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Impacts of drought and manure fertilization on soil and radish resistomes

Fernando Ruiz-Torrubia[1]✉, Carlos Garbisu[1], María T. Gómez-Sagasti[2], Unai Artetxe[2], José M. Becerril[2] & Lur Epelde[1]

Antibiotic resistance is a growing global problem, with agricultural practices and climate change as substantial contributors to the spread of antibiotic resistance genes (ARGs) in the environment. We investigated the effect of drought and fertilization type (organic vs. mineral) on radish crop growth and soil prokaryotic communities, with special emphasis on the radish and soil resistomes, as measured by the relative abundance of ARGs and mobile genetic element (MGE)-linked genes. Manure fertilization significantly increased ARG relative abundances in soil, compared to mineral fertilization. Drought and the presence of radish plants emerged as key variables regulating the association between ARGs and MGE-linked genes. Nonetheless, despite radish being a belowground crop, no direct connection was observed between the soil and crop resistomes. These results suggest that soil moisture and fertilization strategies do not necessarily increase the risk of ARG transfer to human pathogens through crop consumption. Consequently, a robust risk assessment of the environmental resistome must account for all compartments within the transmission chain. Together, our findings highlight the complex interplay between agricultural practices and climatic factors in shaping the soil and crop resistome.

Keywords Agricultural practices, Antibiotic resistance, Climate change, Mobile genetic elements, Soil microbial communities, Drought

The use of antibiotics in humans and farm animals has traditionally been considered the main driver of the emergence and dissemination of antibiotic resistance (AR) worldwide[1]. However, with the advent of the One Health concept, in the last years, increasing attention has been given to the role of the environmental resistome in the spread of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB). Agroecosystems are highly human-impacted environments where human activities, e.g., agricultural practices, are known to shape the structure and function of soil microbial communities[7]. For instance, fertilization has been very frequently associated to changes in the structure, composition, and activity of soil prokaryotic communities[11,33,92] . These changes are often attributed to variations in soil physicochemical properties, such as pH and organic matter (OM) content, among other factors[34,11]. Thus, fertilization can be a driver of both soil and crop resistomes. The use of organic fertilizers derived from animal sources (e.g., manure, slurry) often leads to the incorporation of antibiotic residues, ARB, ARGs, and mobile genetic element-linked (MGE-linked) genes into agricultural soils[7]. As mineral fertilization is concerned, there is contradictory evidence regarding its effects on the soil resistome[6,8,2].

On the other hand, agricultural practices can introduce toxic elements and compounds into soils, such as heavy metals and organic compounds, with concomitant effects on the soil microbiome and, hence, resistome[9,10]. Due to their recalcitrance, heavy metals such as copper (Cu) accumulate in agricultural soils, exerting long-term effects on soil microbial communities[12,18]. Furthermore, heavy metals can influence the soil resistome through a variety of co-selection mechanisms, including cross-resistance, co-resistance, and co-regulation[13]. Similarly, the accumulation of biocides such as glyphosate in soils has been linked with increased ARG abundances[15,19,28], although the underlying mechanisms remain unclear[17].

In the current scenario of climate change, it is crucial to understand how climate change-related variables can interact with agricultural practices regarding their impact on soil microbial communities and, specifically, on the environmental (soil, crop) resistomes. Temperature, in particular, has often been linked to AR[36], though

[1]Department of Conservation of Natural Resources, NEIKER – Basque Institute for Agricultural Research and Development, Basque Research and Technology Alliance (BRTA), Parque Científico y Tecnológico de Bizkaia, Derio P812, 48160, Spain. [2]Department of Plant Biology and Ecology, University of the Basque Country, UPV/EHU, P. O. Box 644, Bilbao 48080, Spain. ✉email: fruiz@neiker.eus

the mechanisms underlying such association remain largely unknown. Studies in the United States, Europe, and China have reported correlations between climate temperature changes and AR at both national and regional scales[73,29,75]. Nonetheless, concerning agroecosystems, changing precipitation patterns are probably more relevant, as soil water content strongly affects soil processes and microbiota[22]. However, there is no consensus on the impact of soil moisture content on the soil resistome. Some studies have reported that soil moisture can regulate ARG abundance and, interestingly, modify the effect of organic amendments on the soil microbiome and resistome[23–25]. By contrast, other authors[21] found that the environmental resistome remained unchanged upon soil moisture variations. Also, soil moisture can be a driver of horizontal gene transfer (HGT) rates between bacteria, as well as of the transfer of bacteria from soil to crops[62,67]. Lastly, droughts can trigger adaptive management responses, such as the use of treated wastewater for irrigation. This practice may inadvertently impact the soil resistome by introducing high levels of AR determinants[93]. These observations are of high relevance, since the transmission of AR determinants between bacteria and between ecological spaces is a crucial aspect behind environmental AR dissemination[44].

Our study aimed to investigate the effect of drought and fertilization type (organic vs. mineral) on radish crop growth and soil prokaryotic communities, with special emphasis on the radish and soil resistomes. The selection of radish, a belowground crop, was motivated by the observation that most studies dealings with the potential links between agricultural practices and the soil and/or crop resistome have been carried out with aboveground crops. Our intention was to assess whether a closer physical contact between the soil and the crop would result in a higher abundance of AR determinants in the food crop. To better reflect realistic agricultural conditions, the soil was amended with Cu and glyphosate prior to the experiment, simulating the typical application of Cu-based fungicides and herbicides. Although these contaminants may modulate the effects of other experimental factors, this interaction was intentionally maintained to capture a real-world scenario where multiple agricultural drivers co-occur and collectively shape soil microbial responses. We hypothesized that: (i) manure fertilization would increase soil ARG abundance, compared to mineral fertilization, with a less pronounced effect under drought conditions; (ii) drought and the presence of radish plants would have a stronger impact on the soil microbiome (e.g., on its composition) than on the soil resistome; and (iii) a transfer of ARGs from soil to radish crop would occur due to close physical contact.

Materials and methods

Soil collection and characterization

Soil was collected from the upper 20 cm of a grassland located in Derio (northern Spain), and sieved to <6 mm for homogenization purposes. A fraction of this soil, intended for soil physicochemical characterization, was air-dried at 30 °C for 48 h, and sieved to <2 mm. Soil pH, extractable potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) were determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) according to standard methods [26]. Olsen phosphorus (P), electrical conductivity (EC), and effective cation exchange capacity (CEC) were determined following ISO 1126 (1994), ISO 11265 (1994), and ISO 23470 (2007), respectively. Total carbon (C) and nitrogen (N) contents were determined following ISO 10694 (1995) and ISO 13878 (1998), respectively, by combustion with a TruSpec CHN analyser (LECO Corporation, Michigan, USA). Soil OM and carbonate content were determined following DIN 19539 (2016). Nitrate concentration was determined using an UV-VIS Spectrophotometer UV-1800 (Shimadzu) at 220 nm[31]. Ammonium concentration was measured following Nelson [32]. Particle size distribution was determined following ISO 13320 (2009).

Aged (>1 year) cow manure was collected from NEIKER facilities in Derio, northern Spain. The fraction designated for the determination of carbon and nitrogen contents (by combustion with a TruSpec CHN analyser) was air-dried at 30 °C for 48 h, and then sieved to <2 mm.

