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Differential impact of swine, bovine and poultry manure on the microbiome and resistome of agricultural grassland

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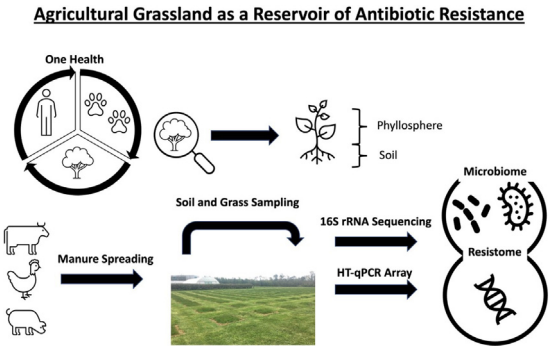
HIGHLIGHTS

- The soil and grass phyllosphere contained a diverse resistome, mobilome and microbiome.
- Bovine, swine and poultry manure altered the soil and grass phyllosphere microbiome and resistome at OTU and ARG level.
- The overall impact on the microbiome and resistome was short term and not significant.
- Antimicrobial resistance genes for critically important antimicrobials were associated with MGEs and OTUs.

GRAPHICAL ABSTRACT



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ABSTRACT

Land spreading of animal manure is an essential process in agriculture. Despite the importance of grassland in global food security the potential of the grass phyllosphere as a reservoir of antimicrobial resistance (AMR) is unknown. Additionally, the comparative risk associated with different manure sources is unclear. Due to the One Health nature of AMR there is an urgent need to fully understand the risk associated with AMR at the agriculture - environmental nexus. We performed a grassland field study to assess and compare the relative and temporal impact of bovine, swine and poultry manure application on the grass phyllosphere and soil microbiome and resistome over a period of four months, using 16S rRNA amplicon sequencing and high-throughput quantitative PCR (HT-qPCR). The soil and grass phyllosphere contained a diverse range of antimicrobial resistance genes (ARGs) and mobile genetic elements (MGEs). Manure treatment was found to introduce ARGs belonging to clinically important antimicrobial classes, such as aminoglycoside and sulphonamide into grass and soil. Temporal analysis of ARGs and MGEs associated with manure treatment indicated ARGs patterns were similar across the different manure types in the manure treated soil and grass phyllosphere. Manure treatment resulted in the enrichment in members of the indigenous microbiota and the introduction of manure associated bacteria, with this impact extending past the recommended six-week exclusion period. However, these bacteria were in low relative abundance and manure treatment was not found to significantly impact the overall composition of the microbiome or resistome. This provides evidence that the current guidelines facilitate reduction of biological risk to livestock. Additionally, in soil and grass samples MGEs correlated with ARGs from clinically important antimicrobial classes, indicating the key role MGEs play in horizontal gene transfer in agricultural grassland. These results demonstrate the role of the grass phyllosphere as an under-studied sink of AMR.

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1. Introduction

According to the World Health organisation (WHO), antimicrobial resistance (AMR) is currently one of the greatest threats to health and food security (WHO, 2021). Antimicrobials are vital in both human and veterinary medicine and plant protection. However, as a result they can be released into the environment through improper disposal or through human and animal excrement (Ventola, 2015). Therefore, antimicrobial resistance is considered a One Health issue due to the links between animals, humans and the environment (Robinson et al., 2016). The environment is a natural reservoir of bacteria, antimicrobial resistance genes (ARGs) and mobile genetic elements (MGEs). It has been found that the indigenous microbiome and resistome (collection of ARGs in an environment) can be impacted by common agricultural practices such as manure land spreading (Lima et al., 2020).

Manure application is essential for waste management and recycling nutrients into agricultural soils, facilitating crop growth (Manyi-Loh et al., 2016). However, despite these benefits manure can introduce and enrich for ARGs, antimicrobial resistant bacteria (ARB) and potential pathogens in the environment (Ding et al., 2014; Udikovic-Kolic et al., 2014; Lopatto et al., 2019; Wang et al., 2020; Macedo et al., 2021). Manure land spreading has been found to enrich for and introduce genes conferring resistance to clinically important antibiotics, such the sulphonamide resistance genes *sul1* (Ruuskanen et al., 2016; Lopatto et al., 2019) and *sul2* (Wang et al., 2020), tetracycline resistance genes *tet(M)* (Ruuskanen et al., 2016), *tet(W)* (Macedo et al., 2020) and *tet(G)* (Wang et al., 2020) and macrolide resistance genes *erm(B)* and *erm(C)* (Lopatto et al., 2019).

Reports of the temporal impact manure land spreading has on agricultural land have varied. Manure land spreading has been found to have a short-term impact (4–12 weeks on average) on soil (Muurinen et al., 2017; Gou et al., 2018; Pérez-Valera et al., 2019; Macedo et al., 2021), with these studies focusing mainly on the impact of bovine manure on these soils. However, long terms effects of manure application on the soil microbiome and resistome have also been observed (Xie et al., 2018; Zhang et al., 2018; Wang et al., 2020). For instance, Wang et al. (2020) identified that agricultural soil repeatedly manured for fifteen years with pig manure had increased diversity and abundance of ARGs and MGEs compared to non-manured soils, illustrating the cumulative and long term impact. Zhang et al. (2018) identified a legacy effect of manure land spreading on the soil microbiome thirteen years after halting manure land spreading. Additionally other factors such as soil type (Blau et al., 2018) and manure origin (Bicudo and Goyal, 2003; Singer et al., 2016) can have differential impacts on the microbiome and resistome. Therefore, a comparative analysis of the temporal impact of manure land spreading from various livestock sources requires concurrent spreading and investigation on the same field site in order to identify only the impact of the manure type on soil and grass.

Manure application can not only impact the soil but previous studies have demonstrated an increased ARG and MGE load on manured crops, such as lettuce (Blau et al., 2019; Sun et al., 2021), rice and wheat (Zhou et al., 2019). Research into the microbiome of the phyllosphere (aerial part of the plant) microbiome has recently identified its importance for plant growth. Globally, the total surface area of the phyllosphere is approximately twice that of land (Vorholt, 2012) and recent findings have shown that the phyllosphere can possess a diverse microbiome and resistome (Yan et al., 2019; Zhang et al., 2020; Sun et al., 2021). However, despite the identification of the phyllosphere as a reservoir of ARGs to date only one study has investigated the impact manure spreading has on the phyllosphere microbiome and resistome of grass; finding it to have a diverse resistome (Do et al., 2023). It has been shown that manure application can increase ARG abundance in rice, wheat and radish phyllospheres (Zhou et al., 2019), but data in other plants of agricultural importance is lacking. Bacteria from manure may be introduced to the aerial part of the plant through aerosols (such as contamination of herbage from splash plate manure spreading). Additionally, soil bacteria have been found to colonise and survive in plant roots (Bulgarelli et al., 2012) as well as colonise the phyllosphere (Grady et al., 2019; Massoni et al., 2021). Do et al. (2023) identified 98.7 % of bacterial genera of grass also in soil providing evidence for the overlapping of these microbiomes. Therefore, these could be potential transmission routes of bacteria, ARGs and MGEs from manure to grass.

Additionally, it has been found that individuals who are in frequent contact with agricultural animals, such as farm workers, are at a higher risk of acquiring ARB (Castillo Neyra et al., 2012). The dissemination of clinically relevant ARGs through horizontal gene transfer (HGT) and through clonal transmission between farm workers, animals and the farm environment has been documented (Deng et al., 2011; Hammerum et al., 2014; Dohmen et al., 2015; Sun et al., 2020). Therefore, as grassland is the dietary basis for grass fed livestock it is vital to understand the potential of the grass phyllosphere as a reservoir of ARB and ARG and the impact of manure spreading practices on the resistome and microbiome.

Of particular interest in this study is the impact of bovine, swine and poultry manure application on the microbiome and resistome of the grass phyllosphere, due to important role grass plays in pasture based livestock systems and in global food security (O'Mara, 2012). Animal husbandry practices and antibiotic usage can differ between livestock, therefore potentially altering the resistome composition of animal manures. Studies have shown varying ARG abundances and resistome composition between pig, cow and chicken manure as well as conflicting reports on their impact on soils (Peng et al., 2017; Zhang et al., 2017; Han et al., 2018; Buta-Hubeny et al., 2022). Additionally, bovine and poultry manure may affect the resistome of lettuce at different compartments of the plant: i) cattle manure impacts the resistome of root endophyte and ii) poultry manure affects the resistome of the rhizosphere, root endophyte and phyllosphere (Zhang et al., 2019). These findings highlight the importance to further investigate the differential impacts of manure sources on soil and crops.

