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Comparison of soil and grass microbiomes and resistomes reveals grass as a greater antimicrobial resistance reservoir than soil

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HIGHLIGHTS

- Grass is a reservoir of antimicrobial resistance genes (ARGs)
- We identify grass as a reservoir of ARGs in the environment
- The grass resistome is more expansive, diverse than soil resistome
- Microbiotas in soil and grass had similar contents but varied in the relative abundances
- The interactions of ARGs, mobile genetic elements and ARGs, and ARGs and microbes differed between soil and grass

GRAPHICAL ABSTRACT

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ABSTRACT

Grasslands cover a large proportion of global agricultural landmass used to feed herbivores and ruminants and link the environment to the food chain via animals onto humans. However, most scientific studies of antimicrobial resistance and microbiomes at the environmental – animal nexus have focused on soil or vegetables rather than grasslands. Based on previous microbiome phyllosphere-soil studies we hypothesised that the microbiome and resistomes across soil and grass would have a core of shared taxa and antimicrobial resistance genes (ARGs), but that in addition each would also have a minority of unique signatures. Our data indicated grass contained a wider variety and higher relative abundance of ARGs and mobile genetic elements (MGEs) than soil with or without slurry amendments. The microbiomes of soil and grass were similar in content but varied in the composition proportionality. While there were commonalities across many of the ARGs present in soil and on grass their correlations with MGEs and bacteria differed, suggesting a source other than soil is also relevant for the resistome of grass. The variations in the relative abundances of ARGs in soil and on grass also indicated that either the MGEs or the bacteria carrying the ARGs comprised a higher relative abundance on grass than in soil. We conclude that while soil may be a source of some of these genes it cannot be the source for all ARGs and MGEs. Our data identifies grass as a more diverse and abundant reservoir of ARGs and MGEs in the environment than soil, which is significant to human and animal health when viewed in the context of grazing food animals.

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1. Introduction

The United Nations declaration on antimicrobial resistance (AMR), which all 193 member states signed and the WHO declaration of priority pathogens, highlights the importance of the AMR problem (WHO, 2014; United Nations, 2016). Each year, AMR results in approximately 1 million disability-adjusted life years (DALYs) lost in EU/EEA countries (OECD, 2018). Antimicrobial resistant bacteria and genes can be transferred from the environment to food animals and via the food chain to humans either in or as food-borne pathogens or commensals. Many different classes of antimicrobials currently used to treat infections in humans and animals were discovered and developed between the 1940s and 1980s. However, since 1990 only three novel classes of antimicrobials have been launched: pleuromutilins, lipoglycopeptides, and oxazolidinones. These new classes have limited or no activity against the gram-negative pathogens such as *Escherichia coli* or *Klebsiella pneumoniae*, which are on the WHO's priority list (Brown and Wright, 2016). Therefore, we need to preserve our current arsenal of antimicrobials. One mode of action is to limit the transfer of AMR genes and bacteria from the environment to animals and humans via the food chain. However, we need to understand the resistome and microbiome of the animal food source before we can limit the transfer.

The phyllosphere (aerial surface of plants) is estimated to cover over 10^9 km^2 and contain 10^{26} bacterial cells, making it one of the largest microbial habitats on earth (Lindow and Brandl, 2003). In Europe, grasslands covering more than a third of the European agricultural area are used to feed herbivores and ruminants and provide important ecosystem services (Schils et al., 2022). Grass in the field is in a constant interactive relationship with the soil in its rhizosphere. The soil likewise is within a constant relationship with the grass. Therefore, neither exists in isolation but in a symbiotic relationship. However, they are rarely studied together. The microbiome and resistome on grasslands are rarely studied (Grady et al., 2019; Massoni et al., 2021). To ensure sustainable agriculture and healthy ecosystems we need to understand the grassland microbiomes and resistomes and how these (both soil and grass) change with the addition of slurry (Laforest-Lapointe et al., 2016; Vandenkoornhuyse et al., 2015). Studies to date analysing the impact of manure on the microbiome and resistome of grassland have focused solely on the soil and have not included the grass (Santamaría et al., 2011; Jechalke et al., 2013; Udikovic-Kolic et al., 2014; Ding et al., 2014; Gonçalo et al., 2020).

Overlaps of microbiomes and resistomes have been detected in the plant-soil ecosystem, suggesting the possibility of dissemination of microbes and antimicrobial resistance genes (ARGs) between soil and plants (Wang et al., 2022). Fresh produce contaminated with enteric pathogens have been frequently reported to originate from environmental sources with wild animals or agricultural activities (Alegbeleye et al., 2018). The microbiome of soil has been found to overlap with those in the leaves and flowers of grape vines and between lettuce roots and soil amended with poultry litter (Zarraonaindia et al., 2015; Zhang et al., 2019). However, the provenance of phyllosphere microorganisms is not yet established (Massoni et al., 2021). A recent study estimated that at least 25 % of the *Arabidopsis thaliana* phyllosphere bacteria reached the phyllosphere from the soil (Massoni et al., 2021). These bacteria represented 40 % of the bacterial taxa detected. However, the microbiomes did not converge between soil and leaves/flowers where the phyllosphere was not in contact with the soil. In contrast a study of two perennial grasses (switchgrass and miscanthus) identified soil as a major reservoir of leaf microorganisms (Grady et al., 2019). These studies however did not investigate the shared resistomes across soil and phyllosphere. Previous phyllosphere studies investigated either the microbiome or targeted antimicrobial resistant bacteria or genes on vegetables (Zhang et al., 2019). Based on previous phyllosphere-soil microbiome studies we hypothesised that the microbiome and resistomes of soil under the grass and grass would have a core of shared taxa and resistance genes, but that in addition each would also have a minority of unique signatures. We introduced pig slurry treated and compared with untreated to understand if the application of the pig slurries would alter the common microbiome and resistome of the soil and grass or the individual elements of each in the short term. This is a unique study, as previous studies have focused on soil and not included grass in relation to resistomes, or have compared only microbiomes across soil and phyllosphere.

2. Materials and methods

2.1. Field side, plot preparation, and slurry application

The field trial was conducted from August to October 2019 in Teagasc Research Facilities in Johnstown Castle, Wexford, Co. Wexford, Ireland. No farm animals were present on this land for the seven months prior to the field trial. The pig slurry samples were described previously (19). Briefly, pig slurry was collected from an Irish pig farm in agreement with the farm owner. The slurry was treated with three methods, storage for 4 months, compost for 8 weeks, and AD for 90 days. Products of slurry treatments and the fresh collected raw slurry were spread on field plots based on the phosphorous content following the EU regulations. One hundred 1 m wide x 1 m long plots were randomly established in the field for control without slurry application and slurry application. Each slurry type was spread on 20 plots, which were designed for sample collection for 5 timepoints with 4 replicates each.

2.2. Soil and grass sampling

Soil cores were collected from four random plots on the field prior to the slurry application for the soil background analysis (T-1). A standard agronomic corer was used to collect 10 cores at the depth of 10 cm along a W-shaped path. These cores were mixed well in a clean plastic sampling bag to make a composite sample.

During the field trial, soil cores were collected from control (no slurry applied) (4 plots) and slurry applied plots (4 plots per treatment) fortnightly for the first 3 timepoints, and at 2.5 months post-T0 for the last time points. Grass samples were randomly collected in the field before trimming to 5 cm for slurry spreading (T-1). During the field trial, grass was harvested to get approximately 200 g from control (no slurry applied) (4 plots) and slurry applied plots (4 plots per treatment) fortnightly for the first 3 timepoints, and at 2.5 months post-T0 for the last time points.

Soil and grass samples were transferred to the laboratory immediately and were processed within 24 h. Soil samples were stored at −80 °C for further molecular analysis. Grass samples were rinsed with PBS buffer (Oxoid) as previously described for leaf washes (Walsh et al., 2011). The resulting buffer was used for microbial testing and to extract microbial DNA for further molecular analysis.