Microcosm experimental design

Two soil moisture levels, e.g., 20 and 80% of the field capacity (FC) were assayed, with the former corresponding to drought conditions. Similarly, two fertilization regimes (i.e., mineral vs. manure fertilization) were tested. Two months before the start of the experiment, the aged cow manure was spiked with oxytetracycline (OTC) to reach a final concentration of 2,000 mg kg^{-1} FW soil (fresh weight, FW). Oxytetracycline was dissolved in Milli-Q water, applied to the manure and thoroughly mixed. While the OTC concentration applied here exceeds levels typically reported in raw manure [94], this dosage was selected to account for the drastic degradation observed during manure maturation. Preliminary findings from a previous study indicated that OTC concentrations decrease substantially during the two-month aging period between manure spiking and soil application.

At the beginning of the experiment, the soil was contaminated with 100 mg Cu kg^{-1} and 25 mg glyphosate kg^{-1} soil, to simulate the use of Cu-based fungicides and herbicide application, respectively. For context, this Cu concentration falls within ranges commonly reported for agricultural soils. The average Cu concentration in European agricultural soils is approximately 31 mg kg^{-1}; however, values close to 100 mg kg^{-1} occur regularly in certain countries, including Spain[87]. Regarding glyphosate, while the applied dose is relatively high compared to typical European soil concentrations, which generally do not exceed 2 mg kg^{-1} [96,95], this level is representative of agricultural soils in the immediate aftermath of application. Briefly, a $Cu(NO_3)_2$ water solution was mixed with sand, dried, and mixed with the soil to reach a final concentration of 100 mg Cu kg^{-1} soil. Glyphosate was diluted in distilled water and applied to the soil to reach a final concentration of 25 mg kg^{-1} soil. At this point, fertilization was applied to the soil. For the manure-fertilized samples, OTC-spiked manure was incorporated to the soil at a rate of 204 t ha^{-1} (14.5% w/w), while mineral fertilized samples received 4.45 t ha^{-1} of a mineral fertilizer (NPK), based on ammonium (15%), phosphorus pentoxide (15%), and potassium oxide (15%). The high fertilization rates were intentionally selected to increase P and K inputs. This decision was based on observations from a

previous, yet unpublished study in which the same fertilizers were applied, and where low P and K contents in the amendment were found to influence the experimental outcomes.

Two kilograms of soil were placed in each pot, with three replicates per treatment. The pots were kept in a greenhouse under the following controlled conditions throughout the rest of the experiment: 16 h photoperiod, 25/22 °C day/night temperature, 60–70% relative humidity, and a minimal photosynthetic photon flux density of 250 µmol photon m^{-2} s^{-1}. Supplemental lighting was provided when natural light fell below this threshold, ensuring at least 16 h of light per day. Seeds of radish (*Raphanus sativus* L.) plants were sown at a rate of 25 seeds per pot in half of the NPK-fertilized pots and half of the manure-fertilized pots. Due to its edible swollen root, which is typically consumed raw, radish serves as a very interesting model for the study of the potential transfer of ARGs from agricultural soil to a food crop. Three weeks later, only six plants per pot were maintained, in order to standardize plant density across treatments. Four weeks after sowing, soils were subjected to different irrigation rates to maintain either 20% or 80% of FC, depending on the specific treatment. These target FC values were established following the approach described by Fuertes-Mendizábal et al.[3] using time domain reflectometry (TDR; Eijkelkamp), which measures soil impedance proportional to soil water potential. Based on these measurements, pots were irrigated with the amount of water required to reach and maintain the designated FC levels. At the flowering stage (10 weeks after sowing), plant material (leaves, swollen roots, and fine roots, separately) was harvested and rinsed with water prior to the analysis of the crop resistome, as well as the determination of a variety of biometric and physiological plant parameters (Sect. 2.6). Simultaneously, soil samples were collected to determine (i) a variety of microbial parameters with potential as bioindicators of soil health (Sect. 2.3); (ii) the relative abundance of ARGs and MGE-linked genes (Sect. 2.4); and (iii) the abundances of soil prokaryotic families (Sect. 2.5). Collected soils were sieved (< 2 mm) and stored at 4 °C for no longer than one month prior to analysis.

Soil microbial parameters and extraction of plant and soil DNA

Soil basal respiration (SBR) was determined by measuring CO_2 evolution in hermetic flasks incubated at 30 °C for 72 h, according to ISO 16072 (2002). Substrate-induced respiration (SIR) was measured following ISO 17155 (2002). Community-level physiological profiles (CLPPs) of cultivable heterotrophic bacteria were determined in soil samples, using Biolog EcoPlates™, as described by Epelde et al[48]. DNA from soil samples was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Carlsbad, CA, USA). For plant DNA extraction, 100 mg of frozen swollen roots were mechanically fragmented and homogenized using a stomacher, followed by extraction using the DNeasy Plant Mini Kit (Qiagen, Carlsbad, CA, USA). Following extraction from both plant and soil samples, DNA quantity and quality were determined using a NanoDrop™ One spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Extracted DNA samples were stored at −20 °C until use. The total abundance of the 16S rRNA gene was determined by real-time qPCR[35]. The primers used for the amplification were Ba519f (CAGCMGCCGCGGTAANWC) and Ba907r (CCGTCAATTCMTTTRAGTT), with an annealing temperature of 50 °C and a resulting amplicon of 424 bp.

Abundance of ARGs and MGE-linked genes

To determine the relative abundances of ARGs and MGE-linked genes, high-throughput quantitative PCR (HT-qPCR) was performed in the Gene Expression Unit of the Genomics Facility of SGIker (University of the Basque Country, Spain), using the BioMark HD Nanofluidic qPCR System combined with 96.96 Dynamic Arrays Integrated Fluidic Circuits (Fluidigm Corporation). Ninety-six validated primer sets[37] were used, 87 targeting ARGs, 8 targeting MGE-linked genes (i.e., transposases and integrases), and 1 targeting the 16S rRNA gene. The primer sets used for amplification are presented in Supplementary Table 2. The assays demonstrated a mean amplification efficiency of 94%. Raw data were processed with the Fluidigm Real-Time PCR Analysis Software, version 4.1.3, to calculate threshold cycle (CT) values. A CT of 27 was established following Muziasari et al[38,39]. and Zhu et al[80]. The mean of the technical replicates with CT values below the established threshold was calculated when at least 3 out of the 4 technical replicates had CT values below such threshold. This value was used to calculate ΔCT values with the $2^{-\Delta CT}$ method, where $\Delta CT = CT_{\text{detected gene}} - CT_{\text{16S rRNA gene}}$. ΔCT values correspond to the relative abundances of a given gene compared with the 16S rRNA gene[41]. Genes were excluded only when all samples failed to meet the quality threshold (quality < 0.65), as indicated by the Fluidigm Real-Time PCR Analysis Software, resulting in the removal of three genes. Furthermore, an additional eight genes were excluded from the analysis as their abundance remained consistently below the detection limits across all samples.

