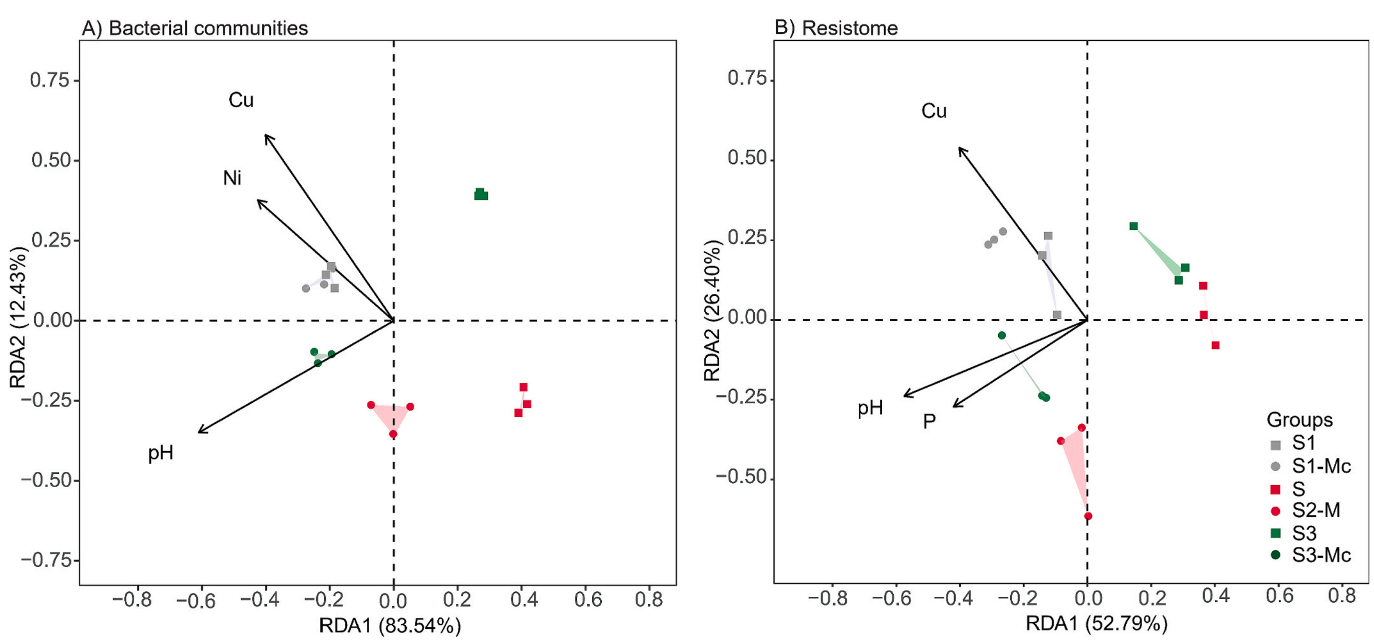

Fig. 3. Redundancy analysis (RDA) of correlations between key soil physicochemical attributes and bacterial community (A) or the resistome (B) in parallel soils with and without composted manure application. Significance of environmental variables were evaluated using Monte Carlo permutation tests ($p \leq 0.05$).

strongly influenced bacterial community structures. Heavy metals, particularly Cu and Ni (Li et al., 2014; Li et al., 2015; Song et al., 2018), are known to shape microbial community (Zhang et al., 2016; Deng et al., 2020; Li et al., 2021b) and can promote resistance gene persistence through selection pressure (Griffiths & Philippot, 2013). Despite this, bacterial diversity remained high, likely due to microbial adaptation mechanisms, including genetic resistance via mutations and horizontal gene transfer (Margesin et al., 2011; Li et al., 2015).

ARG profiles were dominated by multidrug resistance genes, followed by MLS and tetracycline resistance genes, a pattern observed

Table 3
Correlations between metal resistance genes (MRGs) and antibiotic resistance genes (ARGs) for the most relevant classes of antibiotics.