In this study we hypothesise that the soil and grass phyllosphere will contain a diverse microbiome and resistome. Based on studies at the manure-soil interface, we also hypothesise a single manure spreading event will result on a short-term impact on the soil and grass microbiome and resistome. We aim to understand any correlations between members of the microbiome and detected ARGS and MGEs to elucidate the role of soil and the grass phyllosphere in the dissemination of AMR. Additionally, we aimed to further understand any temporal effects the application of bovine, poultry and swine manure have on the microbiome and resistome of agricultural soils.

2. Materials and methods

2.1. Antibiotic usage information of animals

Antibiotic usage data for the farms from which manure was collected for the field trials is described below. In this study, manure refers to both liquid waste as well as solid. The poultry farm was a layer farm and reported no antibiotic usage in the previous year. The swine manure was collected from weaner swine. These weaner swine were fed in-feed antibiotics that consisted of amoxicillin (β-lactam) and tilmicosin phosphate (macrolide) for a period of 10 days. Following this, the swines had medicated feed consisting of amoxicillin and zinc oxide for a period of 10–12 days. Thereafter, the swine had medicated feed supplemented with chlortetracycline hydrochloride (tetracycline) for a period of 8–10 days. The house from which swine manure was collected had a mixture of weaners from all stages of weaning, therefore the manure pit consisted of manure from all weaner stages. Dairy bovine manure was collected from a manure tank. Antibiotic usage data for these bovines was available during the period they were housed and contributing to the manure tank, from January 2019 – March 2019. No routine dosing was carried out during this time and animals were treated on an individual basis for digestive upset and mastitis. Antibiotics used during this time were as follows: amoxicillin (β-lactam), tylosin (macrolide), cefalexin monohydrate (β-lactam, cephalosporin), kanamycin monosuplhate (aminoglycoside) and marbofloxacin (fluoroquinolone).

2.2. Field trial layout and sampling regime

In the summer of 2019, a field trial was performed on a permanent grassland site in Johnstown Castle, Co. Wexford, Ireland (52.294117°N, -6.50102°W), under natural weather conditions (S1). The soil texture of the field was classified as a course loam texture. No animals had been grazed on the field for seven months prior to the trial. In the field, $1m^2$ plots were established in a randomised complete block design. Each plot had a 1.5 m buffer zone at each side to avoid cross contamination between treatments. Plots was designated one of four treatments: untreated (Control), swine manure (SM), bovine manure (BM) and poultry manure (PM) of which each treatment had four biological replicates. A diagram of the field map can be found in S2. Plots were destructively sampled.

Prior to swine, bovine and poultry manure spreading, each manure was mixed to ensure it was homogenous. Bovine and swine manures were spread by hand, mimicking splash plate spreading by using a modified watering can as described by Brennan et al. (2010). Splash plate spreading was chosen due to it being the most used method of bovine slurry application in Ireland (Buckley et al., 2020). Poultry manure, due to its pellet like consistency, was spread by hand evenly over each plot. The amount of manure spread onto each plot was calculated according to application rates outlined in the National Nitrates Action Programme (NAP) regulations (Teagasc Greenbook, 2016). Bovine manure was spread at an application rate of 34 t/ha, swine manure was applied at 40.5 t/ha and poultry manure was spread at an application rate of 15.5 t/ha.

Before manure spreading, grass on the trial area was trimmed to approximately 5 cm in length, to mimic grazing height. Additionally, prior to manure spreading background soil and grass samples were taken (Timepoint BG), as detailed in S3. Following manure spreading soil samples were collected at ten subsequent timepoints and grass samples taken at nine subsequent timepoints (S3). Further details of sampling techniques are described in the supplementary material (S4).

2.3. Sample processing

Sub-samples of manure and soil samples were collected and stored in 5 ml sterile tubes and stored at −80 °C for molecular analysis. Each grass sample (100 g) was placed into a sterile 500 ml centrifuge bottle containing 250 ml of sterile PBS (Gibco). The samples were then sonicated for 5 min to extract the grass microbial biofilm using a modified method based on the protocol by Joyce et al. (2018). Following sonication, the sonication liquid was passed through a sterile sieve to remove any large plant material. Approximately 240 ml of sonication liquid was immediately frozen at −80 °C for molecular analysis.

2.4. DNA extraction

DNA was extracted from three biological replicates for soil ($n = 164$) and manure ($n = 12$) samples for each treatment. DNA was extracted from 0.25 g of soil and 0.25 g of the manure using the DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions. Due to low DNA concentrations obtained from grass samples, the three biological replicates for each treatment at each timepoint were pooled to obtain composite samples with sufficient concentrations of DNA ($n = 37$). Composite grass samples were made by pooling 200 ml of sonication liquid from three biological replicates leading to a total of 600 ml of sonication liquid, which was filtered through a 0.2 μm sterile polycarbonate membranes (Whatman, USA). DNA extraction from the resulting filters was performed using the DNeasy PowerWater kit (Qiagen) according to the manufacturer's instructions. The purity of the extracted DNA was analysed using the NanoDrop spectrophotometer (DeNovix DS-11) and the concentration of the DNA was assessed using the Qubit Broad Range (BR) assay (ThermoFisher) and the Qubit 1.0 fluorometer (ThermoFisher).

2.5. Sample preparation for HT-qPCR analysis

Extracted and normalised DNA samples were sent to Resistomap Oy (Helsinki, Finland) for analysis using a HT-qPCR array. The HT-qPCR array analysed 216 genes, comprising 2 sets of primers for the 16S rRNA gene (AY1, AY600), 28 sets of primers for MGEs/integrons, 3 sets of primers for taxonomic genes and 183 sets of primers targeting ARGs (S5). These primers were validated in previous studies (Pitkänen et al., 2011; Tamminen et al., 2011; Looft et al., 2012; Zhu et al., 2013; Muziasari et al., 2014). DNA from composite grass samples (7 timepoints), soil samples (8 timepoints) and composite manure samples were analysed (S6). Two no template controls (NTC) were also included in the HT-qPCR array experiments.

2.6. Library preparation and sequence processing

16S rRNA amplicon sequencing was performed on all extracted DNA samples. In total there were three poultry manure, three swine manure, three bovine manure, 93 soil and 37 grass samples. A negative control sample (NTC) and mock community DNA Standard (ZymoBIOMICS) were included during each sequencing run as controls. Libraries were prepared using the Nextera XT Index Kit (Illumina). The sequencing error rate for Plate 1 was 1.76051×10^{-4} and Plate 2 was: 7.67098×10^{-5}. The V4 region of the 16S rRNA gene was amplified using the primer set 515F (GTGC CAGCMGCCGCGGTAA) - 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011). The prepared libraries were sent to the Teagasc Food Research Centre, Moorepark for 2×250 bp paired end sequencing, which was performed according to standard Illumina protocols on the Illumina Miseq V2 (500 cycles) (Illumina). Primers were removed from sequences using cutadapt using the Galaxy platform (Galaxy Version 1.16.5) (Martin, 2011). Sequences were processed using mothur on the Galaxy platform (Schloss et al., 2009; Hiltemann et al., 2018).

2.7. Quality control of 16S rRNA amplicon sequence data and data analysis

Following trimming there were a total number of 12,377,074 reads obtained from the samples and an average number of 73,237 reads per sample. Sequences were rarified to a depth of 30,000 reads resulted in the removal of 12 samples from the analysis, resulting in 157 samples remaining in the analysis. Microbiome data visualisation and statistical analysis was performed using R (v4.0.2) (RStudio Team, 2020). Briefly, 16S rRNA amplicon data was analysed for alpha and beta diversity and differential OTU abundances. Details of the microbiome data processing can be found in S7.

2.8. HT-qPCR data clean up and data processing

HT-qPCR data was analysed and visualised using RStudio (v4.0.2). Details of HT-qPCR data clean-up pre analysis is described in the supplementary material (S8) and filtered HT-qPCR data was processed by the Comparative Delta Delta Ct method (Schmittgen and Livak, 2008) (S8).