Metabarcoding of the 16S rRNA gene

In order to assess the impact of the studied treatments on soil prokaryotic communities, amplicon libraries were prepared using the 515 F and 806R barcoded primers, which target the 16S rRNA hypervariable region V4, following the Earth Microbiome Project protocol[42]. For each sample, triplicates of PCR reactions were carried out with the following reaction medium: 12 µL of PCR grade water, 10 µL of 5 µM HotMasterMix (Qiagen), 1 µL of forward primer (5 µM), 1 µL of reverse primer (5 µM), and 1 µL of template DNA. The conditions used for the amplification were a start of 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 60 s, 72 °C for 90 s, and a final elongation at 72 °C for 10 min. The reaction products were pooled, and correct amplification was confirmed by running a 1% agarose gel. Qubit (Invitrogen) was used to quantify the amount of amplified DNA, and 150 ng of DNA from each sample were pooled in a single tube (equimolar concentrations). The pool was cleaned using CleanNGS (CleanNA), following the manufacturer's instructions, and quantified again with Qubit (Invitrogen). Finally, 4 pM were sequenced on the Illumina MiSeq platform (250 bp x 2 pair-end, providing an average sequencing depth of ~ 150,000 reads per sample) at the Genomics Facility of SGIker, University of the Basque Country, Spain.

Forward and reverse EMP16S reads and barcodes were imported into QIIME2 via the qiime tools import plugin and then demultiplexed by their barcodes using the qiime demux emp-paired command. FASTQC[43] was used to read's quality. Primers and low-quality bases were trimmed by Cutadapt [89]. The resulting data were then imported into QIIME2 suite[45] for the read denoising step, using DADA2 plugin[46] and subsequent read taxonomic classification via the qiime feature-classifier classify-sklearn method, using the pre-trained SILVA classifier as supplied by QIIME2 resources web. After denoising and quality filtering, a total of 2,692,194 high-quality reads were retained, which were assigned to 10,968 amplicon sequence variants (ASVs). Raw sequencing data are available in the SRA database (https://www.ncbi.nlm.nih.gov/sra/PRJNA1402202).

Plant biometric and physiological parameters

The leaves and roots of all *R. sativus* plants were collected and their respective fresh weights recorded. The swollen roots (enlarged storage roots, derived from hypocotyl and upper radicle tissues) were collected, weighed fresh, and stored at 4 °C for the analysis of ARGs and MGE-linked genes (Sect. 3.1). Plant samples were then oven-dried at 70 °C for 48 h to determine dry weights. Leaf area was determined by selecting the two youngest fully expanded leaves from each pot, which were scanned and analysed using FIJI Image software.

Chlorophyll fluorescence kinetics were measured using the OJIP[47], a widely employed method to evaluate the photochemical reaction of photosystem II. Measurements were performed with a hand-held FluorPen FP 110 (Czech Republic) to record the OJIP curve. Prior to the measurements, the youngest fully expanded leaves were dark-adapted for at least 30 min at ambient temperature. Key parameters obtained from OJIP curves included: (i) photosynthetic performance index (Pi_{ABS}) as an indicator of crop yield; (ii) excitation energy conversion efficiency (ET_0/RC); (iii) energy dissipation quantum yield (DI_0/RC); and (iv) photon flux absorbed by the antenna complex (ABS/RC)[79]. Regarding pigment and antioxidant profiles, the two youngest fully expanded leaves from each pot were sampled, and then three leaf discs (3 mm diameter) were excised per leaf. Discs were immediately flash-frozen in liquid nitrogen and stored at −80 °C until analysis. The quantification of photosynthetic pigments (chlorophyll a and b), photoprotective pigments [total carotenoids and xanthophyll cycle components: violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z)], and lipophilic antioxidant compounds (total tocopherols) was performed using ultra-performance liquid chromatography (UPLC) with an Acquity™ uHPLC H-Class system (Waters®, Milford, MA, USA), following Lacalle et al[88]..

Statistical analysis

Statistically significant ($p < 0.05$) differences among treatments in terms of (i) the relative abundances of ARGs and MGE-linked genes measured by HT-qPCR; (ii) soil microbial parameters; and (iii) plant parameters, as well as the interactions between factors, were determined in R using a permutation ANOVA with the package *lmPerm*[50]. All data did not comply with the normality assumption. Pairwise comparisons were conducted in R performing a Dunn´s test with the package *dunn.test*[71]. Boxplots were constructed in R to visualize data using the package *ggplot*[52].

Relationships between (i) soil moisture and the other treatments (fertilization type: organic vs. mineral; planted with *R. sativus* vs. unplanted); (ii) normalized abundances of prokaryotic ASVs classified at the family level; and (iii) relative abundance of ARGs and MGE-linked genes, were explored by redundancy analysis (RDA) and variance partition analysis using Canoco 5[53]. Metabarcoding data was previously centered log-ratio transformed, and the number of permutations was unrestricted. When the number of explanatory variables was higher than the number of samples (i.e., when using metabarcoding and ARG relative abundance datasets as explanatory variables), the first 20 principal components of the datasets were calculated and used to conduct the RDAs instead of the complete dataset. Experimental factors were used only as explanatory variables, while metabarcoding data and ARGs and MGE-linked gene relative abundances were used both as explanatory and response variables.

Spearman correlations were calculated between the relative abundances of ARGs, MGE-linked genes, and prokaryotic families in R with the package *compositions*[54]. Prior to this, taxonomic data were loaded into R and centered log-ratio-transformed. Correlation plots representing the correlations between ARGs and MGE-linked genes were built with the package *circlize*[57].

A PERMANOVA analysis was carried out to compare the composition of soil prokaryotic communities (using centered log-ratio transformed abundances of prokaryotic families) and the CLPPs of soil cultivable heterotrophic bacteria among treatments [using average well colour development (AWCD) values from Biolog EcoPlates™ after 40 h of incubation], with the package vegan in R[56]. Two differential analyses were conducted to identify those prokaryotic families whose abundance significantly changed among treatments: ALDEx2 and Maaslin2 tests, with the *aldex2*[5] and the *MaAsLin2*[20] package in R, respectively. A taxonomy barplot was done in R using the package *ggplot2*[52]. Prior to that, correlations were calculated between prokaryotic families and MGE-linked genes, and those with stronger correlations ($R > 0.4$) were highlighted in the plot.

Results

Soil and manure characterization

The soil was characterized as loam, with a pH of 7.97, an OM content of 5%, a total N content of 0.4%, and an Olsen P content of 23.7 mg kg^{-1} (the rest of parameters are shown in Supplementary Table 1). Instead, the manure had a total C content of 10.44% and a total N content of 0.54%.

Fertilization was the primary driver of soil and radish crop resistomes

The effects of soil moisture, plant presence (planted with radish vs. unplanted), and fertilization type (organic vs. mineral) on the soil and radish root resistome (as reflected by the relative abundances of ARGs and MGE-linked genes), as well as on soil prokaryotic composition, along with their interactions, are illustrated in Fig. 1. Among the