ARGs	Metal Resistance Genes (MRGs)					
Classes	Co	Cr	Cu	Ni	Pb	Zn
Aminoglycoside	−0.005[†]	0.243	0.535**	0.252	0.001	0.400*
Bacitracin	−0.124	0.240	0.335*	0.246	−0.167	0.199
B-lactam	−0.081	0.285*	0.315*	0.305*	0.088	0.264
Fluoroquinolone	−0.053	0.153	0.040	−0.004	−0.006	−0.090
Glycopeptide	0.042	0.311*	0.559**	0.238	0.014	0.524*
MLS	−0.126	0.093	0.241	0.258	0.016	0.361*
Nucleoside	0.001	0.459**	0.551**	0.243	0.208	0.329*
Rifamycin	−0.031	0.213	0.410**	0.245*	−0.019	0.523**
Tetracycline	−0.101	0.310*	0.375**	0.223	−0.064	0.416**

[†] Correlation Coefficient – Mantel tests, $p \leq 0.05$ (*) and $p \leq 0.01$(**).

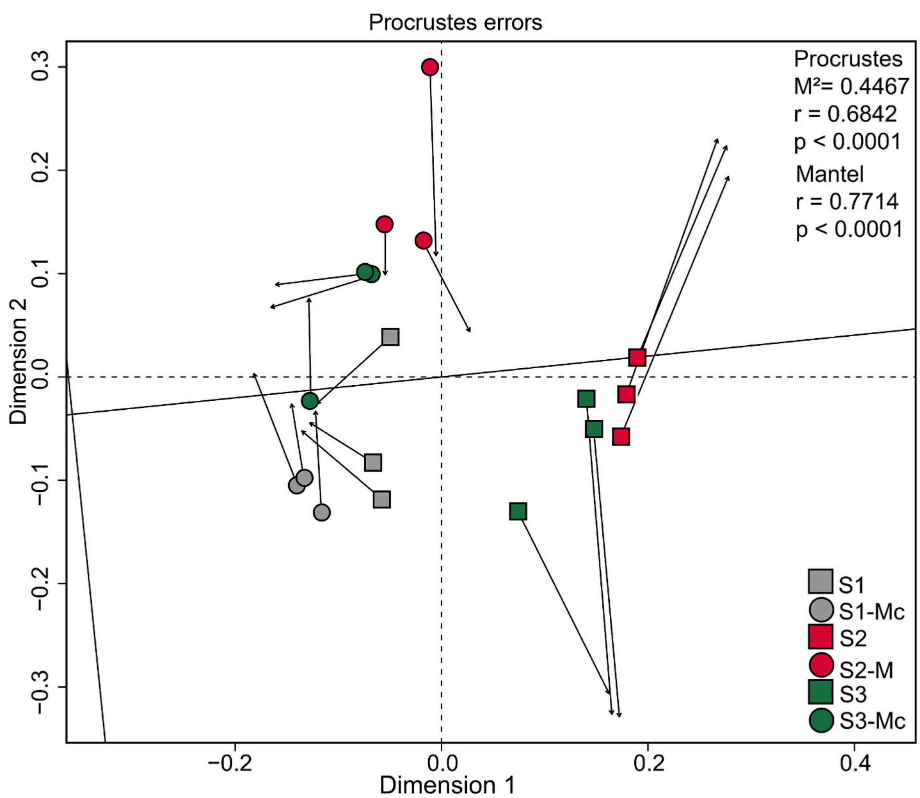

Fig. 4. Procrustes analysis of bacterial community structures and resistome profiles in parallel soils at each site, with and without composted manure application. Squares and circles represent soil sample positions based on the resistome profile, while arrows indicate their corresponding positions based on bacterial community structure.

globally in diverse environments, including the Alaskan tundra and Amazon rainforest (Qian et al., 2021). Liu et al. (2021) reported that multidrug-resistant genes accounted for 54.4–85.3 % of total ARGs in manure-treated soils, while Guo et al. (2018) found them most abundant in pig-manured soils (37.1–49.5 %), followed by fluoroquinolone (11.6–20.8 %), bacitracin (7.8–15.8 %), and sulfonamide (0.5–15.8 %) resistance genes. Despite this global trend, our study found that repeated manure applications did not significantly alter total ARG abundance within each site. This may be due to ARGs suppression during composting or dissipation over time (Keenum et al., 2021). Composting inactivates manure-borne microbes and ARGs through heat and microbial competition, while time interval between compost application and sampling may allow native soil microbiota to re-establish dominance. In all sites, compost was applied annually, but sampling was performed at season end, which may have allowed ARGs to dissipate throughout agricultural year. Increases in resistome abundance after manure application are often transient, with levels returning to baseline within weeks or months (Chen et al., 2019b; Radu et al., 2021; Wang et al., 2021a), as introduced fecal bacteria are gradually outcompeted by native soil microbiota (Zhang et al., 2023a). Supporting this, Chen et al. (2019b) reported no change in ARG abundance and diversity 120 days after applying antibiotic-amended cow manure compost. Wind et al. (2021) also found that ARG levels returned to baseline within 120 days of compost application. Guo et al. (2018) observed a short-term increase in ARGs following compost or raw manure application, but levels normalized after 32 and 60 days, respectively. Even long-term application showed limited impact, as Wang et al. (2018) found no significant ARG accumulation after 26 years of swine manure compost use in dry or paddy soils.