2.9. Correlation analysis

The mantel test using Spearmans rank correlations determined the relationship between the microbiome and resistome and were performed using the *mantel* function from the vegan package (v2.5-7) (Oksanen et al., 2020). For soil samples, the mantel test dissimilarity matrices were constructed from the average relative abundances of biological replicates as there were no biological replicates in the HT-qPCR array. Correlation analysis between the microbial communities and ARGs and ARGs and MGEs were calculated using Spearmans correlation with the SciPy package as described previously (Do et al. (2022). A strong correlation was indicated by Spearman's rank value $|r| > 0.7$ and $p < 0.05$ for all networks except for grass ARG-OTU network which used the parameters $|r| > 0.8$, $p < 0.05$.

3. Results

3.1. Diversity and composition of ARGs and MGEs detected across samples

In total products from 178 primer sets (160 genes) of the 214 primers used in the HT-qPCR array produced results. Products from 167 primer sets were detected in grass phyllosphere samples from 14 different gene classes and 85 in soil samples from 11 gene classes. The manure samples analysed resulted in 104 products in swine manure from 12 gene classes, 114 in bovine manure from 13 gene classes and 63 in poultry manure from 10 gene classes (Fig. 1).

In manure samples the gene classes detected were similar; with aminoglycoside, tetracycline and β-lactam resistance genes being the most commonly detected of the genes tested. Bovine manure contained the widest array of ARG classes ($n = 13$) and poultry manure had the lowest ($n = 10$) (Fig. 1). The manure amended soil and grass phyllosphere samples had higher numbers of genes than the background samples taken from the field before the field trial commenced (S.BG and G.BG) and the untreated control soil and grass samples (S·C and G.C). Only manure treated soil samples were positive for integrons (Fig. 1). Of the genes tested for in the HT-qPCR array, grass and soil overall had similar genes classes detected (Fig. 1).

Sample type (i.e. swine manure, bovine manure, poultry manure, grass or soil) was a significant factor in resistome composition accounting for 22 % of the variance in the data (PERMANOVA, $p < 0.05$, $R^2 = 0.22$, "permutest" ANOVA, $p < 0.05$, $F = 6.16$) (S9). Manure treatment was not a significant factor in the overall resistome composition for either grass or soil samples (Grass PERMANOVA, $p > 0.05$, $R^2 = 0.16$, "permutest", ANOVA, $p > 0.05$, $F = 0.67$) (Soil PERMANOVA, $p > 0.05$, $R^2 = 0.14$, "permutest" ANOVA, $p > 0.05$, $F = 2.9$).

In grass phyllosphere samples, treatment did not have a significant impact on MGE relative abundances (Kruskal-Wallis Test, $p > 0.05$) (Fig. 2-A). However it was found that grass control samples had significantly higher ARG relative abundance than poultry and bovine manured grass (Kruskal-Wallis Test, $p < 0.05$) (Fig. 2 – A) and that swine manured grass had significantly higher ARG relative abundance than bovine manured, poultry manured, and control grass phyllosphere samples (Kruskal-Wallis Test, $p < 0.05$) (Fig. 2 – A). At gene class level, swine manured grass had significantly higher relative abundance of β-lactamase genes in comparison to bovine manured grass and poultry manured grass (Fig. 2-B) and had a significantly higher relative abundances of aminoglycoside resistance genes in comparison to the poultry manure treated grass and bovine manure treated grass (Dunn Test $p < 0.05$), but not the control grass (Dunn Test $p > 0.05$) (Fig. 2-C). Control grass had higher β-lactamase and aminoglycoside resistance gene relative abundance than cow manured grass (Dunn Test $p < 0.05$). Swine manure also had higher abundances of sulphonamide resistance genes than control and poultry manured grass (Dunn Test $p < 0.05$) (Fig. 2-D). Swine manured grass had a higher level of (macrolide, lincosamide, streptogramin B) MLSB resistance genes than bovine and poultry grass samples but not control (Dunn Test $p < 0.05$) (Fig. 2 – E).

Treatment had no effect on the overall soil ARG relative abundances (Kruskal-Wallis Test, $p > 0.05$). However, bovine manured soil had significantly higher relative abundance of β-lactam resistance genes than swine manured soil (Dunn Test $p < 0.05$) (Fig. 3-A). Additionally, swine manured soil had a significantly higher relative abundance of sulphonamide resistance genes than poultry manured soil (Dunn Test $p < 0.05$) (Fig. 3-B).

3.2. Identification and tracking of manure associated genes

There were 24 genes shared across swine manure and swine manure treated grass (S10-A), 13 shared between bovine manure and bovine manure treated grass (S10—B) and 13 genes shared between poultry manure and poultry manured grass (S10—C). These genes were not found in untreated samples therefore they were determined to be of manure origin.

The abundance patterns of these manure originating genes were tracked across time to investigate their enrichment throughout the trial and at what point during the field trial they were no longer detected (Fig. 4-A). For grass phyllosphere samples the ARGs introduced from the manure onto the grass were no longer detected after 18 weeks (T9), with most not detected after 10 weeks post manure spreading (T7). The MGE gene, *tnpA* in bovine manured grass, was found to persist, and was detected 18 weeks following manure application (T9).

For swine manured soil there were 39 genes that were shared between swine treated soil and swine manure (S9 – D), nine shared between bovine manure and bovine manure treated soil (S9 – E) and nine genes shared between poultry manure and poultry manured soil (S9 – F). In soil samples treated with bovine or poultry manure, ARGs were no longer detected 10 weeks following manure application (T7). The MGE genes *intl1* and *tnpA* were detected consistently for 16 weeks (T7) and 18 weeks (T9), respectively, in soils after swine manure application (Fig. 4- B).

3.3. Alpha diversity of the microbiome

Manure treatment had no significant impact on Shannon or Chao1 alpha diversity measurements for grass phyllosphere and soil samples (Kruskal Wallis Test $p > 0.05$) (Fig. 5 A,B). Sample type had a significant

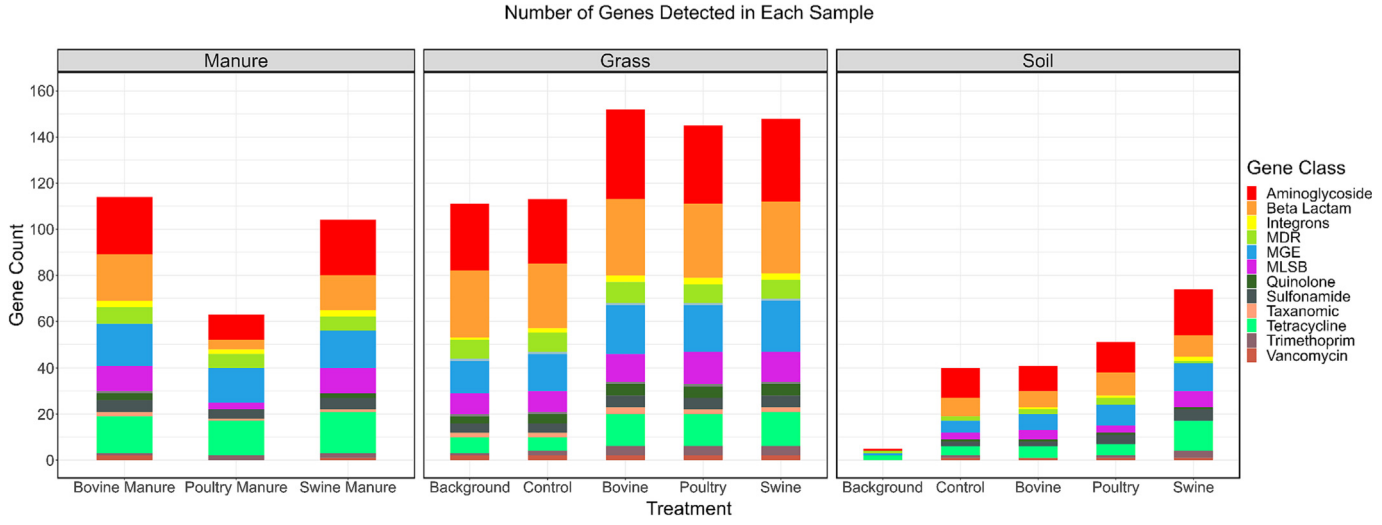

Fig. 1. Barplot displaying the number of target genes detected for each sample type and treatment in the HT-qPCR array. The treatment each sample received is explained on the x axis: Background = Background samples, Control = control samples, Bovine = bovine manure treated samples, Poultry = poultry manured treated samples, Swine = swine manure treated samples. Gene Class Abbreviations: MDR = multidrug resistance, MGE = mobile genetic element, MLSB = macrolide-lincosamide-streptogramin B.