2.3. DNA extraction

Total DNA was extracted from 0.25 g of each soil sample replicate using the DNeasy PowerSoil Kit (Qiagen). The PBS washes of 50 g of each grass sample replicate were centrifuged at 3000*g* for 15 min. The resulting pellets were used for DNA extractions using the DNeasy PowerSoil kit (Qiagen). The quality and quantity of extracted DNA were examined using a DeNovix DS-11 spectrophotometer and Invitrogen Qubit Fluorometer (dsDNA high-sensitivity assay kit) (Waltham, MA). DNA was extracted in triplicate from each sample and extracts were pooled to obtain a single DNA sample per experimental unit at each time point.

2.4. Metagenomic sequencing and HT-qPCR arrays

Extracted DNA from soil and grass samples were prepared using Illumina TruSeq DNA library preparation kits before sequencing on the Illumina NextSeq 500 platform (paired-end, 2 × 150 bp sequencing) in Teagasc Next Generation DNA Sequencing Facility. DNA samples were also used for HT-qPCR arrays. The HT-qPCR arrays were performed using the SmartChip™ Real-Time PCR system (TakaraBio, CA, USA) by Resistomap Oy (Helsinki, Finland). The mix of DNA samples with primer

sets and the qPCR reagents were loaded in each 100 uL reaction well of the SmartChip™ with 5182 wells. A primer set of 216 pairs of primers targeted 186 ARGs conferring resistance to major antibiotic classes, 6 integrons, 22 MGEs, and total bacterial genes 16S rRNA was used in the qPCR array. The melting curves and Ct values were analysed using default parameters of the SmartChip™ qPCR software. The qPCR was conducted in three technical replicates for each DNA sample.

2.5. Data analyses

2.5.1. Metagenomics

The adapter sequences were trimmed from shotgun sequencing raw reads in the fastq format using Cutadaprt (V2.10). We used Sickle (v1.33) with the minimum window of quality score of 20 to remove the low-quality reads with the length <20 bp. The FastQC (v 0.11.9) was used to examine the quality of filtered reads before assembly with Megahit (v1.2.6, −-kmin-1pass –presets meta-large). The assembled contigs were subjected to Kaiju taxonomic classifier (v1.2.6, parameters: –kmin-1pass –presets meta-large) to assign the taxonomy profile for each sample. The microbial communities were analysed in the MicrobiomeAnalyst online platform (Dhariwal et al., 2017).

The microbial genome annotation on filtered reads was carried out using Prokka (v 1.14.6, default settings) (Seemann, 2014). The protein FASTA files resulted from the Prokka software were used to identify the KEGG Orthologs (KOs) by Kofamscan (v 1.3.0) (Aramaki et al., 2020). The KEGG pathways were assigned by MinPath software (v 1.5) based on the Ko's lists (Ye and Doak, 2009). Data were analysed and visualised using Calypso online (Zakrzewski et al., 2017).

2.5.2. qPCR

The qPCR data of the samples were filtered based on the following criteria: (1) a gene was detected in at least two technical replicates; (2) the Ct values ≤27; and (3) the amplification efficiency was in the range of (1.8–2.2). The relative gene copy number was calculated in Eq. (1) in the work of Chen et al., 2016 10. The gene relative abundance was identified by dividing the relative copy numbers by the 16S rRNA gene copy number. The data was then visualised in the MicrobiomeAnalyst online platform (Dhariwal et al., 2017).

$$\text{Relative gene copy number} = 10^{(27-\text{Ct})/(10/3)} \quad (1)$$

2.5.3. Correlation analysis

The interaction between (1) ARGs and MGEs; and (2) microbial communities and ARGs were analysed through Spearman's correlation analysis with the SciPy package (Virtanen et al., 2020). The correlation between ARGs and MGEs was considered strong and significant when Spearman's rank value $|r| > 0.85$ and $p < 0.05$ and $|r| > 0.5$ and $p < 0.05$ between microbial communities and ARGs. The Cytoscape software (v3.8.2) was implemented to build the network based on strong and significant Spearman's correlations (Shannon et al., 2003). The ARG and MGE genes showed high interaction between genes, so we set the threshold $|r| > 0.85$ to build a network with appropriate nodes and edges to manage these properly. However, the ARGs vs Phyla did not build a formative network if $|r|$ was set >0.85 (network with very few nodes and edges). Therefore, the correlation network in soil was formed based on Spearman's correlation $r > 0.5$ and the network in grass based on Spearman's correlation $r > 0.6$. Higher ARG, MGE and phyla abundance were detected in grass samples than in soil, thus bigger, more interactive networks were built in grass than soil. The correlation values are Spearman's rank correlation coefficient (Spearman's correlation: R, rho). These values measure the interaction/association (strength and direction of association) between two variables on at least an ordinal scale. The interaction/association between two variables can be positive (increase together) or negative (one increase, another decrease) in the range $[-1,1]$. The interaction/association is stronger when the absolute value $|r|$ is higher, close to 1.For the current research, an absolute value of the correlation coefficient ≥ 0.3 was estimated as the appropriate threshold. In our case, depending on the interaction, we set the strong correlation at different coefficient values.

3. Results

The dynamics of the microbiomes and resistomes of soil and grass were measured prior to pig slurry application and over a time course up to ten weeks after slurry application in field trial experiments performed to replicate agricultural practices. The applications of four different treated pig slurries were compared with a control to which no slurry was applied. There were no significant changes in the microbiomes nor resistomes of the soil or grass associated with the application of the slurries.

3.1. Soil and grass microbiomes

We detected 6944 OTUs in soil and 6774 OTUs in grass. Almost all genera present on grass (98.7 %) were also detected in the soil microbiomes. However, the relative abundances of the genera varied across grass and soil.

3.2. Microbial compositions of soil and grass over time and slurry treatments

After quality trimming and assembling, metagenomic sequencing reads were assigned to Bacteria, Archaea, Virus and other unclassified organisms with Kaiju (Dataset S1). The most abundant phyla and genera were assigned and compared (Figs. S1, S2).

3.3. Alpha diversity: Chao1 and Shannon PCoA of soil vs grass

The alpha diversities of the microbiomes were compared using the Chao 1 (richness: number of taxonomic groups) and Shannon indexes (evenness: distribution of abundances of the groups) (Fig. 1). Krustall-Wallis was used to determine statistical significance across all samples. Chao 1 richness values varied but were not significantly different across all samples ($p = 0.22249$). The Shannon indexes of evenness levels were significantly higher in soil samples compared with those in grass samples ($p = 0.00032885$). The composition of the microbiomes was also analysed through principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity (Fig. 2, PERMANOVA test, $p < 0.001$). The soil control (without slurry application) and soil treatment (with slurry application) formed absolute overlapping clusters. The grass control and treatment samples also overlapped with each other. However, the soil and grass clusters were completely separated with no overlap.

3.4. Beta-diversity

The composition of the microbial communities was assessed at the phylum level for all samples (Fig. S1). The top three most abundant phyla across the soil and grass sample groups with and without slurry were consistent in taxa but varied in relative abundances across soil and grass; the phyla Proteobacteria and Actinobacteria were dominant in all samples. However, the abundance profiles of the remaining phyla differed. Soil samples were represented by the two most abundant phyla: Proteobacteria and Actinobacteria, while other phyla in the top ten were observed at lower (<6 %) relative abundances. Grass samples were characterised mainly by 3 most abundant phyla: Proteobacteria, Actinobacteria, and Bacteroidetes, other phyla were found at very low abundance levels (<2 %) (Dataset S2). The main phyla on grass were the same as on *Galium album* (Aydogan et al., 2018) and perennial grasses switchgrass (*Panicum virgatum L.*) and miscanthus (*Miscanthus x giganteus*): Proteobacteria and Bacteroidetes (Grady et al., 2019). These also agree with several other phyllosphere communities (Bodenhausen et al., 2013; Kinkel, 1997; Knief et al., 2010; Lindow and Brandl, 2003; Rastogi et al., 2013; Vorholt, 2012). The soil phyla were also similar to those previously determined (Janssen, 2006; Mhete et al., 2020). The application of different treated slurry did not impact the bacterial compositions of soil or grass.