The most diverse ARG classes were multidrug, beta-lactam, MLS, aminoglycoside, and tetracycline resistance genes, a pattern also observed globally across manure-amended soils in subtropical, humid continental, and cold desert climates (Cheng et al., 2019; Muurinen et al., 2017; Sun et al., 2023). Multidrug resistance genes were generally

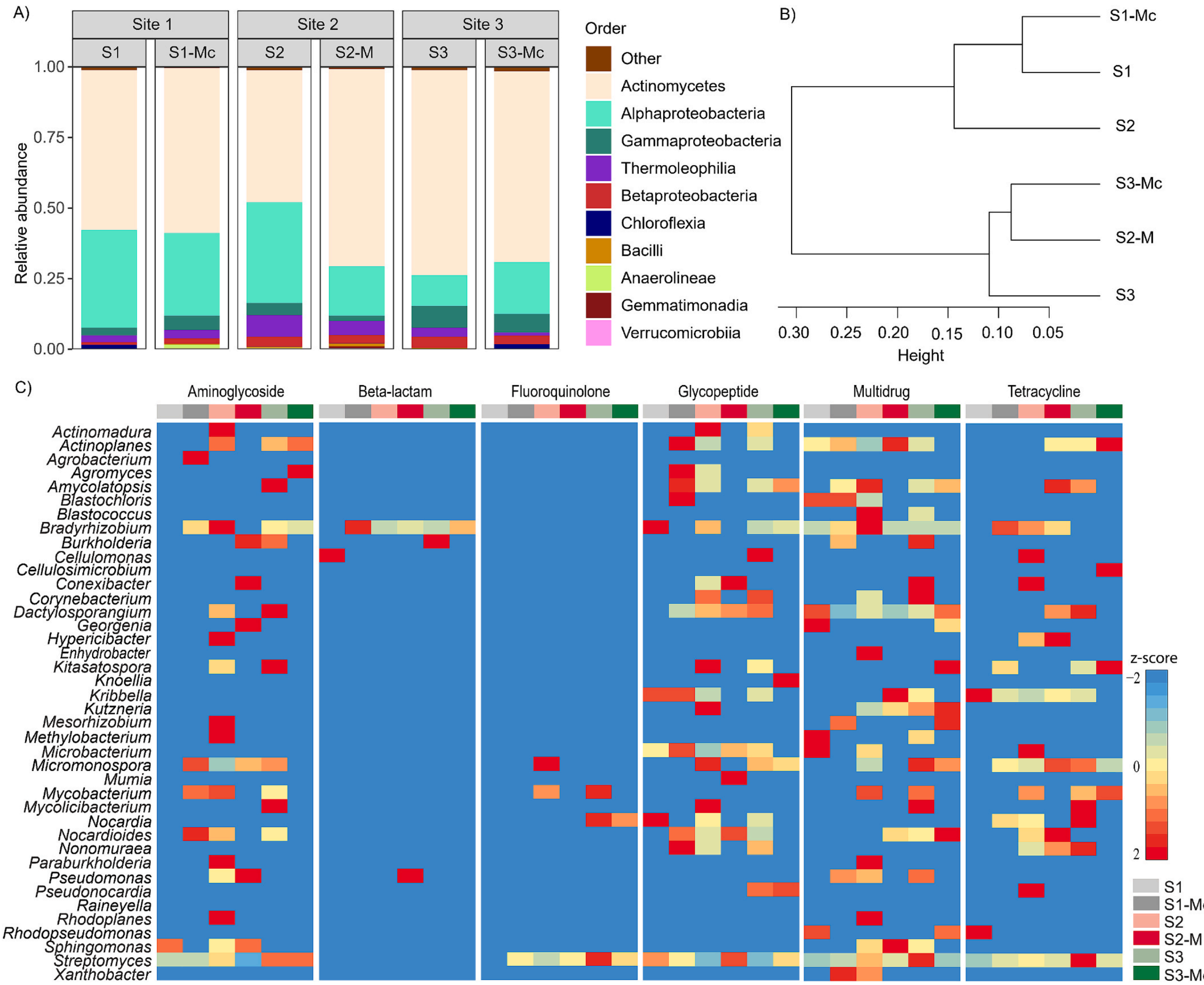

Fig. 5. Taxonomic composition of ARG hosts among the ten dominant taxonomic classes (A), dendrogram illustrating taxonomic relationships of ARG hosts (B), and heatmap showing the most abundant microbial genera hosting ARGs (C) in parallel soils at each site, with and without composted manure applications.

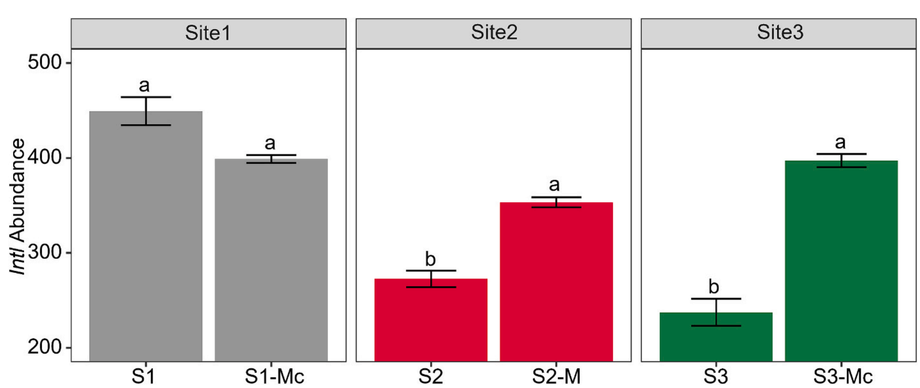

Fig. 6. Rarefied abundance of integrase (*intI*) genes in parallel soils from each site, with and without composted manure applications. Values represent the mean ± standard error (n = 3); Welch's *t*-test ($p \leq 0.05$).

the most abundant, particularly in subtropical regions of China and the USA (Guo et al., 2018; Yang et al., 2020; Liu et al., 2022). These ARGs originate naturally in soils and can be present even in environments with minimal anthropogenic influence (Walsh & Wright, 2005; Van Goethem et al., 2018; Song et al., 2021) due to natural presence of antimicrobials in soils (McSorley et al., 2018) resulted from microbial competition or signaling between microbial cells (Linares et al., 2006; Nesme et al., 2015; Niehus et al., 2021). Multidrug ARGs play multifunctional roles beyond antibiotic resistance, including detoxification, virulence, and signal trafficking (Lubelski et al., 2007; Martínez, 2008).

In this study, ARGs abundance increased in uncontaminated soils (sites 2 and mainly 3) but not in heavy metal-rich soils (site 1), suggesting that metal contamination exerted a stronger influence on soil resistome than manure application. Han et al. (2018) observed introduction of 67, 82, and 73 unique ARGs into soils amended with swine, poultry, and cattle manures, respectively, none of which were composted. In high Arctic soils, lithology (metal content) determined ARGs abundance in unimpacted sites, whereas fecal inputs from wildlife were the dominant source of ARGs in impacted areas (McCann et al., 2019). However, ARGs tend to exhibit higher relative abundance in tropical regions compared to boreal and temperate zones (Khalid et al., 2023). Tropical conditions likely influence ARGs distribution and host profiles, promoting greater microbial abundance. Warmer climate may enhance genetic exchange among bacteria, including more frequent conjugative transfers of antibiotic resistance plasmids (Devarajan et al., 2017). We also observed that clinically relevant ARGs were unaffected by manure, consistent with previous findings that composted manure has minimal impact on resistome composition (Chen et al., 2019b; Deng et al., 2020).