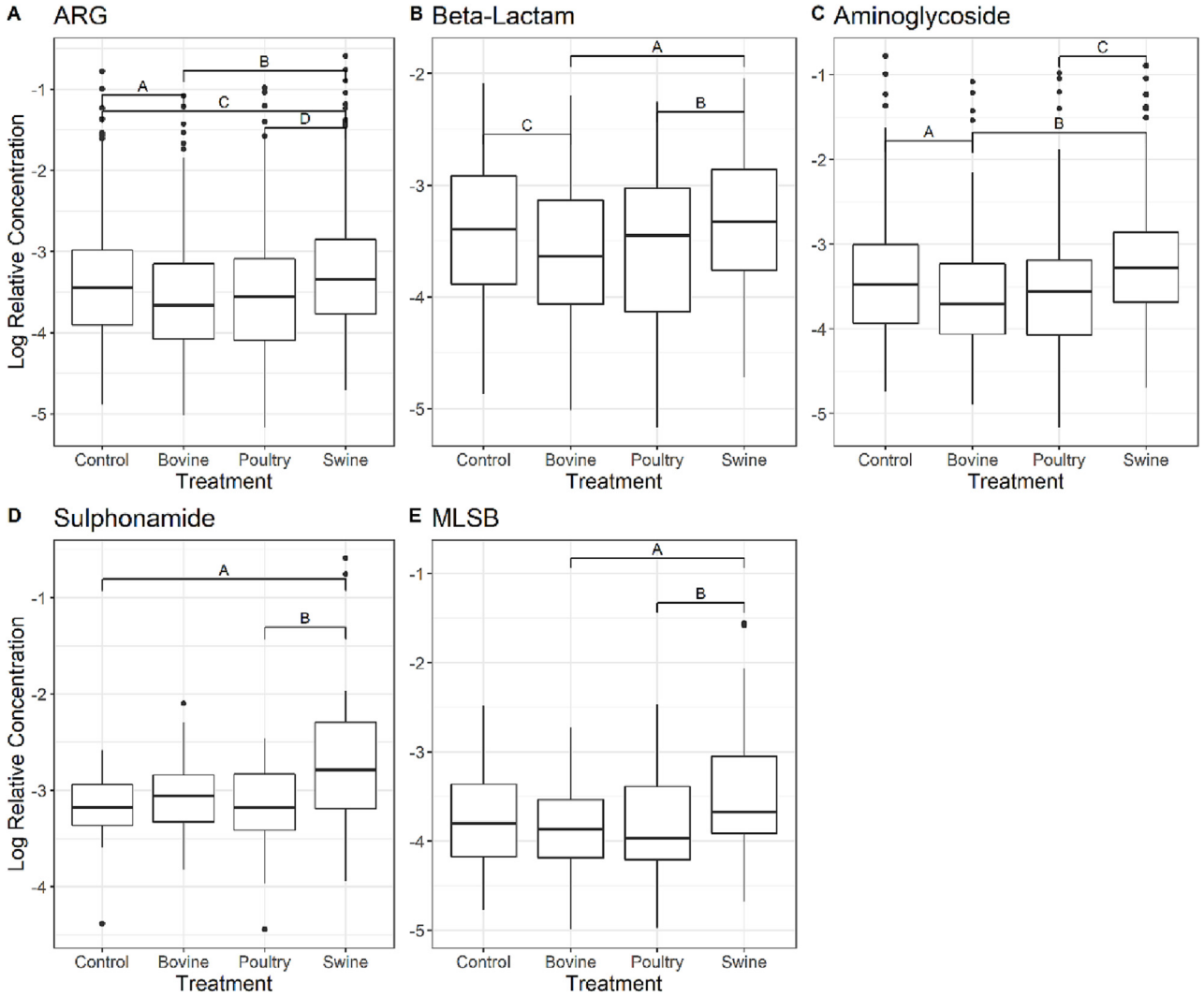

Fig. 2. Grass Samples - Bar charts illustrating the relative abundance of (A) ARGs (B) MGEs (C) β-lactam Resistance Genes (D) Aminoglycoside Resistance Genes (E) Sulphonamide Resistance Genes (F) MLSB Resistance Genes in Grass. The letters A,B,C and D indicate significance between groups ($p < 0.05$).

impact on both Shannon and Chao1 alpha diversity measurements with soil alpha diversity being significantly higher than bovine manure, swine manure and grass alpha diversities (Kruskal Wallis Test $p < 0.05$).

3.4. Beta-diversity of the microbiome

Sample type (Soil, Grass, Bovine Manure, Swine Manure and Poultry Manure) was found to have a significant impact on microbiome composition ($p < 0.05$) and accounted for 55 % of the variation in the data (PERMANOVA, $p < 0.05$, $R^2 = 0.55$). The permutest function to test for the non-homogenous dispersion of the data indicated a positive result ("permutest" ANOVA $p < 0.05$, $F = 50.021$), but as indicated by the separate clustering on the NMDS plot (Fig. 5), sample type had an obvious impact on the microbial composition.

Treatment had a significant impact on the soil microbial composition, however this only accounted for 4 % variance in the data (PERMANOVA, $p < 0.05$, $R^2 = 0.04$;"permutest" ANOVA $p > 0.05$, $F = 1.33$). Pairwise PERMANOVA testing of the treatments indicated that swine manured soil and poultry manured soil had significantly different microbial compositions than control soil (PERMANOVA $p < 0.05$). Additionally, poultry manured soil and swine manured soil had significantly different microbial compositions from each other (PERMANOVA $p < 0.05$). The combined effect of treatment and timepoint was significant, explaining 23 % of the variation in the data (PERMANOVA $p < 0.05$, $R^2 = 0.23$). Treatment was not found to have a significant impact on the grass phyllosphere microbiome composition (PERMANOVA $p > 0.05$, $R^2 = 0.14$; "permutest" ANOVA, $p > 0.05$, $F = 0.9$). Therefore overall, sample type was deemed to have an impact on the microbiome and manure treatment had a small yet significant impact on soil β-diversity but not on grass sample β-diversity.

3.5. Differential OTU testing using DESeq2

Soil resulted in a larger amount of differentially abundant OTUs than grass samples and all manure treatments in both grass and soil resulted in differentially abundant OTUs in comparison to the control grass and soil samples, respectively (S11 A-F). All manure treated grass and soil samples contained bacterial families that were found in high abundance in manure and in their respective treatments but were not detected or were present in low abundance in control grass. Additionally, these introduced or enriched members of the microbiota were found to be at low abundance or not detected by at least 10 weeks following manure application in grass (Fig. 6 A-C) and between week 10–14 post manure application in soil (Fig. 7 A-C).