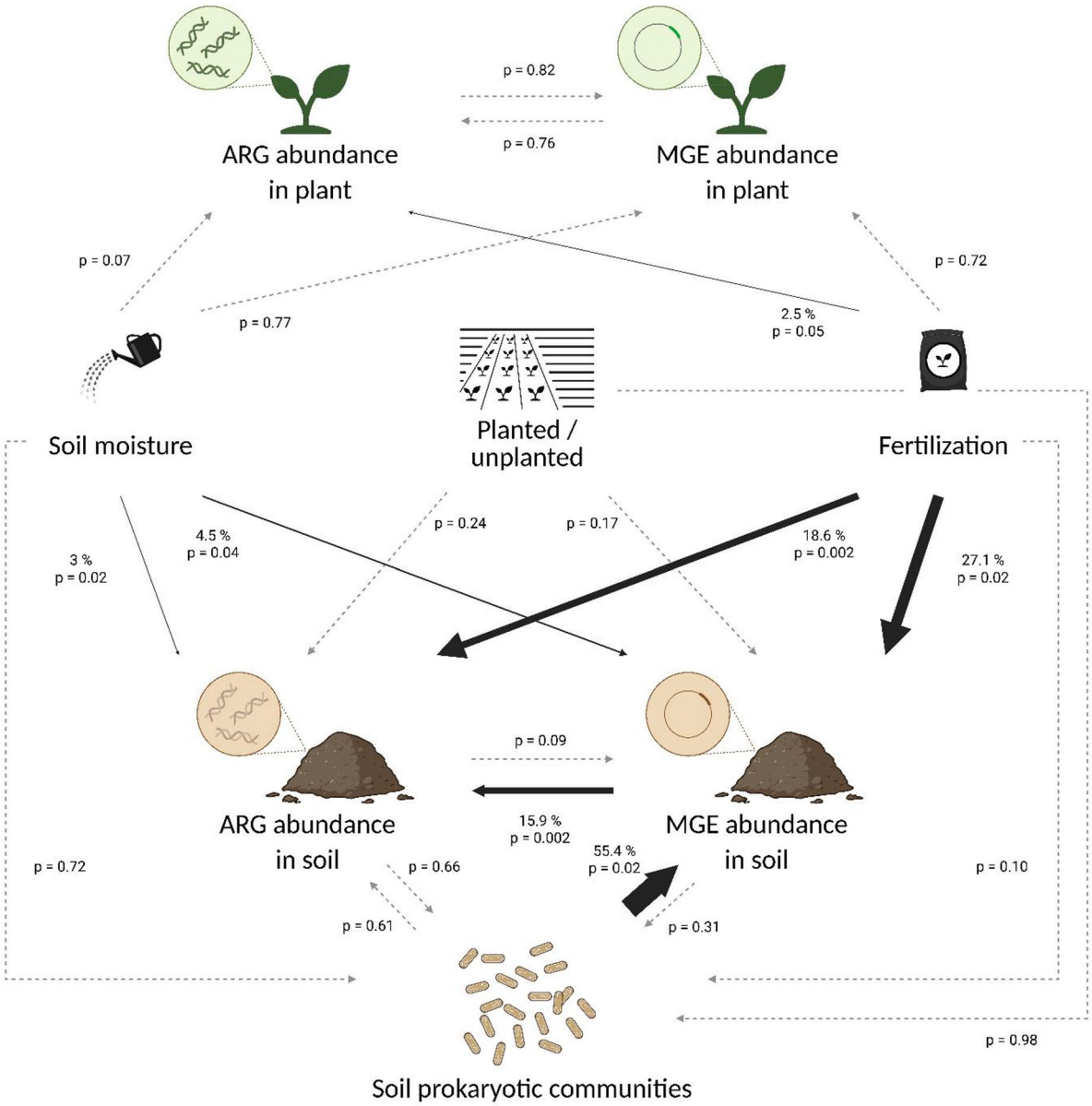


Fig. 1. Summary of the variation in antibiotic resistance genes (ARGs) and mobile genetic element (MGE)-linked gene relative abundances and soil prokaryotic community composition explained by the experimental factors, as determined by redundancy analysis (RDA). Solid lines indicate significant effects ($p < 0.05$), while dotted lines represent non-significant effects. The percentage of absolute variation explained by each factor is shown next to the solid lines when the RDA analysis was significant. Figure created in https://BioRender.com.

experimental factors, fertilization accounted for the largest proportion of the variation in the relative abundance values of soil ARGs (18.6%) and MGE-linked genes (27.1%), followed by soil moisture, which explained 3.0 and 4.5% of the variation in ARGs and MGE-linked genes, respectively. As indicated by the RDA, the presence of radish plants was not a significant factor in explaining the observed variations in ARGs or MGE-linked genes abundances. The composition of the soil prokaryotic community explained a greater proportion of the variation of the relative abundances of MGE-linked genes (55.4%), compared to any of the other experimental factors (by contrast, it was not a significant determinant of ARG relative abundances). However, none of the experimental factors (soil moisture, plant presence, fertilization type) significantly explained the observed variations in soil prokaryotic community composition. The relative abundances of MGE-linked genes accounted for 15.9% of the variation in soil ARGs relative abundances. Regarding radish crop samples, only fertilization type significantly affected the relative abundance of ARGs, though to a much lesser extent than in soil samples (2.5% of the variation explained). The remaining unexplained proportion of the variation of the relative abundances of ARGs, MGE-linked genes and the soil prokaryotic community composition may be attributable to stochastic processes, methodological noise or other factors not captured by the experimental design.

These findings align with the permutational ANOVA results, which indicate that the relative abundances of 32, out of the 87, ARGs differed significantly ($p < 0.05$) among soil samples subjected to different fertilization treatments, compared to 13 ARGs for the presence of plants and 8 ARGs for the soil moisture content (Supplementary Table 3). Among those soil ARGs whose relative abundance was affected by fertilization type, 31 exhibited higher abundance values in manure-fertilized soils, while only the *aadE* gene showed higher abundance in minerally-fertilized soils (Supplementary Fig. 1). The effect of fertilization on ARG relative abundances in soil samples was largely independent of the other experimental factors, as significant interactions with plant presence were detected for only 12% of the affected genes (4 out of 32) and for 16% with moisture level (5 out of 32). Out of the 13 genes significantly influenced by the presence of plants, 11 showed higher relative abundance values in the absence of plants (unplanted controls). Similarly, the 8 genes significantly affected by soil moisture level showed higher relative abundances under high moisture level (80% of the FC).

With regard to radish root samples, the permutational ANOVA showed a lower effect of the experimental factors on the radish resistome, compared to soil samples. Only the relative abundance of 5 (*aadE*, *ermB*, *mphA*, *penA*, and *strB*) and 4 (*ermB*, *KPC*, *mdtG* and *tetM*) genes was found to be significantly different ($p < 0.05$, Supplementary Table 3) between the different fertilization treatments and moisture levels, respectively. The effect of soil moisture was dependent on fertilization type in three of the genes whose relative abundance varied between moisture levels. Compared to soil samples, the relative abundances of ARGs and MGE-linked genes in radish crop samples were, on average, 2–5 times lower. These findings indicate a low connectivity between the soil and the radish crop resistomes. Among all the radish ARGs whose relative abundances varied across treatments, only one gene (*strB* gene) showed a treatment-dependent response that matched the response observed in soil samples.

Prokaryotic families more correlated with MGE-linked genes were among the least abundant

Consistent with the RDA results, the PERMANOVA analysis indicated that none of the experimental factors significantly influenced prokaryotic community composition (Supplementary Table 4). ALDEx2 and MaAsLin2 analyses revealed no significant changes in the abundance of any prokaryotic family among samples with different moisture levels (similarly, the presence of radish plants resulted in no significant changes). The only significant change was observed for the *Flavobacteriaceae* family, which varied between fertilization treatments (ALDEx2 corrected $p < 0.05$, MaAsLin2 corrected $p < 0.2$).