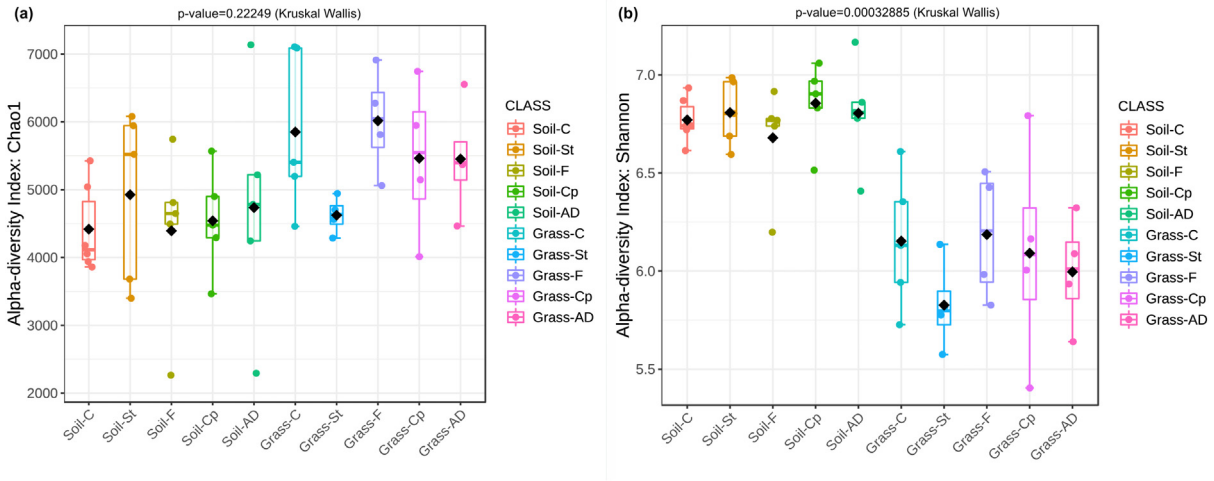

Fig. 1. Microbial Diversity a) Chao 1 (richness) values vary but are not significantly different across all samples; b) The Shannon indexes (evenness) were significantly higher in soil samples compared with those in grass samples.
Grass and soil samples were divided into 5 groups: Control (Soil-C and Grass-C) contained soil and grass samples collected from field plots without pig slurry application; Storage (Soil-St and Grass-St): samples collected from field plots with application of pig slurry product of storage treatment, Fresh (Soil-F and Grass-F) samples collected from field plots with application of raw pig slurry without treatment, Compost (Soil-Cp and Grass-Cp) samples collected from field plots with application of pig slurry compost, and AD (Soil-AD and Grass-AD) samples collected from filed plots with application of AD digestate of pig slurry.

The relative abundance profiles at genus level were highly similar between slurry treated and untreated samples in both soil and grass across the timepoints (Fig. S2) . The abundance profile was notably different between soil and grass samples. In contrast to the abundance order at the phylum level, soil samples were predominated by the genus *Bradyrhizobium*, while other genera were detected at a very low relative abundance level. Among grass samples, the two most abundant genera were *Pseudomonas* and *Sphingomonas*, other genera in the top ten were found at the lower relative abundances but at values greater than in soil. The predominance of *Pseudomonas* and *Sphingomonas* was consistent with findings of other phyllosphere microbiomes (Bodenhausen et al., 2013; Grady et al., 2019; Kinkel, 1997; Knief et al., 2010; Lindow and Brandl, 2003; Rastogi et al., 2013; Vorholt, 2012).

3.5. Characteristics of the resistomes and mobile genetic elements (MGEs) of soil and grass over time and under slurry treatments

A total of 140 different ARGs and 24 different MGEs were detected across all samples (Dataset S3). The detected ARGs were divided into 11 main antibiotic classes to which they conferred resistance: aminoglycoside,

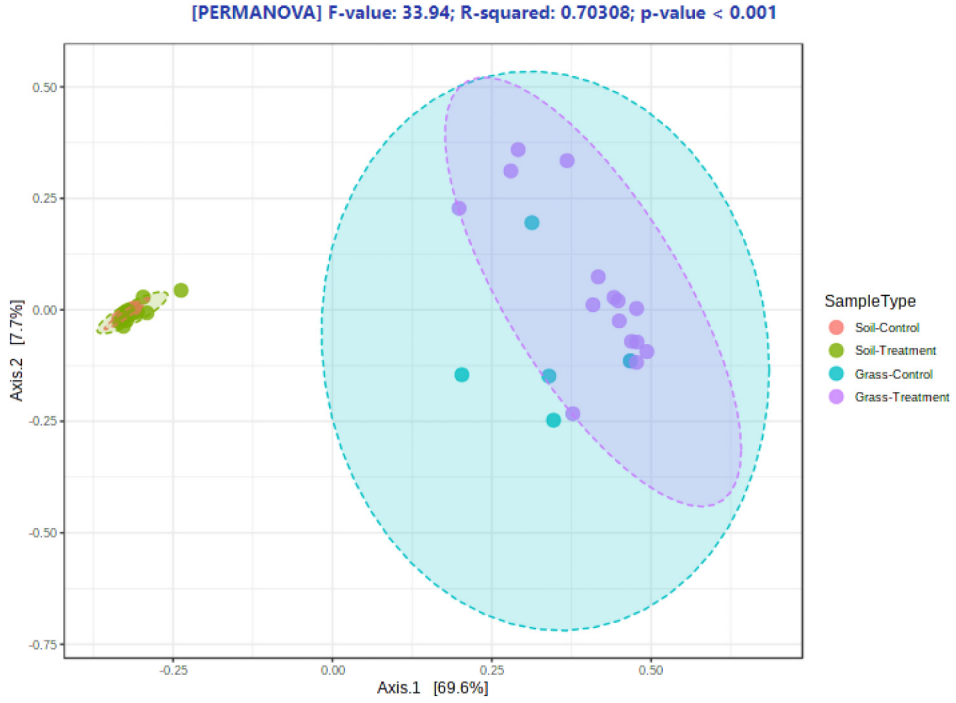

Fig. 2. PERMANOVA PCoA: The soil control (without slurry application) and soil treatment (with slurry application) form absolute overlapping clusters. The grass control and treatment samples also absolutely overlap with each other. However, soil clusters are completely separated from grass clusters.
Soil and Grass controls contained soil and grass samples collected from the field plots without pig slurry application. Soil and Grass treatments contained soil and grass samples collected from the field plots where different treated products of pig slurry (storage, compost, and AD) were spread onto.

beta-lactam, multi-drug resistance (MDR), macrolide-lincosamide-streptogramin B (MLSB), colistin, phenicol, quinolone, sulfonamides, tetracycline, trimethoprim, and vancomycin. Four MGE groups were identified comprising integrons, transposons, insertional sequences, and plasmid-associated genes. In total 116 genes (ARG and MGE) were detected across the soil and within at least one soil sample and 158 genes across the grass and within at least one grass sample. The relative abundance of total ARGs in grass samples was consistently higher in all samples than in soil samples (Fig. S3). Soil at time 0 prior to the application of composted slurry had an unusually high ARG relative abundance, not consistent across the other soil samples. All ARGs and MGEs present in soil were detected in grass samples. Those absent from soil but present in grass comprised aminoglycoside, beta-lactam, carbapenem, chloramphenicol, quinolone, tetracycline, and vancomycin resistance genes, in addition several MGEs were detected only in grass. As grass contained a wider variety and higher relative abundance of ARGs and MGEs we conclude that while soil may be a source of some of these genes it cannot be the source for all ARGs and MGEs as some are absent from the soil.