3.6. Relationship of ARGs, MGEs and the bacterial community

The microbiome and resistome of grass phyllosphere samples were strongly correlated based on Bray-Curtis distances using the mantel test

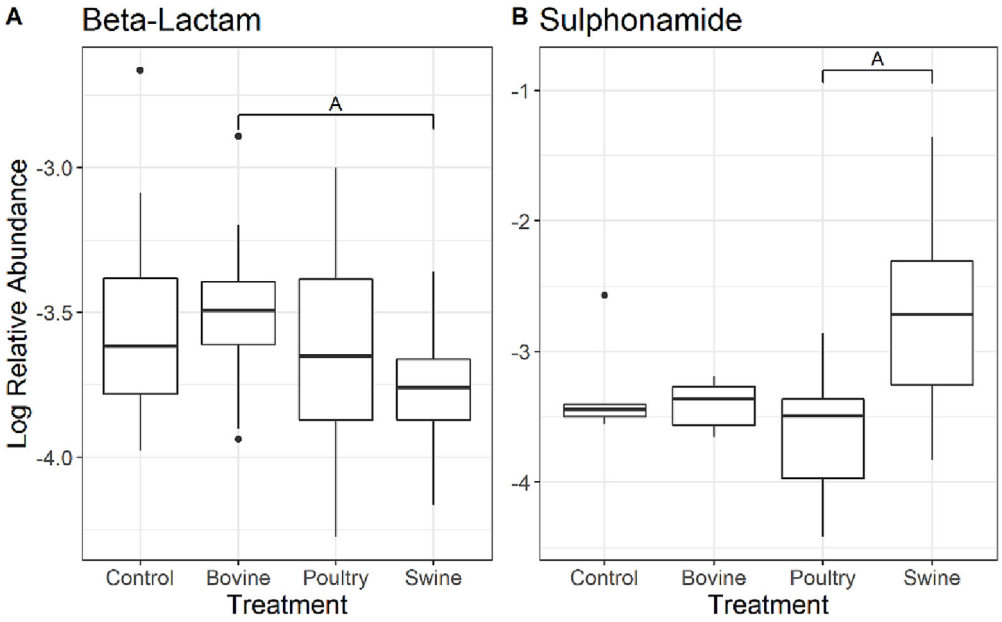

Fig. 3. Soil Samples - Boxplots illustrating the log relative abundance of (A) Beta Lactamase Genes (B) Sulphonamide Resistance Genes in Soil Samples. The letter A indicates significance between groups ($p < 0.05$).

(Spearman, $r = 0.59$, $p < 0.05$). The soil microbiome and resistome had weak but significant correlation (Spearman, $r = 0.2399$, $p < 0.05$).

3.6.1. Network analysis

3.6.1.1. Relationship between ARGs and MGEs in grass and soil. In both soil and grass MGEs play a key role in network formation illustrating the important role of HGT of ARGs in these environments. In the soil samples only positive interactions (red edges) were detected in strong correlation analysis ($|r| > 0.7$, $p < 0.05$) (S14). The network contained 55 nodes (42 ARGs and 13 MGEs) and 104 positive edges. In soil the MGEs, Tp614, IS613, TnpA and IS1133 displayed the strongest interactions with ARGs. ARGs from multiple clinically important gene classes were associated with MGEs such as resistance to aminoglycosides, sulphonamides, MLSB and tetracyclines. Additionally, the third-generation cephalosporin resistance gene, $bla_{\text{CTX-M}}$, was associated with the MGEs: IS1133, Incl1 and IncQ-oriT.

The grass phyllosphere network contained 103 nodes (consisting of 84 ARGs and 19 MGEs) and 189 edges (2 negative interactions and 187 positive interactions) (S15). The insertion sequence IS1133 had a strong interaction with multiple ARGs. Additionally, in the grass phyllosphere Incl1_repl1, IncN-rep, intl2 and orf37-IS26 played key roles in network

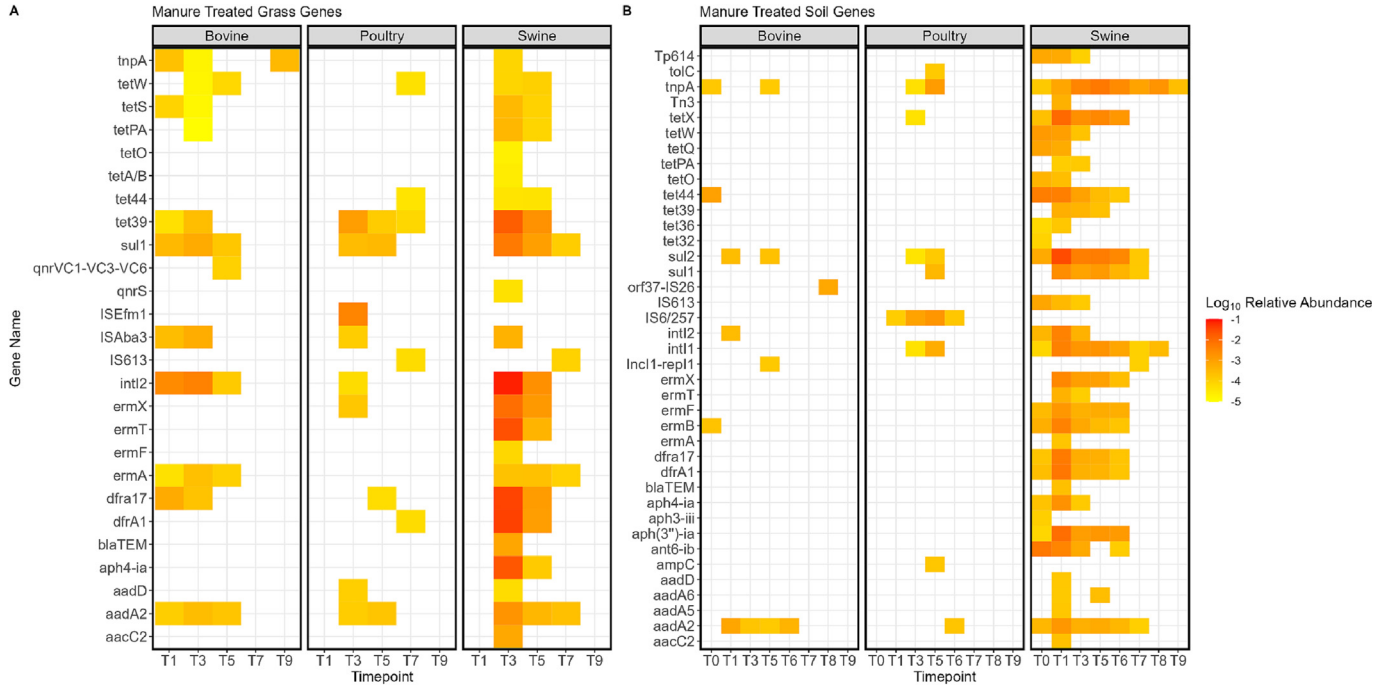

Fig. 4. Heatmap displaying the $\log_{10}$ relative abundances of the manure associated genes that were enriched in each treatment in grass (A) and soil (B) samples. Timepoints are indicated by codes: T1 = Week 1, T3 = Week 3, T5 = Week 5, T6 = Week 6, T7 = Week 10, T8 = Week 14, T9 = Week 18.