However, soil prokaryotic community composition was a significant factor explaining the variation in the relative abundances of MGE-linked genes, as indicated by the RDA. A Spearman correlation analysis between prokaryotic families and MGE-linked genes was performed to identify potential bacterial hosts of MGE-linked genes. No significant ($p < 0.05$) correlations with a high correlation coefficient ($R > 0.7$) were found. However, 107 significant ($p < 0.05$) correlations were found with correlation factors ranging from −0.56 to +0.51. The RDA analysis showed that those prokaryotic families with higher positive correlation factors ($R > 0.45$) explained 26.4% of the total MGE-linked genes variation ($p = 0.002$). The abundance of those families (namely, *Peptostreptococcaceae*, *Flavobacteriaceae*, *Polyangiaceae*, *Methylophilaceae*, *Anaerolineaceae*, *Chthonomonadales*, *Fimbriimonadaceae*, *Ruminococcaceae*, the SO85 family from the Dehalococcoidia class, the mle1-27 family from the Polyangia class, and an unknown family from the *Planctomycetales* order) accounted for 0.25% of the total prokaryotic abundance (Fig. 2).

Spearman correlations between prokaryotic families and ARGs were also calculated to identify potential bacterial hosts of ARGs. Only one significant ($p < 0.05$) correlation with a high correlation coefficient ($R > 0.7$) was detected.

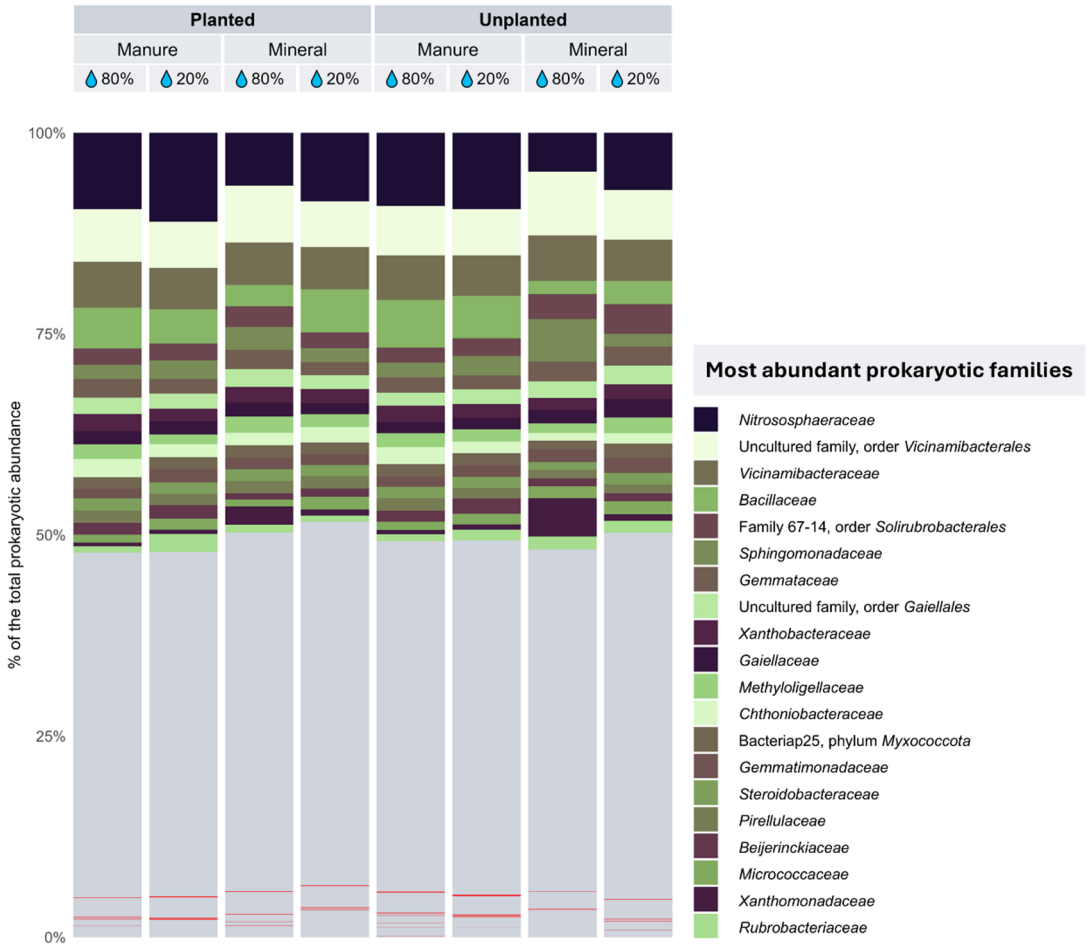
Soil ARGs were more correlated with MGE-linked genes in planted pots and in low moisture content soils

To better estimate the potential risk of ARG dissemination from environmental bacteria to potential human pathogens, we examined whether any experimental factors increased the association between ARGs and MGE-linked genes in soils. Spearman correlation analysis revealed that the number of significant ($p < 0.05$) correlations with a high correlation coefficient ($R > 0.7$) between soil ARGs and MGE-linked genes was more than twice as high in planted pots and low moisture soil, compared to unplanted pots and soils at 80% FC, respectively (Fig. 3, Supplementary Table 5). Minerally-fertilized soils exhibited more significant ($p < 0.05$, $R > 0.7$) correlations between ARGs and MGE-linked genes, compared to manure-amended soils (Supplementary Table 5). However, only one and three significant correlations were found in manure- and mineral-fertilized samples, respectively. The low number of significant correlations observed under both treatments limits the ability to draw robust conclusions.

The presence of manure and radish plants are the main drivers of soil microbial parameter values

Permutational ANOVA indicated significant ($p < 0.05$) differences in SBR values between samples with different fertilization types and between planted vs. unplanted soils (Supplementary Fig. 2, Supplementary Table 6). Values of SBR were highest in planted soils subjected to manure fertilization and 20% FC, and lowest in unplanted soils subjected to mineral fertilization and 80% FC. Significant ($p < 0.05$) differences in SIR values were observed between soil samples with different moisture contents and between planted vs. unplanted soils, with the highest SIR value being observed in unplanted soil with a 20% FC, and the lowest in planted soil with a 80% FC. The abundance of the 16S rRNA gene varied significantly ($p < 0.05$) with fertilization type, being highest in manure-amended soil samples (except for unplanted soil with a 20% FC) and lowest in minerally-fertilized unplanted soil with 80% FC.

A



B

	p value	F-statistic
Fertilization type	0,068	1,52
Moisture level	0,440	0,96
Plant presence	0,987	0,61
Fertilization type : Moisture level	0,388	1,00
Fertilization type : Plant presence	0,592	0,86
Plant presence : Moisture level	0,427	0,97
Fertilization type : Plant presence : Moisture level	0,863	0,72

Fig. 2. (**A**) Stacked bar plot of prokaryotic families relative abundances. Families with significant positive Spearman correlations with MGE-linked genes ($p < 0.05$) with an R statistic > 0.45 are highlighted in red. Mineral: mineral fertilization; Manure: manure fertilization; 20%: moisture level of 20% of the field capacity; 80%: moisture level of 80% of the field capacity. (**B**) the p values and F-statistics derived from PERMANOVA indicate the influence of experimental factors on the relative abundance of prokaryotic families.

The PERMANOVA analysis revealed that the CLPPs of soil cultivable heterotrophic bacteria (i.e., Biolog EcoPlates™ data) differed significantly ($p < 0.05$) between samples with different moisture levels, fertilization type, and planted vs. unplanted soil (Supplementary Table 4, Supplementary Fig. 3). The presence of radish plants was the main driver of the CLPP results, as indicated by the F statistic.