Long-term manure applications can increase microbial and ARGs diversity (Wang et al., 2017; Zhang et al., 2021b). Some studies suggest that manure introduces unique ARGs via MGEs (Han et al., 2018; Liu et al., 2021), while others report no long-term impact on ARGs diversity (Muurinen et al., 2017; Cheng et al., 2019). Our results indicate that composted manure enhanced bacterial diversity of metal deprived soils (sites 2 and 3) but did not significantly increase resistome diversity of all sites, likely mitigating ARG introduction through microbial competition (Cheng et al., 2019; Wang et al., 2021a). This reinforces that composting may be an effective strategy to mitigate the introduction of extraneous ARGs present in manure.

Bacterial community structures were major determinants of resistome profiles (Forsberg et al., 2014; Su et al., 2015). At site 1, high heavy metal contents, especially Cu and Ni, drove bacterial clustering, while at sites 2 and 3, manure-induced pH changes dictated bacterial shifts. Despite differences in geographical locations and manure treatment, soils from sites 2 and 3 showed similar bacterial and ARG profiles, highlighting the influence of soil physicochemical properties in shaping the resistome (Su et al., 2015; Chen et al., 2016). Therefore, pH for bacterial community and pH and P for the resistome were key drivers of bacterial and resistome shifts post-manure application in these soils (sites 2 and 3), as previously reported (Cadena et al., 2018; McCann et al., 2019; Wang et al., 2020b; Zhang et al., 2021c). Animal waste applications are known to elevate soil pH and nutrient content (Li et al., 2012; Zhang et al., 2017), influencing bacterial community and ARG modulation, particularly in P-deficient tropical soils (Cadena et al., 2018; Mendes et al., 2021).

In contrast, site 1′s resistome was primarily shaped by high Cu concentrations rather than manure application. Although an essential metal for microbiota, in high concentrations, Cu becomes toxic to soil organisms, damaging the cell macromolecules (Seiler and Berendonk 2012; Kang et al. 2018). Its low sorption compared to antibiotics like tetracyclines increases soil bioavailability (Gillieatt & Coleman, 2024). Cu also interacts with antibiotics, affecting their mobility and stability, and enhancing microbial efflux pump activity (Poole, 2017). Among trace metals, Cu exerts the strongest selective pressure on ARGs, exceeding Fe, Ni, Pb, and Zn (Kang et al., 2018; Glibota et al., 2020). This role has been demonstrated in rhizosphere (Pan et al., 2023), subsurface (Wang et al., 2021c), mining (Zou et al., 2025), urban (Knapp et al., 2017; Zhao et al., 2019), and agricultural soils amended with sludge (Urra et al., 2019) or manure (Peng et al., 2017; Guo et al., 2018; Zhang et al., 2023a). In a microcosm study, Kang et al. (2018) showed that even a short 2-month exposure to Cu altered ARG composition, with stronger effects at higher concentrations. Long-term field studies have also shown that Cu can affect ARG abundance and diversity, depending on soil type and Cu levels (Hu et al., 2016).

Unlike antibiotics, which degrade over time, heavy metals persist in soils and impose sustained selective pressure on microbial communities and ARGs (Ji et al., 2012; Chen et al., 2019a). This pressure facilitates ARGs enrichment through co-selection with metal resistance genes (MRGs), particularly when both gene types co-occur on MGEs (Guo et al., 2018). Zhou et al. (2016) reported a positive correlation between ARG and MRG richness. Under metal stress, MRGs contribute to bacterial survival and indirectly sustain antibiotic resistance (Li et al., 2022a), a process intensified by the genetic linkage of ARGs, MRGs, and MGEs, which facilitates horizontal transfer of resistance clusters (Wu et al., 2024).

In our study, Cu and Zn resistance genes were strongly correlated with the overall resistome structure, with those of Cr and Ni also contributing significantly, supporting known genetic linkages indicative of co-resistance (Farias et al., 2015; Zhang et al., 2021a). Genetic associations between Zn, especially Cu, and ARGs have been repeatedly observed in mobile genetic elements (MGEs) such as plasmids and transposons (Poole, 2017). Soil bacteria have been found carrying Cu and Zn resistance genes and ARGs for tetracyclines and β-lactams, within the same genome (Glibota et al., 2020), with suck linkages occurring on both chromosomes and plasmids (Martins et al., 2014; Poole, 2017). Silveira et al. (2014) reported that Cu resistance genes and ARGs co-occurring in *Enterococcus* can be co-transferred to other isolates. High levels of Cu and Zn (or other metals) may promote plasmid conjugation via co-resistance, as both gene types often reside on the same plasmid (Xu et al., 2017). Co-selection may also result from cross-resistance, where a single gene confers resistance to both antibiotics and metals (Kang et al., 2018; Zhang et al., 2021a). For instance, RND efflux pumps like MdtABC mediate resistance to antibiotics, Cu, and Zn (Nishino et al., 2007; Gillieatt & Coleman, 2024).