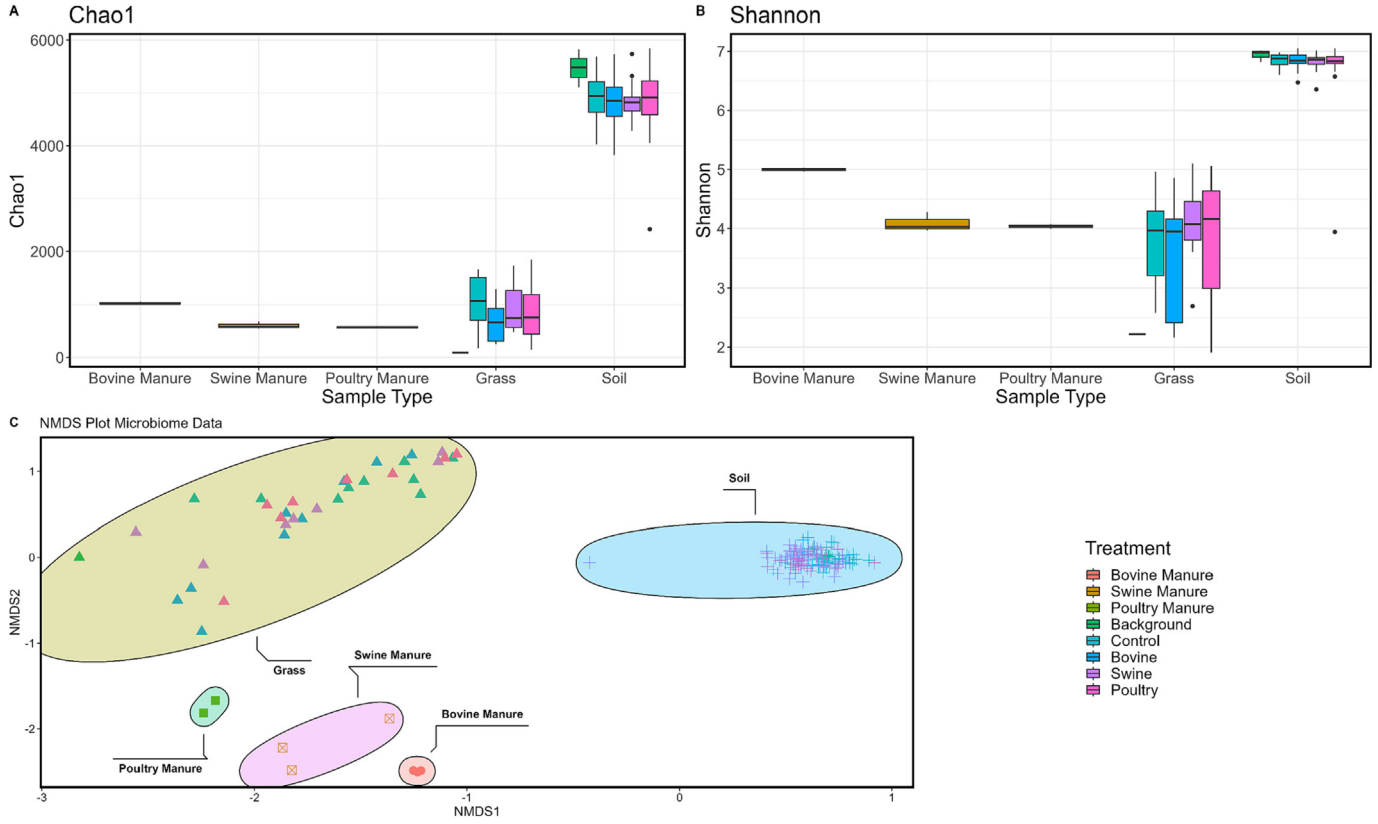

Fig. 5. (A) Alpha diversity using the Chao1 index (B) Alpha Diversity using the Shannon index. Sample codes are as follows: CM = bovine manure, SM = swine manure, PM = poultry manure (C) Non-metric multidimensional scaling (NMDS) ordination plot of 16S rRNA amplicon data. K = 2, stress value = 0.06485712 using Bray – Curtis distances.

formation. In grass phyllosphere networks, like soil networks, aminoglycoside and sulphonamide resistance genes were commonly associated with MGEs.

3.6.1.2. Relationship between ARGs and OTUs in grass and soil. Network analysis was conducted to investigate the relationship between ARGs and OTUs in soil (S16, S17) and grass (S18, S19) to predicate the potential microbial hosts of the ARGs in the environment or the close association of ARGs with specific bacteria. In both networks OTUs played a key role in network formation illustrating the predicted ARG primary hosts in these environments.

In soil the network consisted of 33 nodes (22 ARGs and 9 OTUs) and 65 positive edges. The bacterial families the *Flavobacteriacea* (OTU01246, OTU02552, OTU00967), *Alcaligenacea* (OTU01144) and *Pseudomonadaceae* (OTU00254) co-occurred most frequently with ARGs. These bacteria were associated with a myriad of resistance genes across the classes of tetracycline, sulphonamide and aminoglycoside resistance genes (S16, S17).

The grass phyllosphere networks consisted of 125 nodes (28 ARGs and 97 OTUs) and 124 edges (118 positive and 6 negative interactions). In grass samples, ARGs showed the central contribution with higher interaction compared with OTUs illustrating that these ARGs were harboured by multiple hosts. The metallo-β-lactamase bla_{IMP} was found to be highly associated with 55 OTUs across 24 families. Other clinically relevant antimicrobial resistance genes such as the cephalosporin resistance gene $bla_{\mathrm{CTX\text{-}M}}$ and the erythromycin resistance gene *ermB* were found to be associated with multiple OTUs across 5 and 7 families, respectively. Similar to soil, grass networks showed significant interactions with ARGs associated with aminoglycoside, sulphonamide and tetracycline resistance (S19).

4. Discussion

4.1. Resistome composition of samples

Due to its importance as a food source for livestock as well as its physical contact with livestock, the impact manure spreading has on the grass phyllosphere was of particular interest in this study. Manure, soil, and grass phyllosphere samples all contained a diverse range of clinically relevant ARGs. The resistome composition of pig, cow and chicken manure was found to be largely similar and was consisting of aminoglycoside and tetracyline resistance genes which has been previously reported (Muurinen et al., 2017; Zhang et al., 2020; Qiu et al., 2022). Grass had 167 genes across 14 gene classes identified; the highest diversity of genes detected. Similarly, both Yan et al. (2019) and (Do et al., 2023) identified the grass phyllosphere to be rich in ARGs.

4.2. Effect of manure landspreading on grassland resistome

Manure land spreading has been shown to alter the resistome of agricultural soils by the introduction of manure derived ARGs and MGEs and by enriching the natural resistome for indigenous ARGs (Chen et al., 2017; Gou et al., 2018; Wang et al., 2020). For the grass phyllosphere in particular this aspect of the study was vital as there is, to date, little data regarding the impact agricultural practices, such as manure application, have on the grass phyllosphere microbiome and resistome.

In this study, the grass phyllosphere and soil resistome were disturbed due to the direct introduction of manure originating ARGs and MGEs as a result of manure application. Manure treatment had no significant impact on overall soil ARG relative abundance in manure treated samples when compared to control soil samples or on the overall resistome composition,

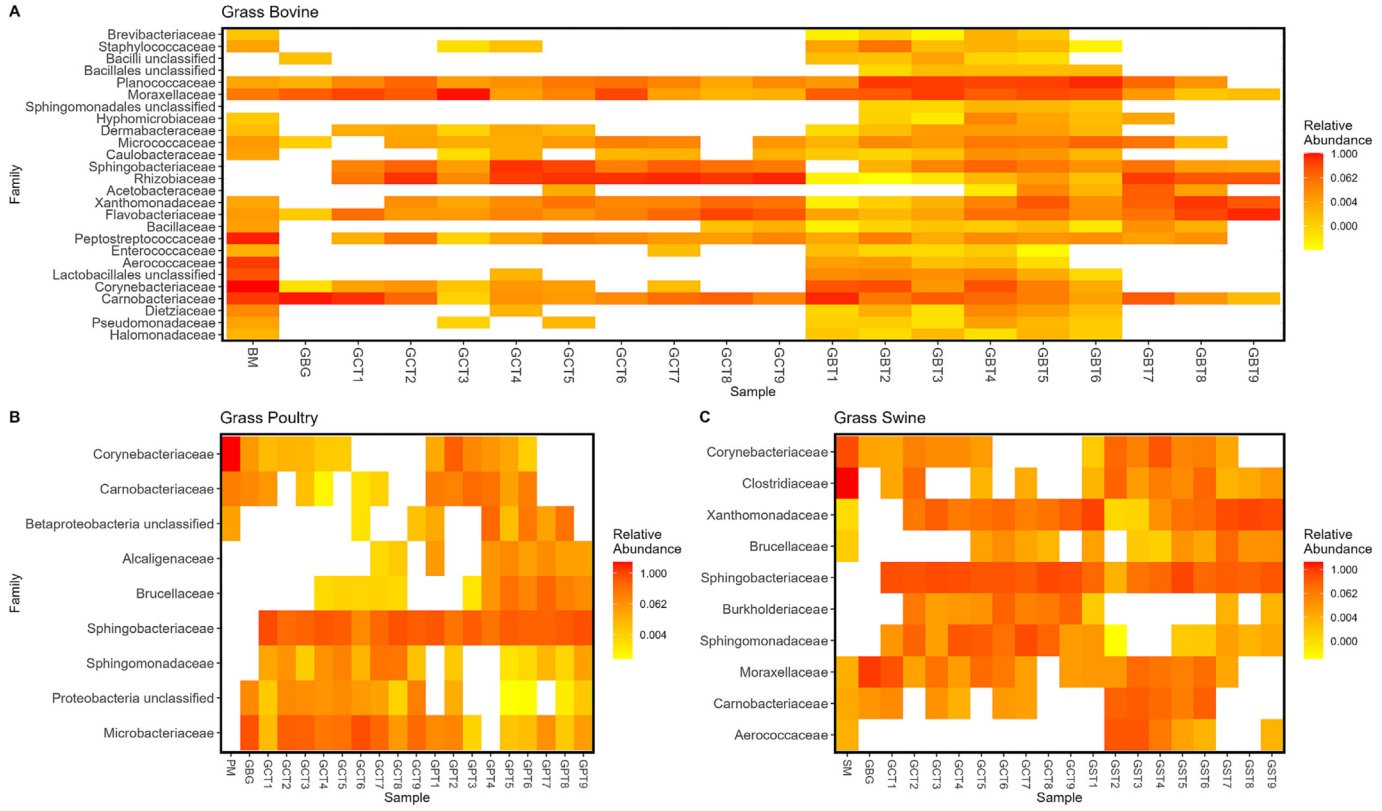

Fig. 6. Heatmaps of the relative abundances of differentially abundant OTUs determined by DESeq2 analysis ($p < 0.01$) in (A) Bovine manure amended grass (B) Poultry manure amended grass (C) Swine manure amended grass. Sample codes indicate treatment (GC = grass control, GB = grass amended with bovine manure, GP = grass amended with poultry manure and GS = grass amended with swine manure). Timepoints are indicated by codes (BG = background, T1 = Timepoint1, T2 = Timepoint 2, T3 = Timepoint 3, T4 = Timepoint 4, T5 = Timepoint 5, T6 = Timepoint 6, T7 = Timepoint 7, T8 = Timepoint 8, T9 = Timepoint 9.)