Manure reduced plant performance but led to more drought-tolerant plants

Minerally-fertilized radish plants showed significantly higher aboveground biomass values, compared to manure-fertilized plants (Supplementary Fig. 4, Supplementary Table 7). However, a lower soil moisture level led to a significant decline in aboveground biomass under mineral fertilization. In contrast, plants grown in

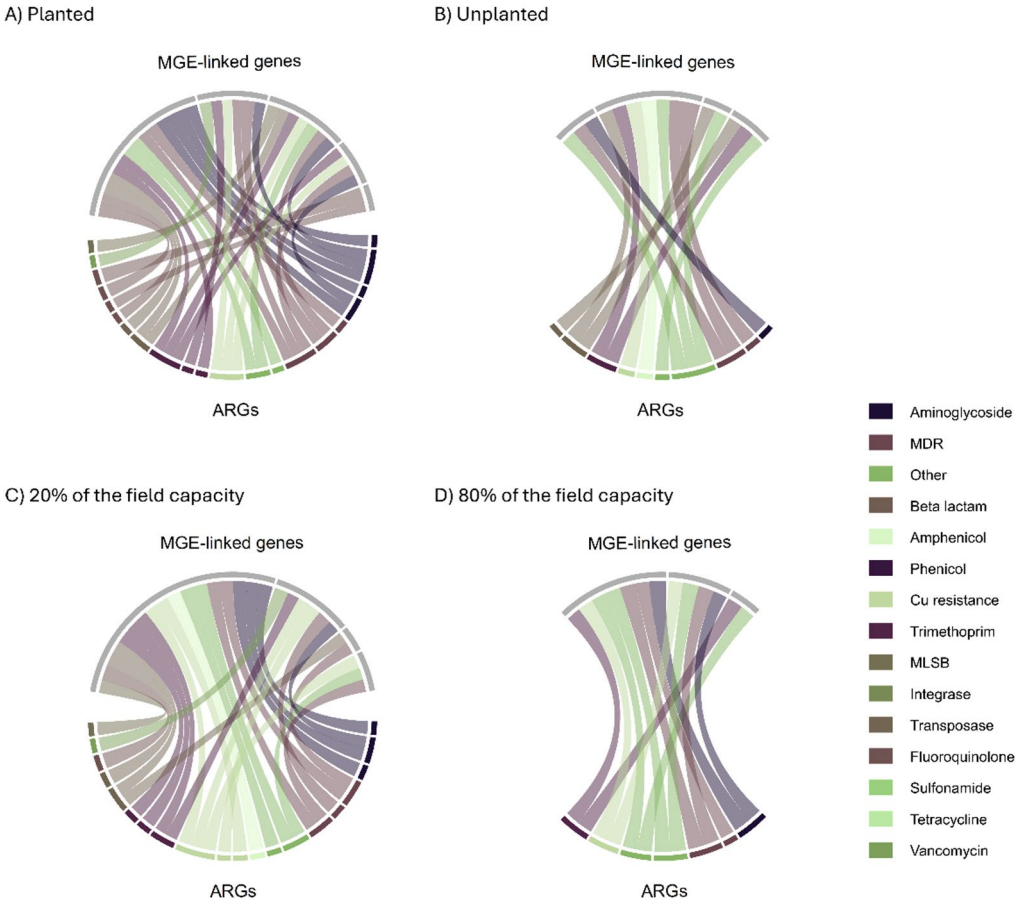


Fig. 3. Correlation network analysis of antibiotic resistance genes (ARGs) and mobile genetic element (MGE)-linked genes in (**A**) planted samples, (**B**) unplanted samples, (**C**) samples with a moisture of 20% of the field capacity (drought conditions), and (**D**) samples with a moisture of 80% of the field capacity. Spearman's rank correlation statistics were used. Only significant positive correlations ($p < 0.05$) with an R statistic > 0.7 between MGE-linked genes and ARGs are shown.

manure-amended soils did not exhibit a significant biomass decline when grown under the low soil moisture content (20% FC). The biomass of the swollen root was not significantly affected by either fertilization type or soil moisture.

Fertilization type only affected significantly the photosynthetic performance index (Pi_{ABS}), while variations in soil moisture had no significant impact on any of the fluorescence parameters. Remarkably, radish plants grown in minerally-fertilized soils under the low moisture content (20% FC) exhibited a significant ($p < 0.05$) increase in Pi_{ABS}, with values being 4-fold higher than those observed in plants grown in manure-fertilized or mineral-fertilized soils under 80% FC (Supplementary Fig. 4B, Supplementary Table 7).

No significant differences were observed for chlorophyll, carotene, or xanthophyll concentrations in response to either fertilization type or soil moisture content (Supplementary Fig. 4C, Supplementary Table 7). However, plants grown in manure-fertilized soils consistently tended to accumulate higher levels of tocopherols compared to those cultivated under mineral fertilization, with this difference reaching statistical significance under 80% FC.

Discussion

Effect of treatments on soil ARG relative abundances

Our results indicate that manure application increased the relative abundance of ARGs, in agreement with many previous works[60,61,4,16]. This increase has been attributed to the (i) transfer of ARGs from manure-associated bacteria to soil bacteria; and (ii) introduction of selective agents, such as antibiotics or heavy metals present in the manure, which may favour the proliferation of pre-existing soil ARGs[63]. In our study, co-selection with heavy metals is unlikely to be the primary driver, as all soils were contaminated with 100 mg Cu kg^{-1}, a concentration exceeding typical Cu levels in cattle manure[64,65]. However, the presence of the applied oxytetracycline, as well as other potential antibiotic residues not degraded during the manure ageing period, could select for AR following manure application. Previous works have shown that the increase in soil ARG relative abundances following manure application depends on antibiotic concentrations in the applied manure[66]. Additionally, antibiotics may facilitate the horizontal transfer of ARGs from manure-borne bacteria to soil bacteria[59], suggesting that

antibiotic-induced selection could further enhance ARG abundance in manure-amended soils. Nevertheless, the impact of manure application on the soil resistome has been reported to be transient[68,69], with an initial increase in soil ARG abundance immediately after manure application, followed by a later decline to pre-fertilization levels. The duration of this transient effect depends on various factors, including soil type and manure origin[68,69]. In our study, the increase in ARG abundances observed in manure-treated soil was, most likely, not caused by the transference of ARB from manure to soil, since no changes in soil prokaryotic community composition were detected as a result of manure application. The manure-induced increase in soil ARG relative abundances observed here could be attributed to the transference of manure-borne ARGs to the soil and/or the introduction of selective agents (possibly antibiotics, not heavy metals).

Regarding the effect of soil moisture, the relative abundance of the majority of the ARGs and MGE-linked genes remained unaffected by the drought-simulated condition (20% FC). Furthermore, no changes in the soil prokaryotic community composition were detected in response to simulated drought, nor were there significant differences in the relative abundance of specific bacterial families between the distinct soil moisture levels. The amount of bacteria, as estimated by the total abundance of the 16S rRNA gene, and the overall soil microbial activity, as indicated by the SBR values, remained unchanged in the 20% FC soil, compared to the 80% FC soil. Furthermore, the abundances of the detected prokaryotic families remained consistent across soil samples with different moisture contents. The main effects of drought on soil microbial communities and, in general, on soil processes, appear to be caused by the limited diffusion of both nutrients and microorganisms[22]. Under drought conditions, the larger water-filled pores dry out first and, then, the hydrological connectivity of the soil declines[70]. Water is a key transport medium within the soil matrix, and thus the disruption of the water continuum in soil hampers diffusion of both nutrients and microorganisms[70]. Soil microorganisms, and particularly bacteria, may adapt to drought conditions by changing their resource use, prioritizing survival over reproduction, entering dormancy, or producing extracellular polymeric substances to alleviate stress[22]. However, we did not observe a reduction in soil microbial activity at 20% FC, which points out to a lack of drought-induced stress on soil microbial communities. In our study, only the soil microbial biomass, estimated from SIR values, was higher under drought conditions, which could be attributed to lower predation rates due to limited mobility of microbial predators in dry soils[22,72,30]. Rewetting of the soil can decrease soil microbial biomass, while microbial activity can peak as a consequence of the reactivation of predation in the rewetted soil[22,72,30]. This raises the question of how rewetting after a drought period might affect ARG abundance. As abovementioned, the relative abundance of most of the studied genes remained unaffected by drought (at 20% FC), but those genes whose abundance did change showed higher abundances values at 80% FC. Since we did not observe any drought-stress indicators, it is most likely that gene abundances were affected by soil hydrologic connectivity. Thus, increased connectivity following soil rewetting could create a window of opportunity for ARG proliferation.