Clinically important plasmid mediated resistance genes detected included several $bla_{\text{CTX-M}}$ genes (in soil and grass), carbapenem resistance genes (bla_{NDM}, bla_{IMP}, bla_{VIM} and $bla_{\text{OXA-48}}$ in both soil and grass, and bla_{KPC}, $bla_{\text{OXA-51}}$ in grass), colistin resistance genes *mcr1* in soil and grass and *mcr4* in one grass sample type and the quinolone resistance genes *qepA* in all soil and grass and *qnrB* in all grass samples. At the threshold of over 20 % prevalence and over 0.01 relative abundance, 13 genes were found in core resistome shared across all soil and grass samples (Fig. S4). Among them, only three genes (*tetG*, *qepA*, and *intI1*_1) were found at 100 % prevalence in all samples. The five most abundant ARGs conferred resistance to aminoglycosides, beta-lactams, quinolones, tetracyclines, or vancomycin. The total relative abundances of the detected ARGs in soil and grass samples did not significantly increase due to slurry application (Fig. S3). The highest relative abundance within the grass samples, but not the soil samples occurred at timepoint 4 (ten weeks following slurry application). The control samples also contained this increase, suggesting a factor other than slurry is the contributing factor(s).

The alpha diversities of the resistomes and the MGEs were compared separately using the Chao 1 and Shannon indexes (Fig. 3). Krustall-Wallis was used to determine statistical significance across all samples. Richness values for ARGs and MGEs were significantly different across all samples (p = 3.0132e-05 and 9.7868e-05, respectively), with higher values for the grass samples than the soil. The evenness levels for ARGs but not MGEs were significantly higher in grass samples compared with those in

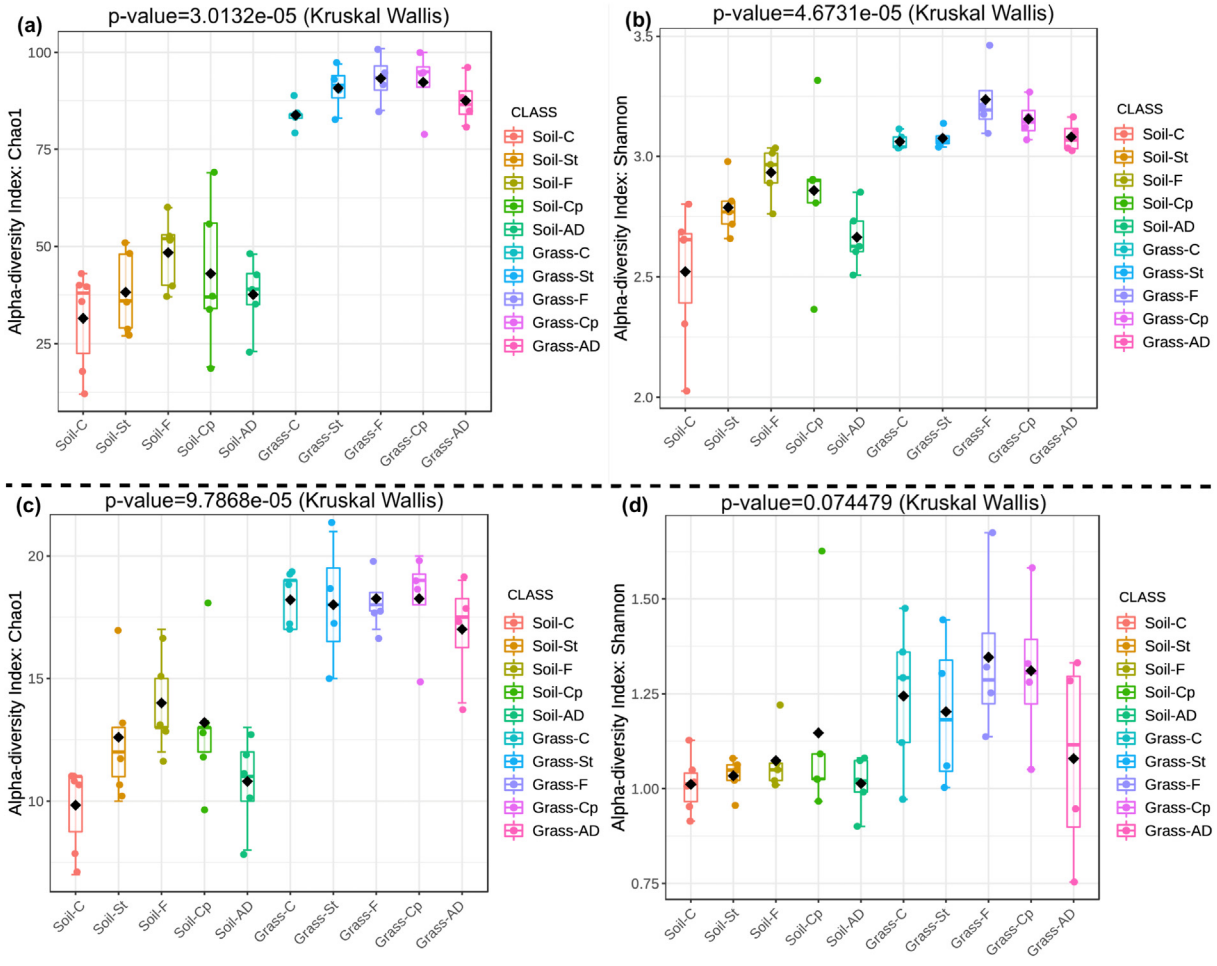

Fig. 3. Resistome alpha diversity (a and b). The resistome alpha diversity (Chao 1 (a) and Shannon (b) indexes) is significantly higher in the grass samples than in soil samples. All treatment samples in both soil and grass have notable higher values of Chao 1 and Shannon indexes in comparison with control samples.
The MGE alpha diversity (c and d). Grass sample groups have a significantly higher richness (c) (Chao 1) of MGE than soil groups. The evenness (d) is also higher in grass compared with soil; however, the differences are not significant.
Grass and soil samples were divided into 5 groups: Control (Soil-C and Grass-C) contained soil and grass samples collected from field plots without pig slurry application; Storage (Soil-St and Grass-St): samples collected from field plots with application of pig slurry product of storage treatment, Fresh (Soil-F and Grass-F) samples collected from field plots with application of raw pig slurry without treatment, Compost (Soil-Cp and Grass-Cp) samples collected from field plots with application of pig slurry compost, and AD (Soil-AD and Grass-AD) samples collected from filed plots with application of AD digestate of pig slurry.

soil samples (p = 4.6731e-05). The composition of resistomes and MGEs was analysed through principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity (Fig. S5, PERMANOVA test, $p < 0.001$). The ARGs and MGEs detected in control soils (without slurry application) and treated (with slurry application) formed overlapping clusters. The ARGs and MGEs grass control and treatment samples also overlapped with each other. This indicates a similar resistance gene profile in soil with/without treatments, as well as in grass with/without treatment. The majority of soil clusters were separated from grass clusters for ARGs with overlaps in four grass treated samples and five soil treated samples (total sample n = 50). Further separation and overlap (n = 15 soil with 15 grass samples) was observed in relation to the MGEs across soil and grass samples.

3.6. Network analysis

3.6.1. Association between ARGs and MGEs

The network analysis based on strong and significant Spearman's correlations ($r > 0.85$ and $p < 0.05$) between ARGs and MGEs was employed to understand the co-occurrence of ARGs and MGEs across all the grass and soil samples (Fig. 4, Dataset S4). There were only positive interactions found (i.e. no negative correlations) when analysing the network between ARGs and MGEs in soil and grass samples. These results indicate the co-location or co-association of these ARGs with MGEs and the potential for dissemination of these genes via horizontal gene transfer within soil and grass microbial communities. The larger the size of the nodes the greater the degree of the interactions. Within the soil networks six different clusters were identified comprising one large and five smaller clusters. The ARGs of greatest clinical significance were contained within the large cluster with the MGEs (predominantly *repA*, *IncP*, *IncW*, *tnpA*, *orf37-IS26*) acting as the central linking components. The grass networks formed two clusters, one large and one small. Similarly, to the soil the MGEs (*repA*, *IncW*, *IncN*, *tnpA*, *IncF*, *IncQ* and *int1*) act as the main linkers of the ARGs, but some of the MGEs differed from the soil (*repA*, *IncW*, *tnpA*). While some ARGs were present across all samples they were not always clustered with the same MGE e.g., *qepA* correlated with *intI3_2*, *IncW_trwAB*, *IncN_rep*, *tnpA_3*, *IncQ_oriT*, *intI1_4* and *repA* in the grass and *IS1111*, *repA*, *tnpA_3* and *orf37-IS26* in the soil samples. Thus, while the ARG was detected across all samples it may be moving either on the same MGEs (*repA*, *tnpA*) or on different MGEs across the samples, indicating multiple potential modes of mobility for the same genes within and across different samples.