which is in contrast to data from other studies of soil and the episphere of other plants (Han et al., 2018; Zhang et al., 2019; Liu et al., 2021; Sun et al., 2021). Manured soil and manured grass phyllosphere samples both had a wider range of ARGs detected than non-manured comparators, which has previously been reported only for soil (Wang et al., 2020; Liu et al., 2021) but to date, not for the grass phyllosphere. Manure application was found to introduce ARGs and MGEs into soil and grass phyllosphere, with swine manure introducing the greatest number of genes into soil and grass in comparison to the bovine and poultry manure. Genes conferring resistance to antibiotic classes considered highly or critically important, such as sulphonamide, tetracycline and macrolide resistance genes were found to be introduced by manure application to the soil (WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), 2019). The enrichment of soil for sulphonamide, tetracycline and macrolide resistance genes has been reported previously (Ruuskanen et al., 2016; Lopatto et al., 2019; Macedo et al., 2020).

A differential impact was found in terms of overall ARG abundance in the grass phyllosphere, with swine manured grass harbouring higher ARG abundance than control grass phyllosphere samples, bovine manured or poultry manured grass. Swine manured grass contained higher relative abundances of β-lactam, MLSB and aminoglycoside resistance genes compared to other manure amended grass samples and higher sulphonamide resistance genes compared to control grass. This difference may correspond to the β-lactam and macrolide use in the swine from which manure was collected.

In soil samples, integrons were detected only in manured soil and manures, illustrating how the application of manure can introduce genes associated with ARG mobilisation into agricultural grassland. An increase in MGEs due to manure application in soil has been previously reported (Nõlvak et al., 2016; Han et al., 2018; Wolters et al., 2018), but to date, not in the grass phyllosphere resistome. However, manure application has been found to increase the abundance of ARGs and MGEs in other crops (Blau et al., 2019; Zhang et al., 2019; Zhou et al., 2019; Sun et al., 2021) and notably in the phyllosphere resistome of lettuce (Zhang et al., 2019) and the mobilome of rice and wheat (Zhou et al., 2019). The increase and identification of genes associated with mobilisation indicates that manured soil and grass may act as hotspots for HGT, resulting in increased dissemination of ARGs and also their persistence in agricultural land due to their possible integration into indigenous members of the environmental microbiome (Heuer et al., 2011).

4.3. Effect of manure landspreading on grassland microbiome

Similar to the impact manure land spreading has on the resistome of agricultural land, manure can alter the environmental microbiome through the introduction of bacteria from the manure (Lopatto et al., 2019) and also through the enrichment of the natural bacterial microbiota (Udikovic-Kolic et al., 2014). Manure treatment has been shown to affect the soil bacterial community composition (Udikovic-Kolic et al., 2014; Wang et al., 2020; Sun et al., 2021). Through NMDS plots and PERMANOVA testing, manure treatment was found to have a small yet significant effect on the soil β-diversity, but this was not the same for grass. Swine manured soil and poultry manured soil microbiomes were significantly different to control samples, whereas bovine manured soils did not significantly differ from control soils. Despite manure treatment not having an overall significant impact on the grass β-diversity, both grass and soil samples contained OTUs that differed in abundance between manure treated samples and control samples. DESeq2 analysis revealed that bovine manured grass resulted in the greatest number of differentially abundant bacteria, while in soil swine manure had the greatest impact. Heatmap construction of the differentially abundant families found that manured grass and soil samples

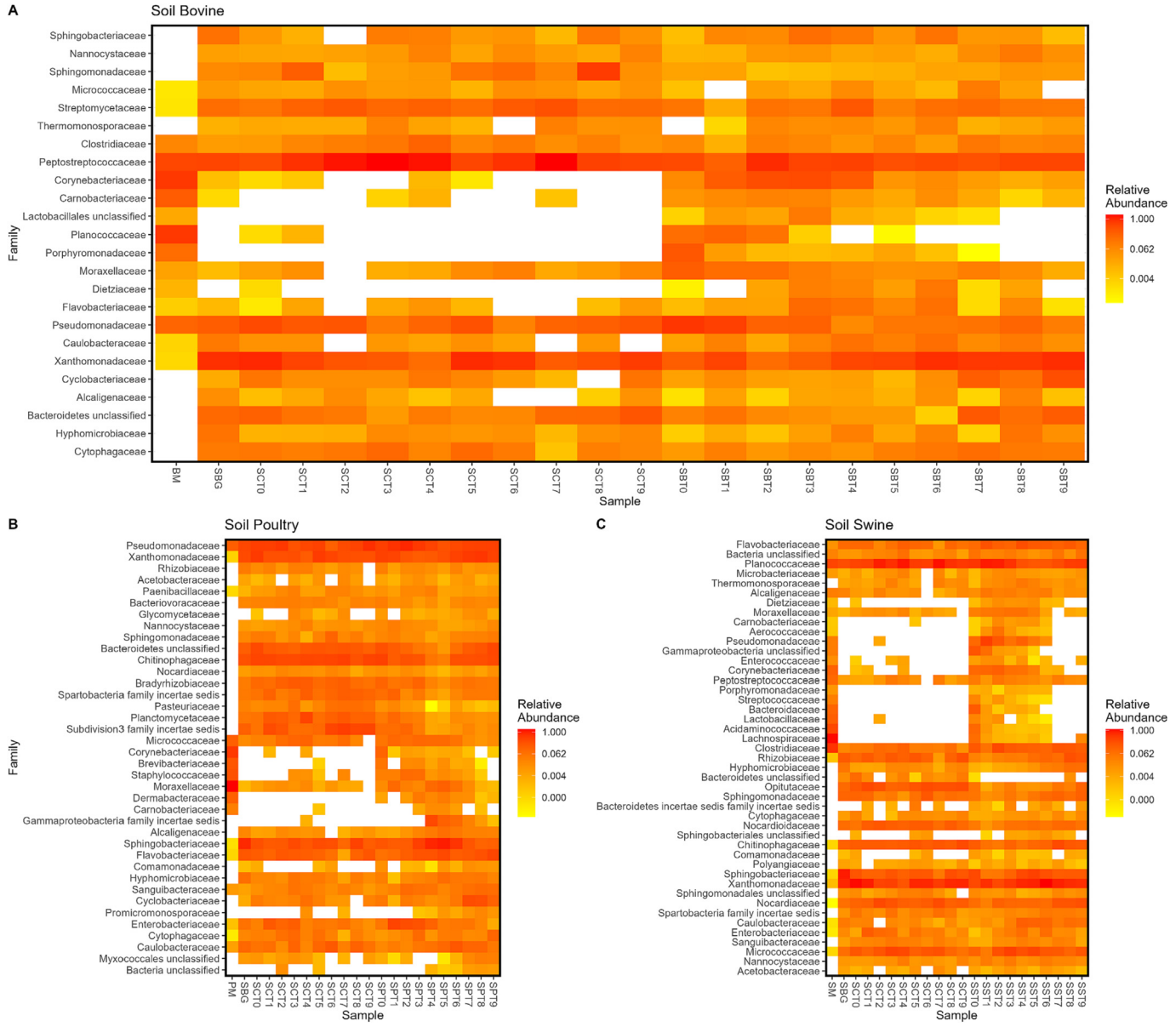

Fig. 7. Heatmaps of the relative abundances of differentially abundant OTUs determined by DESeq2 analysis ($p < 0.01$) in (A) Bovine manure amended soil(B) Poultry manure amended soil (C) Swine manure amended soil. Sample codes indicate treatment (SC = soil control, SB = soil amended with bovine manure, SP = soil amended with poultry manure and SS = soil amended with swine manure). Timepoints are indicated by codes (BG = background, T1 = Timepoint1, T2 = Timepoint 2, T3 = Timepoint 3, T4 = Timepoint 4, T5 = Timepoint 5, T6 = Timepoint 6, T7 = Timepoint 7, T8 = Timepoint 8, T9 = Timepoint 9.