We hypothesized that the effect of antibiotic-containing manure on soil microbial communities could be moisture-dependent. However, we observed that fertilization effects were independent of soil moisture, and elevated ARG relative abundances were also observed in mineral-fertilized wet soils (80% FC). Since there was a time lapse of 3 months between manure application and the start of the drought-simulated conditions, it is possible that the effects caused by manure application had already occurred and did not change significantly during the drought period. It is also possible that this observation is linked to the type of soil used in our experiment, as loam soil has been reported to exhibit weaker moisture-driven ARG variations, compared to clay and sandy soil. Also, the relationship between soil water content and water potential, and hence hydrological connectivity, can depend on soil texture and OM content[7485].

Link between the soil and radish crop resistome

The transfer between environmental compartments (e.g., from soil to crop) is a crucial step in ARG transmission from agroecosystems to humans. The transmission of an ARG from an environmental bacterium to a human bacterial pathogen is a highly complex process involving multiple critical steps. Understanding them is essential for assessing the risk that environmental AR truly poses to human health[49]. First, the ARG must be capable of mobilization within the genome, followed by HGT between bacterial cells, and ultimately, physical transfer across environmental compartments (i.e., from the environmental microbiome to the human microbiome)[49]. At some point in this process, the ARG must be acquired by a human bacterial pathogen to present a direct risk to human health[4449]. Therefore, when using molecular methods targeting DNA to evaluate AR-linked risks (particularly, ARGs), it is crucial to consider the (i) ARG genomic context; (ii) association of ARGs with MGE-linked genes; (iii) bacterial host in which they reside; and (iv) ecological spaces where they are found. In this study, radish was selected as a model plant to evaluate the risk of ARGs from soil to food crops (and, hence, humans), since radish root is typically consumed raw. Besides, as abovementioned, the selection of radish, a belowground crop, was motivated by the observation that most studies dealing with the links between agricultural practices and the soil and/or crop resistome have been performed with aboveground crops. Our intention was to assess whether a closer physical contact between the soil and the crop would result in a higher abundance of AR determinants in the food crop. To simulate the agroecosystem-to-human transmission pathway, DNA extraction was performed on whole radish roots rinsed with water, reflecting the portion of the vegetable typically consumed. Across the different treatments, the relative abundance of ARGs and MGE-linked genes in radish crop was 2 to 5 times lower than in soils. Besides, the number of genes that were not detected in any sample (or were detected only in 1 replicate) was two times higher in radish samples compared to soil samples. Fertilization was the main driver of ARG abundance variation in radish samples, but its effect was much lower on the radish vs. soil resistome, consistent with previous studies reporting a limited soil-to-plant connectivity in terms of AR[44]. These findings indicate that, under our experimental conditions, the crop represents a bottleneck for the transfer of antibiotic resistance genes from soils to humans. More importantly, our results suggest that the drivers shaping the crop resistome may differ from those of the soil resistome. Consequently, agricultural practices such as manure-

based fertilization, which have been shown to increase ARG abundances in soils following application, may not necessarily translate into an increased risk of exposure to the environmental resistome through food crops.

Soil moisture and plant presence can drive ARG mobilization

The study of ARG-MGE associations is crucial for a better assessment of the risk of ARG dissemination. On the other hand, drought can disrupt the soil water continuum, impeding the diffusion of microorganisms and, it could be argued, the dissemination of ARGs. However, Tecon et al. observed that, in low-moisture soils, spatially isolated aqueous microhabitats are formed, where bacterial cell-to-cell interactions are more frequent due to close contact [70]. In our study, we observed a stronger correlation between ARGs and MGE-linked genes in drier soils (at 20% FC). Whether this results from microhabitat formation remains unclear, but a stronger ARG-MGE association could suggest that ARG mobilization is higher under drought conditions. A higher mobilization of ARGs, especially in conditions with an enhanced cell-to-cell contact, could facilitate the horizontal transfer of genes between bacteria, increasing the risk of AR transfer to human bacterial pathogens. This aspect may be even more relevant under drought-rewetting cycles. Following drought, rewetting induces a sharp increase in microbial activity that may favour bacterial replication and the horizontal transference of ARGs[76,77]. The re-establishment of soil pore connectivity due to water infiltration can facilitate the mobilization of ARG-harbouring bacteria and the diffusion of root exudates and other nutrients, which are known to act as triggers of HGT[78]. Even though we did not detect any effect of the drought condition (20% FC) on the impact of manure on the soil resistome, it is unclear whether drought could also promote the association of manure-borne ARGs with MGEs. Drought-rewetting cycles could potentially induce the transfer and dissemination of ARGs among soil bacteria.

An increased association of ARGs with MGE-linked genes was also observed in planted vs. unplanted soils. The rhizosphere is known to be a hotspot for HGT compared to bulk soil, due to root growth and exudate production[78] exerting a stimulatory effect on microbial activity [51]and plasmid mobilization[40]. In our study, the presence of radish plants enhanced soil microbial activity and was the main driver of the observed changes in CLPP data. A greater number of correlations between ARGs and MGE-linked genes in the rhizosphere suggests a higher risk of environmental AR spread, particularly taking into consideration that HGT often occurs at higher rates in this ecological space than in bulk soil.

Regarding potential bacterial hosts of ARGs or MGE-linked genes, we did not identify any, but a correlation analysis between MGE-linked genes and prokaryotic families indicated that the families most linked with AR determinants were among the least abundant. This aligns with findings from Zheng et al[81]., who reported that while 21% of prokaryotic species harboured ARGs or MGEs, they accounted for only 1% of the total prokaryotic abundance. In our study, no prokaryotic taxa exhibited significant abundance shifts in response to the experimental factors, suggesting that either unidentified drivers could be shaping soil microbial communities or the full diversity of soil prokaryotic communities was not captured in our analysis. While metabarcoding is a powerful tool for microbial identification, the complexity of soil microbiomes suggests that metagenomics may provide a more comprehensive perspective, particularly for identifying less abundant bacterial families[82,83]. Zheng et al[81]. identified *Enterobacterales* and *Pseudomonadales* as core ARGs and MGE-linked genes hosts in soil ecosystems, yet only two of the 619 detected families in our study belonged to these bacterial orders.