3.6.2. Correlation analysis between microbial taxa and ARGs

The relationship and interaction between microbial phyla and ARGs were investigated in the network based on Spearman's correlation analysis (Fig. 4B). The network in soil comprised 44 nodes (from 14 microbial phyla and 30 ARGs), and 42 edges (built from 14 negative and 28 positive correlations). A positive correlation indicates the presence of both items together, a negative correlation indicates the presence of one item but the absence of the other. The network in grass consisted of 70 nodes (27 phyla and 43 ARGs), and 179 edges (built from 15 negative and 164 positive correlations). Thus, the soil networks were simpler than the grass networks. In soil Fusobacteria and Proteobacteria had the most positive interactions with ARGs. These results indicated their role as primary ARG hosts. Actinobacteria had positive correlations with *tetM* and negative interactions with bla_{KPC_2}. These phyla did not produce the same results in the grass samples. The grass networks were more complex and included many more ARGs and phyla interactions, most of which were positive. In contrast to soil, Proteobacteria were relatively low in interactions with ARGs, their only interaction with ARGs was a negative interaction with *dfrA12*. The relative abundances of Proteobacteria in soil and grass samples was not significantly different (Fig. S1). The main grass phyla with ARG networks were Spirochaetes and Aquificae, neither of which were correlated with ARGs in the pig slurry (Do et al., 2022). Therefore, the slurry was not the source of these bacteria.

4. Discussion

While studies have suggested that soil is an important source of bacterial transfer to the phyllosphere (Bodenhausen et al., 2013; Copeland et al., 2015; Grady et al., 2019; Massoni et al., 2021; Tkacz et al., 2020; Vorholt, 2012; Wei and Ashman, 2018; Zarraonaindia et al., 2015), no studies have analysed the commonality of the total resistome between soil and the phyllosphere and we have yet to identify any studies that have analysed the total resistome of grass. This study showed that the grass phyllosphere contained both a wider array of ARGs and MGEs and a larger relative abundance of these genes than the soil. Soil is a recognised and well-studied reservoir and source of a wide range and relative abundance of ARGs (Cytryn, 2013; Forsberg et al., 2012; Van Goethem et al., 2018). While the classes of antimicrobials to which the ARGs conferred resistance were present both on grass and in soil there were unique many ARGs only present in the grass samples. There was also significantly higher evenness and richness in the grass resistomes than the soil resistomes. The grass phyllosphere contained ARGs and MGEs not detected in soil e.g. *qnrB* and *IncF*, respectively, and multiple versions of the same ARG types e.g. $bla_{CTX\text{-}M\ 1–6}$ in grass relative to $bla_{CTX\text{-}M\ 1–4}$ in soil. Network analysis identified that where the same ARGs and MGE genes were detected in soil and grass samples the correlation between these ARGs and MGEs did not occur in both grass and soil. Thus, while some ARGs were present across grass and soil the specific mobile elements capable of moving the ARGs between different bacteria were significantly different. These data suggests that the ARGs either did not move from soil or that they moved into new MGEs once present on grass. The former is more likely due to the number of movements required across the wide range of ARGs. In addition, the correlation between ARGs or MGEs and bacteria were different in soil and grass, indicating that the common ARGs and MGEs either moved from the bacteria in soil to different bacteria when on grass or that the common ARGs and MGEs were mobilised by bacteria from sources other than soil. Our data indicates that while there are commonalities across many of the ARGs present in soil and on grass their modes of movement, correlations with MGEs and bacteria differ, suggesting a source other than soil is also relevant for the resistome of grass. The variations in the relative abundances of ARGs in soil and on grass also indicate that either the MGEs or the bacteria carrying the ARGs comprise a higher relative abundance on grass than in soil.

Our data identifies grass as a more diverse and more abundant reservoir of ARGs and MGEs in the environment than soil. As the microbiome of the grass samples was consistent over time and with other studies we suggest that these resistome findings may also be representative of grass globally (Aydogan et al., 2020, 2018; Ding and Melcher, 2016; Doherty et al., 2021; Grady et al., 2019; Hestrin et al., 2021) but at least the resistome is not due to a unique grass microbiome. However, this requires further verification. In Europe, grasslands covering more than a third of the European agricultural area are used to feed herbivores and ruminants. Should our findings be reproduced across grasslands then this represents a very large reservoir of ARGs that is connected directly to the food chain.

Our study has identified commonalities across the phyla of soil and grass, which were not significantly impacted by slurry. A number of studies have demonstrated that slurry-derived ARB and their ARGs may persist in soil for a few weeks and up to years and that these ARGs can be horizontally transferred into native soil bacteria (Binh et al., 2009; Chee-Sanford et al., 2001; Heuer et al., 2011; Heuer et al., 2008; Heuer and Smalla, 2007; Jechalke et al., 2013; Schmitt et al., 2006; Udikovic-Kolic et al., 2014; Wichmann et al., 2014). Previous studies have examined the impact of slurry application on the levels of ARGs in the phyllosphere of leafy vegetables (Chen et al., 2016, 2017), or changes in ARGs abundance and dissemination (Marti et al., 2013; Murray et al., 2019; Tien et al., 2017). Mobile genetic elements such as *int*I1 and genes encoding transposase have been detected in leaf endophytes, as well as in the phyllosphere of lettuce (Wang et al., 2015; Zhu et al., 2017), maize (Chen et al., 2016), *Brassica chinensis* L (Chen et al., 2019a), and *Coriandrum sativum* L (Chen et al., 2019b). Slurry-amended soils have been associated with increased detection of ARB and ARGs on lettuce and root vegetables; however, this has