Evidence of co-selection via co– and cross-resistance also extends to Cr and Ni. Cr resistance plasmids have been found to carry ampicillin resistance genes (Vats et al., 2022), and Cr exposure can induce efflux pump expression and plasmid conjugation (Wu et al., 2024). Cr has also been linked to increase abundance of sulfonamide, MLSB, β-lactamase, and aminoglycoside resistance genes, along with upregulation of related efflux pumps (Zhang et al., 2023b). When combined with sulfamethoxazole, Cr further amplified *sul1* and *sul2* gene abundances (Xu et al., 2023). In parallel, Ni has been linked to ARG co-selection, including in aquatic environments (Stepanauskas et al., 2006). This may result from cross-resistance via shared efflux pump mechanisms between multidrug and Ni resistance genes (Zhang et al., 2021a). Co-resistance has been directly observed in plasmids carrying both Ni MRGs and ARGs (Raja and Selvam, 2009; Zhai et al., 2016).

Actinomycetota and Pseudomonadota were the predominant ARG-hosting phyla, reflecting their well-documented capacity to both produce and resist multiple antibiotics (Alam et al., 2022; Park et al., 2019), consistent with findings from previous studies (Han et al., 2022). *Streptomyces* (Actinomycetota) alone produces over 70 % of naturally derived antibiotics, including tetracyclines, aminoglycosides, macrolides, and β-lactams, while members of Pseudomonadota, such as *Pseudomonas* and *Burkholderia*, synthesize phenazines and polyketides. Likewise, *Streptomyces* and *Bradyrhizobium* (Pseudomonadota) correspond to genera carrying the highest ARGs diversity. Although *Bradyrhizobium* is primarily known for N fixation rather than antibiotic production, it may acquire ARGs through horizontal gene transfer mechanisms, such as integrons and gene cassettes. These mobile genetic elements facilitate rapid adaptation by capturing and expressing novel resistance genes, potentially facilitating the spread of ARGs to other environmental or pathogenic bacteria (Ormeño-Orrillo & Martínez-Romero, 2019).

The above phyla dominance aligns with the high diversity of multidrug resistance gene hosts reported by Han et al. (2022). Multidrug resistance genes, including those for tetracyclines, MLS, beta-lactams, and glycopeptides, originate naturally in soils (McSorley et al., 2018), where antibiotic-producing microbes use these compounds for competition and signaling, while others acquire ARGs for protection (Linares et al., 2006; Niehus et al., 2021). However, human activities accelerate their spread, even to remote locations like Antarctica (Wang et al., 2016). ARGs are both intrinsic to soil ecosystems and shaped by environmental pressures, making it difficult to distinguish between natural and anthropogenic sources, contributing to a dynamic resistome (Khalid et al., 2023). These authors mention that microbiome changes due to environmental factors may exert selective pressures on microbial communities, resulting in resistome expansion. Therefore, distinguishing allochthonous from autochthonous ARGs is difficult due to widespread human, agricultural, and industrial pollution, with few truly "pristine" environments remaining (McCann et al., 2019). Although not significantly observed in this study, these genes could be mobilized via lateral gene transfer and potentially disseminate into clinically relevant pathogens, contributing to global antibiotic resistance (Larsson & Flach, 2022).