Besides, as the associations between ARGs, MGE-linked genes and taxa in this study are based on correlations, they should be interpreted with caution. These correlations do not provide direct evidence of physical linkage or HGT, which would require confirmation through gene co-localization or direct monitoring of HGT events. Future research utilizing metagenomic approaches (particularly long-read sequencing and plasmidome analysis) could offer more direct insights into the genomic localization of ARGs. Such methods would help overcome the inherent limitations of HT-qPCR-based assessments in resolving ARG-MGE associations ([8491]).

Effect of treatments on plant parameters

The enhanced aboveground biomass observed in radish plants under mineral fertilization is consistent with the high bioavailability of essential nutrients, particularly N, in mineral fertilizers, which facilitates rapid vegetative growth through increased C assimilation and cell expansion. However, under low soil moisture (20% FC), the marked reduction in shoot biomass highlights their vulnerability to drought. This suggests that while mineral fertilization supports vigorous growth under optimal conditions, it may compromise plant resilience under drought stress, likely due to limited improvements in soil water retention.

Conversely, plants grown in manure-amended soils maintained a stable aboveground biomass regardless of soil moisture content, suggesting a protective effect of soil OM on plant performance under drought. Organic amendments are known to improve soil structure, water-holding capacity, and microbial activity [90], all of which contributing to a more buffered rhizosphere environment[8614]. These benefits can mitigate water deficit impacts by enhancing water and nutrient uptake efficiency, as well as root-soil interactions. Interestingly, the biomass of the swollen root remained constant across treatments, suggesting that swollen root development in radish is under more conservative molecular and physiological control[55,27]. The swollen root may function as a strategic C sink, regulated independently of shoot growth and more resilient to short-term environmental fluctuations.

The enhanced photosynthetic performance observed under water-limited conditions in minerally-fertilized plants was likely driven by the greater N bioavailability associated with mineral inputs, which supports the maintenance of photosynthetic machinery and facilitates osmotic adjustment under drought. This response may also reflect a compensatory physiological adjustment of the photosynthetic apparatus aimed at optimizing light energy use. However, the long-term sustainability of this response remains unclear, as it may incur metabolic costs or lead to increased vulnerability if stress persists. Photosynthetic pigment concentrations remained stable across radish plants grown in minerally-fertilized soils, suggesting that the pigment pool, and thus the core light-harvesting capacity, was preserved under our experimental conditions.

Although radish plants grown in manure-amended soils maintained a stable biomass across moisture levels, their overall growth was consistently lower than that observed in minerally-fertilized plants. This reduced growth, coupled with a trend toward higher tocopherol accumulation, particularly at 80% FC, may reflect a state of moderate physiological stress. The elevated tocopherol levels can indicate an oxidative imbalance, potentially due to lower N bioavailability. This suggests that while organic fertilization may buffer water stress, it can impose a distinct set of metabolic constraints that trigger antioxidant responses, even in the absence of visible stress symptoms. In any case, the increased level of tocopherol has an additional benefit from a nutritional point of view, as vitamin E is an excellent lipophilic antioxidant for humans.

Limitations of the experimental design and future perspectives

First, the experiment was conducted using a single soil type; consequently, these results should be extrapolated to alternative pedological environments with caution. This is particularly significant given that the impact of manure application on ARG dynamics is often soil-type dependent[69]. Future research should employ multi-soil comparisons to determine how varying physicochemical properties, such as pH and clay content, modulate the resistome's response to organic amendments.

Second, this study utilized a single type of mineral fertilizer, whereas modern agriculture relies on a diverse array of chemical formulations. While the specific fertilizer selected may have influenced the comparative analysis between organic and mineral treatments, existing literature consistently reports that the impact of synthetic fertilizers on the soil resistome is significantly lower than that of manure, regardless of the specific mineral formulation used[2].

Third, the single-point sampling strategy at harvest constitutes a temporal limitation. Although monitoring the successional evolution of AR determinants would provide deeper mechanistic insights into microbial dynamics, the harvest stage was prioritized as the most critical time point for assessing transmission risk via food crops. Transient fluctuations occurring during earlier growth stages that do not persist until harvest are generally considered less critical for evaluating final consumer exposure.

Finally, it must be acknowledged that microcosm experiments are inherent simplifications and may not fully replicate the complexity of field conditions. While these systems provide a controlled, cost-effective, and statistically robust environment [97], they lack the stochasticity of natural ecosystems. Although we mitigated this by incorporating agricultural contaminants to simulate realistic soil conditions, transitioning to long-term field trials remains a vital next step to validate these findings under fluctuating climatic and environmental pressures.

Conclusions

This study aligns with a growing body of evidence indicating that certain agricultural practices, such as manure fertilization, act as drivers of the soil resistome by modulating the abundance of ARGs in agroecosystems. Drought conditions and the presence of radish plants emerged as key variables regulating the association between ARGs and MGE-linked genes. This provides a foundation for further research concerning the effects of drought-rewetting cycles on the environmental resistome, which is of particular relevance in the context of climate change. We observed a low ecological connectivity between the soil and radish resistomes, indicating that agricultural practices that elevate the abundance of AR determinants in soil may not alter the risk that the environmental resistome poses to human health through food crops. Mineral fertilization enhanced radish growth but heightened drought sensitivity, whereas manure fertilization appeared to buffer plant biomass stability under water stress. Our study highlights the critical need to understand the complex interactions among agricultural practices, climatic factors, and microbial dynamics in evaluating the risk of AR dissemination from agroecosystems to humans. It is important to take into consideration that, in our study, environmental AR was addressed mainly by studying the abundance of ARGs, with its concomitant limitations. In order to circumvent the biases and limitations of bacterial culturing, environmental AR is nowadays typically assessed using molecular methods that target DNA, and less frequently RNA or proteins[84], but we must not forget that AR is a phenotypic trait. Antibiotic resistance evaluation using molecular methods relies on detecting genetic determinants linked with AR, but generally without confirming their functionality[58]. This approach may lead to an underestimation of AR, as not all resistance mechanisms depend on ARGs. Conversely, reliance on ARG analysis may also overestimate AR, as ARG detection does not indicate functional resistance. Moreover, it must be emphasized that not all ARGs pose a serious threat to public health (many genes believed to confer AR are ubiquitous in bacteria where they fulfill different roles). Particular attention must be paid to the identification of high-risk ARGs, those with high enrichment in human-associated environments, high mobility, and, finally host pathogenicity. Lastly, establishing a causal connection between an environmental ARG and a clinical infection with an ARB in humans is highly complex, as it involves multiple transmission barriers and ecological bottlenecks. Here, we observed a poor ecological connectivity between the soil and crop resistomes, exemplifying how extrapolating public health risks from environmental (i.e., soil) ARG observations can be misleading.

Data availability

Raw sequencing data are available in the SRA database (https://www.ncbi.nlm.nih.gov/sra/PRJNA1402202).

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Author contributions

The contributions of each author are reported according to the CRediT (Contributor Roles Taxonomy) guidelines. Conceptualization: Fernando Ruiz-Torrubia, Carlos Garbisu, José M. Becerril, Lur Epelde; Formal analysis and investigation: Fernando Ruiz-Torrubia, Unai Artetxe, Maria T. Gómez-Sagasti; Writing – original draft preparation: Fernando Ruiz-Torrubia; Writing – review and editing: Carlos Garbisu, Unai Artetxe, Maria T. Gómez-Sagasti, José M. Becerril, Lur Epelde; Funding acquisition: Carlos Garbisu, José M. Becerril, Lur Epelde.

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Declarations

Competing interests

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Additional information

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Correspondence and requests for materials should be addressed to F.R.-T.