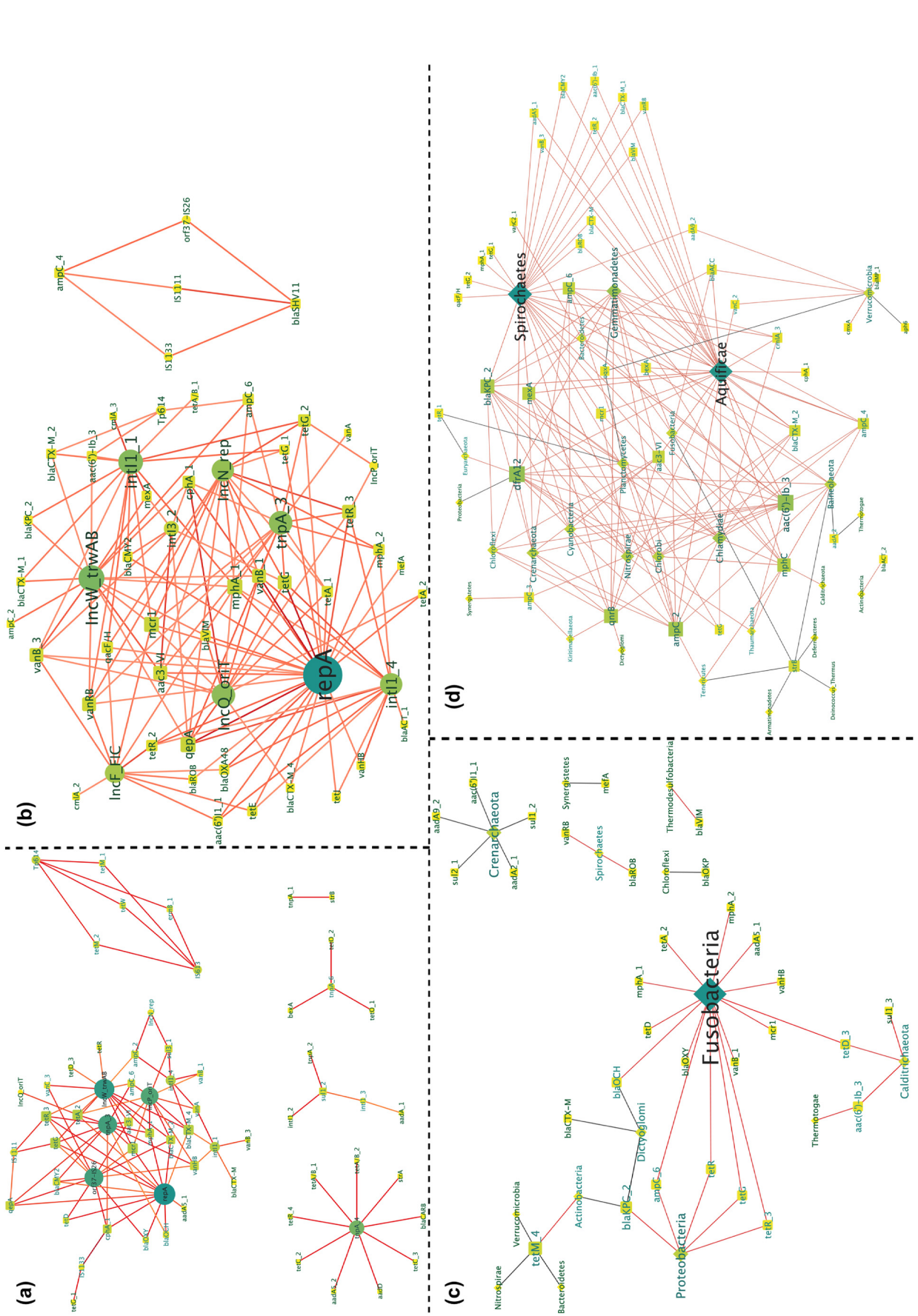

Fig. 4. Network analysis between ARG and MGE (a and b), and ARG and microbial phyla (c and d).
ARG and MGE network based on the interaction of ARGs and MGEs, (a) in soil samples, (b) in grass samples. There were only positive interactions found when analysing the network between ARGs and MEGs in soil and grass samples with Spearman's correlation $r > 0.85$ and $p < 0.05$. These results indicate the location of these genes on MGEs →the dissemination of these genes via HGT within soil and grass microbial communities.
ARGs and microbial phyla network built on the interactions between microbial phyla and ARGs in soil (c) and grass (d) samples. Network in soil was formed based on Spearman's correlation $r > 0.5$, $p < 0.05$. Network in grass, Spearman's correlation $r > 0.6$, $p < 0.05$. Red edges: positive interaction, blue edges: negative interactions.

not been associated with all crops or ARGs (Marti et al., 2013; Rahube et al., 2014; Wang et al., 2015). Our results agree with the latter studies, although many ARGs were detected. The results contrast with our own previous studies on soil and lettuce (Gekenidis et al., 2020). This raises the question of whether this is unique to grass rhizosphere and phyllosphere.

The microbiome compositions, the evenness and the PCoA were significantly different between soil and grass. This finding is in agreement with Vorholt et al., (Massoni et al., 2021) who found that the communities on leaves not in contact with the soil did not converge with the soil communities. However, other environmental factors (but not slurry) may have contributed to these significant differences. Few studies have compared the microbiomes of soil and grass phyllosphere. Yan et al., 2020 compared soil and grass across Australian urban and national park environments, but not agricultural use (Yan et al., 2020). They identified an overlap of 87.6 % in the total genera identified in the grass phyllosphere with the soil genera. Our study identified a 98.7 % overlap in genera across grass and soil. The Shannon index and the PCoA of Yan et al., demonstrated statistically significant differences between both, which agrees with the data in this study (Yan et al., 2020). While there were overlaps in the genera detected across grass and soil in this study the relative abundances varied considerably. This suggests that while genera are shared across soil and grass, the phyllosphere provides a selective environment for the relative abundances of genera different to the soil to flourish and that filtering of the composition occurs on the grass. *Bradyrhizobium* (soil genera high, grass low) are an example of a large proportion of the soil genera composition, which was not detected on grass. They are nitrogen-fixing symbiotes, with a niche in the roots of plants. Thus, survival on plant surfaces is unlikely. The major grass genera included *Pseudomonas* and *Sphingomonas*, which have been previously identified as major genera on plants and grass specifically. *Acinetobacter* spp. was also detected on grass in the top 10 abundances; these are potential opportunistic pathogens of humans. Other genera detected in relatively high abundances on grass by Shade et al., e.g. *Methylbacterium* or *Bacillus* were not detected in the ten most abundant genera in this study (Grady et al., 2019).

5. Conclusions

While scientists have focused much of the environmental resistome analysis on soil as a resistome reservoir and link between the animal and environment this study identified the importance of grass as a reservoir of ARGs in addition to the soil resistome. The data identifies grass as a more expansive, diverse and persistent reservoir and source of ARGs, MGEs and differential abundance of microbiomes to soil. Grass and plants must be included as they directly link the environment back to the animal or human eating the plant and we have demonstrated the wide array of ARGs linked to MGEs present on grass, which is greater than in soil.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159179.

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Data and materials availability

The sequencing data have been submitted in the NCBI Sequencing Read Archive (SRA) under the bio-project PRJNA773121. Relative abundances of microbial taxa, ARGs, MGEs; and correlation analysis data as well as HT-qPCR array data are available as supplementary material (Supplementary Data).

CRediT authorship contribution statement

TTD and FW: Design and/or interpretation of the reported experiments, acquisition and/or analysis of data, Drafting and revising the manuscript, Administrative, technical, or supervisory support. **CS**: Acquisition and/or analysis of data. **FC:** Acquisition and/or analysis of data. **CB and FB:** Drafting and revising the manuscript, Administrative, technical or supervisory support.

Data availability

The location of the raw data is described in the manuscript. The sequencing data have been submitted in the NCBI Sequencing Read Archive (SRA) under the bio-project PRJNA773121.

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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References

Alegbeleye, O.O., Singleton, I., Sant'Ana, A.S., 2018. Sources and contamination routes of microbial pathogens to fresh produce during field cultivation: a review. Food Microbiol. 73, 177–208. https://doi.org/10.1016/j.fm.2018.01.003.

Aramaki, T., Blanc-Mathieu, R., Endo, H., Ohkubo, K., Kanehisa, M., Goto, S., Ogata, H., 2020. KofamKOALA: KEGG ortholog assignment based on profile HMM and adaptive score threshold. Bioinformatics 36, 2251–2252. https://doi.org/10.1093/bioinformatics/btz859.

Aydogan, E.L., Moser, G., Müller, C., Kämpfer, P., Glaeser, S.P., 2018. Long-term warming shifts the composition of bacterial communities in the phyllosphere of Galium album in a permanent grassland field-experiment. Front. Microbiol. 9, 144. https://doi.org/10.3389/fmicb.2018.00144.

Aydogan, E.L., Budich, O., Hardt, M., Choi, Y.H., Jansen-Willems, A.B., Moser, G., Müller, C., Kämpfer, P., Glaeser, S.P., 2020. Global warming shifts the composition of the abundant bacterial phyllosphere microbiota as indicated by a cultivation-dependent and -independent study of the grassland phyllosphere of a long-term warming field experiment. FEMS Microbiol. Ecol. 96, fiaa087. https://doi.org/10.1093/femsec/fiaa087.

Binh, C.T.T., Heuer, H., Kaupenjohann, M., Smalla, K., 2009. Diverse aadA gene cassettes on class 1 integrons introduced into soil via spread manure. Res. Microbiol. 160, 427–433. https://doi.org/10.1016/j.resmic.2009.06.005.

Bodenhausen, N., Horton, M.W., Bergelson, J., 2013. Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. PLOS ONE 8, e56329. https://doi.org/10.1371/journal.pone.0056329.