had enriched bacterial families that were in high abundance in manure samples and either not detected or detected in low abundance in control samples, illustrating that manure alters the grassland microbiota through the direct introduction of bacteria from manure to grassland, in addition to the enrichment of the indigenous microbiota. This enrichment of the natural microbiome is attributed to the addition of nutrients from manure to the soil (Udikovic-Kolic et al., 2014). In both grass and soil manure application resulted in an increase of both plant and soil commensal bacteria and also of some families that can be potential pathogens to humans such as *Enterococcaceae, Brucellaceae, Staphylococcaceae, Enterobacteriaceae, and to plants: Xanthomondaceae.* This increase in opportunistic pathogens in manured soil has been previously reported by Ding et al. (2014) who reported increases in *Stenotrophomonas* spp. and *Clostridium* spp. However, deeper sequencing to understand these patterns at genus level would be required to adequately assess these patterns.

4.4. Co-occurrence of OTUS, ARGs and MGEs

The microbiome and resistome of both grass and soil were correlated and network analysis revealed that OTUs and ARGs were correlated in both grass and soil. The bacterial families *Flavobacteriacea, Alcaligenacea* and the *Pseudomonadaceae* were highly associated with a wide variety of ARGs in soil. The identification of *Pseudomonadaceae as ARG hosts has been identified previously in rice and wheat* (Zhou et al., 2019). Additionally, in both grass and soil MGEs played a key role in network formation, highlighting the role HGT plays in the dissemination of ARGs in agricultural land. Antimicrobial resistance genes conferring resistance to the WHO listed critically important antimicrobials such as the aminoglycosides, third generation cephalosporins and macrolides, were associated with MGEs and OTUs in both grass and soil. These results therefore illustrate the important role grass and soil

play in the dispersal and maintenance of clinically relevant antimicrobial resistance in the agricultural environment.

4.5. The temporal impact of manure landspreading on the grassland resistome and microbiome

The impact that manure application has is thought to be short term; there have been conflicting reports however regarding the exact time it takes for the soil to return to a pre-manured state (Fahrenfeld et al., 2014; Chen et al., 2017; Muurinen et al., 2017; Lima et al., 2020; Macedo et al., 2021). To investigate the temporal effects of manure, soil and grass were analysed for 18 weeks post manure application and compared with non-manured soil and grass over the same timeframe.

The temporal trends of manure application on both the resistome and microbiome were similar. Manure application resulted in a short-term impact on the soil and grass resistome, as most of the manure introduced genes were no longer detected ten weeks following manure application with the exception of the MGE genes *tnpA* in bovine manured grass, and *intl13* and *tnpA* in swine manure amended soils which were detected between ten and 18 weeks following manure application. Similarly, most manure associated bacteria were no longer detected 10 weeks post manure application. Additionally, some OTUs were present in both manure treated and control samples and were therefore associated with the indigenous microbiota were detected at elevated relative abundances at the end of the trial – 18 weeks post manure application. This similarity in temporal trends between the microbiome and resistome indicate that the change in the resistome is possibly due to the die off of the manure associated bacteria that may be hosting these ARGs and MGEs. This supports previous studies which showed that the impact manure land spreading has on the soil microbiome is short term due to manure originating bacteria not being well adapted to the soil and therefore being outcompeted by the indigenous microbiome (Chen et al., 2017; Muurinen et al., 2017; Gou et al., 2018; Pérez-Valera et al., 2019). Additionally, these results demonstrate the ability of manure application to alter the grass phyllosphere microbiome.

Manure appeared to have a more pronounced impact on the soil microbiome than grass, with more differentially abundant OTUs identified in manure treated soil samples than grass samples. The reduced temporal effect of manure treatment on the grass phyllosphere samples in comparison to soil may reflect the dynamic and complex nature of the phyllosphere environment due to its exposure to both abiotic and biotic factors, such as UV radiation, temperature fluxes and invading plant pathogens (Lindow and Brandl, 2003; Compant et al., 2019; Sivakumar et al., 2020). Other studies investigating the time dependent impact of manure estimated that the temporal effect of manure land spreading lasts for approximately two months in soil (Fahrenfeld et al., 2014; Muurinen et al., 2017; Gou et al., 2018), which correspond with the results of this study.

Following manure land spreading there is often an exclusion period of the land to minimise the risk of grazing animals ingesting manure contaminated herbage. This land exclusion period has varying recommendations, ranging from 3 weeks (Tucker, 2015) up to 8 weeks (ADAS, 2001) and is dependent on a multitude of factors such as the manure type and if the manure has been pre-treated such as by composting (CRÉ- Compost Ireland, 2007) or digestion (Nolan et al., 2020). In Ireland, a 6-week exclusion period is often recommended for splash plate manure spreading, which was used in this study. Resistome data showed that 10 weeks post manure application most manure associated ARGs were found in low relative abundance or below the detection limit. Manure application resulted in indigenous microbiota and manure associated OTUs being detected in manure treated samples past the 6-week exclusion period. However, these were in low relative abundance. Therefore, this supports the current manure landspreading guidelines in mitigating the risk to livestock. In terms of the comparative risk associated with swine, bovine and poultry manure, all manure types altered the microbiome and resistome of soil and the grass phyllosphere, however all were found to not have a minimal impact on soil and the grass phyllosphere. Further research is required to examine how variables such as manure storage, farming practices and antimicrobial usage in livestock may contribute to differing exclusion times for different manure types.

5. Conclusion

This study demonstrates the impact manure application from different livestock sources has over time on the microbiome and resistome of soil and the grass phyllosphere. The findings highlight the role of the grass phyllosphere as a diverse reservoir of MGEs and ARGs conferring resistance to clinically important antimicrobials. The presence of clinically important ARGs in grassland may lead to their transfer to livestock through ingestion or through direct contact. Therefore, the potential rate of transfer between grass ARGs and bacteria to livestock needs be assessed to fully elucidate the role the grass phyllosphere plays in the dissemination of antimicrobial resistance. Despite the lack of overall significant effect of manure application on soil resistome and grass microbiome compositions, and its small effect on soil microbial composition, manure both introduced and enriched the ARGs, MGEs and members of the bacterial microbiome in soil and grass. Manure application resulted in short term alteration of the grass and soil microbiomes and resistomes at OTU and gene level, respectively. Key aspects of this study were the temporal and differential impact of bovine, swine, and poultry manure on the microbiome and resistome of grass and soil. Overall, manure landspreading had minimal impact on the overall compositions of the grass resistome and microbiome, and the soil resistome. Additionally, the impact of manure landspreading at OTU, ARG and MGE level was short term and therefore aligned with recommended agricultural guidelines. This data highlights the need to fully elucidate the risk and role of the phyllosphere in the maintenance and dissemination of antimicrobial resistance on plants and their potential transfer to other organisms.

CRediT authorship contribution statement

Ciara Tyrrell: Investigation, Formal analysis, Visualization, Writing – original draft. **Thi Thuy Do:** Software, Formal analysis, Visualization, Writing – review & editing. **Robert J. Leigh:** Software, Formal analysis. **Catherine M. Burgess:** Conceptualization, Writing – review & editing, Project administration, Supervision, Funding acquisition. **Fiona P. Brennan:** Conceptualization, Writing – review & editing, Project administration, Supervision, Funding acquisition. **Fiona Walsh:** Conceptualization, Writing – review & editing, Project administration, Supervision, Funding acquisition.

Data availability

The sequencing data has been submitted in the NCBI Sequencing Read Archive (SRA) under the bioproject PRJNA929919. HT-qPCR data can be found at https://github.com/CiaraT/Grassland_ARG.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.163926.

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