Integrons are MGEs that facilitate horizontal gene transfer and play a key role in ARGs dissemination as well as microbial community modulation in the environment. They enhance bacterial adaptability to diverse stressors, including antibiotics, heavy metals, and other pollutants, and their presence in contaminated settings accelerates the spread of multidrug-resistant bacteria, posing risks to human, animal, and ecosystem health. In sites 2 and 3, composted manure application increased abundance integrase genes (*intI*), consistent with previous studies (Nõlvak et al., 2016; Li et al., 2017; Peng et al., 2017). This increase may result either from direct introduction of integron-carrying microbes via manure or from changes in soil physicochemical properties induced by manuring (Nõlvak et al., 2016). Poultry and swine manure commonly contain not only antibiotics but also heavy metals, pesticides, and disinfectants (Gosling et al., 2017; Kyakuwaire et al., 2019; Souza et al., 2019; Soares et al., 2021; Li et al., 2025; Zhao et al., 2025), which persist in soils after application (Qian et al., 2018b; Xie et al., 2018; Xiao et al., 2022; Zeng et al., 2024). Composting reduces but does not fully eliminate these pollutants (Congilosi & Aga, 2021), and subinhibitory concentrations may select for microbial defense mechanisms (Gillings, 2014), including integrons proliferation (Dealtry et al., 2014; Gillings et al., 2015).

Integrons are often associated with other MGEs, such as transposons and plasmids, that carry ARGs and pollutant degradation genes, promoting co-selection (Gillings et al., 2015; Mulder et al., 2018). This process is intensified when pollutants select for both ARGs and MRGs via cross-resistance or co-resistance mechanisms (Paul et al., 2019; Zeng et al., 2024). In site 1, *intI* abundance was not affected by manure but remained higher than in other sites, likely due to high heavy metal concentrations, particularly Cu (Hu et al., 2016), Zn (Tongyi et al., 2020), Cr (Xu et al., 2023; Zhang et al., 2023b), Ni (Hu et al., 2017), and Co (Di Cesare et al., 2016), all of which are associated with integrons proliferation. The co-occurrence of integrons and MRGs on the same transposons or plasmids could have masked any manure-induced increases as observed at other sites (Vats et al., 2022; Gillieatt & Coleman, 2024).

5. Conclusions

This study provides novel field-based insights into how successive composted manure applications and heavy metal contamination interact to shape soil bacteriome and resistome profiles under tropical conditions. Across all sites, multidrug resistance genes dominated the resistome, followed by MLS and tetracycline resistance genes, with Actinomycetota and Pseudomonadota as the main host phyla, consistent with their natural roles in antibiotic production and resistance. Manure application increased soil pH and P availability, enhancing bacterial diversity and influencing resistome composition in metal-depleted soils. However, compost amendment had little impact on total ARG abundance, resistome diversity, or clinically relevant ARGs, indicating partial mitigation of ARG inputs. In the metal-contaminated site, Cu primarily shaped the resistome, while both Cu and Ni influenced bacteriome structure. Strong correlations between ARGs and MRGs for Cr, Ni, Cu, and Zn suggest co-selection between resistance genes. Elevated levels of integrons, indicate that metals may sustain and facilitate the horizontal transfer of ARGs independently of antibiotic pressure. While composted manure increased integrons abundance in uncontaminated soils, this effect was not observed in the metal-rich site, likely due to pre-existing high integrons levels driven by metal-induced selective pressure. This highlights the importance of background soil contamination in modulating the impacts of manure inputs. Overall, these findings show the need for improved manure management practices tailored to local soil conditions, particularly in tropical regions where high antibiotic use and large manure volumes converge. Future studies should investigate ARG dynamics across temporal scales, ideally spanning a full agricultural cycle and accounting for seasonal variation (dry and wet seasons) in tropical soils, while conducting long-term tracking studies on different types of soil and fertilization management strategies to further explore changes in antibiotic resistance genes. Evaluating alternative management practices and integrating metagenome-assembled genomes (MAGs) analysis will be critical for identifying host-specific ARG-MRG-MGE associations and elucidating mechanisms of genetic co-selection, especially in metal-reach soils. Such efforts are essential to advance our understanding of ARG persistence, mobility, and environmental risks in tropical agroecosystems.

CRediT authorship contribution statement

Julio Flavio Osti: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Rafael Marques Pereira Leal:** Writing – review & editing, Investigation. **Adijailton José de Souza:** Writing – review & editing, Funding acquisition. **Luiz Gustavo Paulon Rezende:** Funding acquisition. **Douglas Gomes Viana:** Funding acquisition. **Fernando Dini Andreote:** Writing – review & editing. **Rafael Santana Mendonça:** Writing – review & editing. **Adriano Jakelaitis:** Funding acquisition. **Jussara Borges Regitano:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109783.

Data availability

Data will be made available on request.

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