Brown, E.D., Wright, G.D., 2016. Antibacterial drug discovery in the resistance era. Nature 529, 336–343.

Chee-Sanford, J.C., Aminov, R.I., Krapac, I.J., Garrigues-Jeanjean, N., Mackie, R.I., 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. Appl. Environ. Microbiol. 67, 1494–1502. https://doi.org/10.1128/AEM.67.4.1494-1502.2001.

Chen, Q., An, X., Li, H., Su, J., Ma, Y., Zhu, Y.-G., 2016. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. Environ. Int. 92–93, 1–10. https://doi.org/10.1016/j.envint.2016.03.026.

Chen, Q.-L., An, X.-L., Zhu, Y.-G., Su, J.-Q., Gillings, M.R., Ye, Z.-L., Cui, L., 2017. Application of struvite alters the antibiotic resistome in soil, rhizosphere, and phyllosphere. Environ. Sci. Technol. 51, 8149–8157. https://doi.org/10.1021/acs.est.7b01420.

Chen, Q.-L., Cui, H.-L., Su, J.-Q., Penuelas, J., Zhu, Y.-G., 2019a. Antibiotic resistomes in plant microbiomes. Trends Plant Sci. 24, 530–541. https://doi.org/10.1016/j.tplants.2019.02.010.

Chen, Q.-L., Zhu, D., An, X.-L., Ding, J., Zhu, Y.-G., Cui, L., 2019b. Does nano silver promote the selection of antibiotic resistance genes in soil and plant? Environ. Int. 128, 399–406. https://doi.org/10.1016/j.envint.2019.04.061.

Copeland, J.K., Yuan, L., Layeghifard, M., Wang, P.W., Guttman, D.S., 2015. Seasonal community succession of the phyllosphere microbiome. Mol. Plant-Microbe Interact. 28, 274–285. https://doi.org/10.1094/MPMI-10-14-0331-FI.

Cytryn, E., 2013. The soil resistome: the anthropogenic, the native, and the unknown. Soil Biol. Biochem. 63, 18–23. https://doi.org/10.1016/j.soilbio.2013.03.017.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B., Xia, J., 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res. 45, W180–W188. https://doi.org/10.1093/nar/gkx295.

Ding, T., Melcher, U., 2016. Influences of plant species, season and location on leaf endophytic bacterial communities of non-cultivated plants. PLOS ONE 11, e0150895. https://doi.org/10.1371/journal.pone.0150895.
Ding, G.C., Radl, V., Schloter-Hai, B., Jechalke, S., Heuer, H., Smalla, K., et al., 2014. Dynamics of soil bacterial communities in response to repeated application of manure containing sulfadiazine. PLoS ONE 9, e92958. https://doi.org/10.1371/journal.pone.0092958.
Do, T.T., Nolan, S., Hayes, N., O'Flaherty, V., Burgess, C., Brennan, F., Walsh, F., 2022. Metagenomic and HT-qPCR analysis reveal the microbiome and resistome in pig slurry under storage, composting, and anaerobic digestion. Environ. Pollut. 305, 119271. https://doi.org/10.1016/j.envpol.2022.119271.
Doherty, J.R., Crouch, J.A., Roberts, J.A., 2021. Plant age influences microbiome communities more than plant compartment in greenhouse-grown creeping bentgrass. Phytobiomes J. 5, 373–381. https://doi.org/10.1094/PBIOMES-03-21-0021-R.
Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., Dantas, G., 2012. The shared antibiotic resistome of soil bacteria and human pathogens. Science 337, 1107–1111. https://doi.org/10.1126/science.1220761.
Gekenidis, M.-T., Rigotti, S., Hummerjohann, J., Walsh, F., Drissner, D., 2020. Long-term persistence of blaCTX-M-15 in soil and lettuce after introducing extended-spectrum β-lactamase (ESBL)-producing Escherichia coli via manure or water. Microorganisms 8, E1646. https://doi.org/10.3390/microorganisms8111646.
Gonçalo, M., Hernandez-Leal, L., van der Maas, P., Heederik, D., Mevius, D., Schmitt, H., 2020. The impact of manure and soil texture on antimicrobial resistance gene levels in farmlands and adjacent ditches. Sci. Total Environ. 737, 139563. https://doi.org/10.1016/j.scitotenv.2020.139563.
Grady, K.L., Sorensen, J.W., Stopnisek, N., Guittar, J., Shade, A., 2019. Assembly and seasonality of core phyllosphere microbiota on perennial biofuel crops. Nat. Commun. 10, 4135. https://doi.org/10.1038/s41467-019-11974-4.
Hestrin, R., Lee, M.R., Whitaker, B.K., Pett-Ridge, J., 2021. The switchgrass microbiome: a review of structure, function, and taxonomic distribution. Phytobiomes J. 5, 14–28. https://doi.org/10.1094/PBIOMES-04-20-0029-FI.
Heuer, H., Smalla, K., 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ. Microbiol. 9, 657–666. https://doi.org/10.1111/j.1462-2920.2006.01185.x.
Heuer, H., Focks, A., Lamshöft, M., Smalla, K., Matthies, M., Spiteller, M., 2008. Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. Soil Biol. Biochem. 40, 1892–1900. https://doi.org/10.1016/j.soilbio.2008.03.014.
Heuer, H., Schmitt, H., Smalla, K., 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr. Opin. Microbiol. 14, 236–243. https://doi.org/10.1016/j.mib.2011.04.009.
Janssen, P.H., 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. 72, 1719–1728. https://doi.org/10.1128/AEM.72.3.1719-1728.2006.
Jechalke, S., Kopmann, C., Rosendahl, I., Groeneweg, J., Weichelt, V., Krögerrecklenfort, E., Brandes, N., Nordwig, M., Ding, G.-C., Siemens, J., Heuer, H., Smalla, K., 2013. Increased abundance and transferability of resistance genes after field application of manure from sulfadiazine-treated pigs. Appl. Environ. Microbiol. 79, 1704–1711. https://doi.org/10.1128/AEM.03172-12.
Kinkel, L.L., 1997. Microbial population dynamics on leaves. Annu. Rev. Phytopathol. 35, 327–347. https://doi.org/10.1146/annurev.phyto.35.1.327.
Knief, C., Ramette, A., Frances, L., Alonso-Blanco, C., Vorholt, J.A., 2010. Site and plant species are important determinants of the Methylobacterium community composition in the plant phyllosphere. ISME J. 4, 719–728. https://doi.org/10.1038/ismej.2010.9.
Laforest-Lapointe, I., Messier, C., Kembel, S.W., 2016. Tree phyllosphere bacterial communities: exploring the magnitude of intra- and inter-individual variation among host species. PeerJ 4, e2367. https://doi.org/10.7717/peerj.2367.
Lindow, S.E., Brandl, M.T., 2003. Microbiology of the phyllosphere. Appl. Environ. Microbiol. 69, 1875–1883. https://doi.org/10.1128/AEM.69.4.1875-1883.2003.
Marti, R., Scott, A., Tien, Y.-C., Murray, R., Sabourin, L., Zhang, Y., Topp, E., 2013. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Appl. Environ. Microbiol. 79, 5701–5709. https://doi.org/10.1128/AEM.01682-13.
Massoni, J., Bortfeld-Miller, M., Widmer, A., Vorholt, J.A., 2021. Capacity of soil bacteria to reach the phyllosphere and convergence of floral communities despite soil microbiota variation. Proc. Natl. Acad. Sci. 118, e2100150118. https://doi.org/10.1073/pnas.2100150118.
Mhete, M., Eze, P.N., Rahube, T.O., Akinyemi, F.O., 2020. Soil properties influence bacterial abundance and diversity under different land-use regimes in semi-arid environments. Sci. Afr. 7, e00246. https://doi.org/10.1016/j.sciaf.2019.e00246.
Murray, R., Tien, Y.-C., Scott, A., Topp, E., 2019. The impact of municipal sewage sludge stabilization processes on the abundance, field persistence, and transmission of antibiotic resistant bacteria and antibiotic resistance genes to vegetables at harvest. Sci. Total Environ. 651, 1680–1687. https://doi.org/10.1016/j.scitotenv.2018.10.030.
OECD, 2018. Stemming the Superbug Tide: Just A Few Dollars More, OECD Health Policy Studies. OECD Publishing, Paris https://doi.org/10.1787/9789264307599-en.
Rahube, T.O., Marti, R., Scott, A., Tien, Y.-C., Murray, R., Sabourin, L., Zhang, Y., Duenk, P., Lapen, D.R., Topp, E., 2014. Impact of fertilizing with raw or anaerobically digested sewage sludge on the abundance of antibiotic-resistant coliforms, antibiotic resistance genes, and pathogenic bacteria in soil and on vegetables at harvest. Appl. Environ. Microbiol. 80, 6898–6907. https://doi.org/10.1128/AEM.02389-14.
Rastogi, G., Coaker, G.L., Leveau, J.H.J., 2013. New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. FEMS Microbiol. Lett. 348, 1–10. https://doi.org/10.1111/1574-6968.12225.
Santamaría, J., et al., 2011. Detection and diversity evaluation of tetracycline resistance genes in grassland-based production systems in Colombia,South America. Front. Microbiol. 2, 252. https://doi.org/10.3389/fmicb.2011.00252.
Schils, R.L.M., Bufe, C., Rhymer, C.M., Francksen, R.M., Klaus, V.H., Abdalla, M., Milazzo, F., Lellei-Kovács, E., ten Berge, H., Bertora, C., Chodkiewicz, A., Dămătîrcă, C., Feigenwinter, I., Fernández-Rebollo, P., Ghiasi, S., Hejduk, S., Hiron, M., Janicka, M., Pellaton, R., Smith, K.E., Thorman, R., Vanwalleghem, T., Williams, J., Zavattaro, L., Kampen, J., Derkx, R., Smith, P., Whittingham, M.J., Buchmann, N., Price, J.P.N., 2022. Permanent grasslands in Europe: land use change and intensification decrease their multifunctionality. Agric. Ecosyst. Environ. 330, 107891. https://doi.org/10.1016/j.agee.2022.107891.
Schmitt, H., Stoob, K., Hamscher, G., Smit, E., Seinen, W., 2006. Tetracyclines and tetracycline resistance in agricultural soils: microcosm and field studies. Microb. Ecol. 51, 267–276. https://doi.org/10.1007/s00248-006-9035-y.
Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504. https://doi.org/10.1101/gr.1239303.
Tien, Y.-C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., Marti, R., Topp, E., 2017. Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. Sci. Total Environ. 581–582, 32–39. https://doi.org/10.1016/j.scitotenv.2016.12.138.
Tkacz, A., Bestion, E., Bo, Z., Hortala, M., Poole, P.S., 2020. Influence of plant fraction, soil, and plant species on microbiota: a multikingdom comparison. mBio 11, e02785-19. https://doi.org/10.1128/mBio.02785-19.
Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J., 2014. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. PNAS 111, 15202–15207. https://doi.org/10.1073/pnas.1409836111.
United Nations, 2016. http://www.un.org/pga/71/wp-content/uploads/sites/40/2016/09/DGACM_GAEAD_ESCAB-AMR-Draft-Political-Declaration-1616108E.pdf.
Van Goethem, M.W., Pierneef, R., Bezuidt, O.K.I., Van De Peer, Y., Cowan, D.A., Makhalanyane, T.P., 2018. A reservoir of 'historical' antibiotic resistance genes in remote pristine Antarctic soils. Microbiome 6, 40. https://doi.org/10.1186/s40168-018-0424-5.
Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., Dufresne, A., 2015. The importance of the microbiome of the plant holobiont. New Phytol. 206, 1196–1206. https://doi.org/10.1111/nph.13312.
Virtanen, P., Gommers, R., Oliphant, T.E., Haberland, M., Reddy, T., Cournapeau, D., Burovski, E., Peterson, P., Weckesser, W., Bright, J., van der Walt, S.J., Brett, M., Wilson, J., Millman, K.J., Mayorov, N., Nelson, A.R.J., Jones, E., Kern, R., Larson, E., Carey, C.J., Polat, İ., Feng, Y., Moore, E.W., VanderPlas, J., Laxalde, D., Perktold, J., Cimrman, R., Henriksen, I., Quintero, E.A., Harris, C.R., Archibald, A.M., Ribeiro, A.H., Pedregosa, F., van Mulbregt, P., 2020. SciPy 1.0: fundamental algorithms for scientific computing in Python. Nat. Methods 17, 261–272. https://doi.org/10.1038/s41592-019-0686-2.
Vorholt, J.A., 2012. Microbial life in the phyllosphere. Nat. Rev. Microbiol. 10, 828–840. https://doi.org/10.1038/nrmicro2910.
Walsh et al., n.d. F. Walsh A. Ingenfeld M. Zampicolli M. Hilber-Bodmer J.E. Frey B. Duffy Real-time PCR methods for quantitative monitoring of streptomycin and tetracycline resistance genes in agricultural ecosystems. Journal of Microbiological Methods 86, 150-155.n.d. doi:10.1016/j.mimet.2011.04.011
Wang, F.-H., Qiao, M., Chen, Z., Su, J.-Q., Zhu, Y.-G., 2015. Antibiotic resistance genes in manure-amended soil and vegetables at harvest. J. Hazard. Mater. 299, 215–221. https://doi.org/10.1016/j.jhazmat.2015.05.028.
Wang, F., Sun, R., Hu, H., Duan, G., Meng, L., Qiao, M., 2022. The overlap of soil and vegetable microbes drives the transfer of antibiotic resistance genes from manure-amended soil to vegetables. Sci. Total Environ. 828, 154463. https://doi.org/10.1016/j.scitotenv.2022.154463.
Wei, N., Ashman, T.-L., 2018. The effects of host species and sexual dimorphism differ among root, leaf and flower microbiomes of wild strawberries in situ. Sci. Rep. 8, 5195. https://doi.org/10.1038/s41598-018-23518-9.
Wichmann, F., Udikovic-Kolic, N., Andrew, S., Handelsman, J., 2014. Diverse antibiotic resistance genes in dairy cow manure. mBio 5, e01017. https://doi.org/10.1128/mBio.01017-13.
World Health Organization, 2014World Health Organization. 2014. ISBN: 978 92 4 156474 8.
Yan, Z.-Z., Chen, Q.-L., Zhang, Y.-J., He, J.-Z., Hu, H.-W., 2020. Industrial development as a key factor explaining variances in soil and grass phyllosphere microbiomes in urban green spaces. Environ. Pollut. 261, 114201. https://doi.org/10.1016/j.envpol.2020.114201.
Ye, Y., Doak, T.G., 2009. A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. PLoS Comput. Biol. 5, e1000465. https://doi.org/10.1371/journal.pcbi.1000465.
Zakrzewski, M., Proietti, C., Ellis, J.J., Hasan, S., Brion, M.-J., Berger, B., Krause, L., 2017. Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions. Bioinformatics 33, 782–783. https://doi.org/10.1093/bioinformatics/btw725.
Zarraonaindia, I., Owens, S.M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., Bokulich, N.A., Mills, D.A., Martin, G., Taghavi, S., van der Lelie, D., Gilbert, J.A., 2015. The soil microbiome influences grapevine-associated microbiota. mBio 6, e02527-14. https://doi.org/10.1128/mBio.02527-14.
Zhang, Y.-J., Hu, H.-W., Chen, Q.-L., Singh, B.K., Yan, H., Chen, D., He, J.-Z., 2019. Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. Environ. Int. 130, 104912. https://doi.org/10.1016/j.envint.2019.104912.
Zhu, B., Chen, Q., Chen, S., Zhu, Y.-G., 2017. Does organically produced lettuce harbor higher abundance of antibiotic resistance genes than conventionally produced? Environ. Int. 98, 152–159. https://doi.org/10.1016/j.envint.2016.11.001.