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Research Article

Soil-specific responses in the antibiotic resistome of culturable *Acinetobacter* spp. and other non-fermentative Gram-negative bacteria following experimental manure application

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Abstract

Acinetobacter spp. and other non-fermenting Gram-negative bacteria (NFGNB) represent an important group of opportunistic pathogens due to their propensity for multiple, intrinsic, or acquired antimicrobial resistance (AMR). Antimicrobial resistant bacteria and their genes can spread to the environment through livestock manure. This study investigated the effects of fresh manure from dairy cows under antibiotic prophylaxis on the antibiotic resistome and AMR hosts in microcosms using pasture soil. We specifically focused on culturable *Acinetobacter* spp. and other NFGNB using CHROMagar Acinetobacter. We conducted two 28-days incubation experiments to simulate natural deposition of fresh manure on pasture soil and evaluated the effects on antibiotic resistance genes (ARGs) and bacterial hosts through shotgun metagenomics. We found that manure application altered the abundance and composition of ARGs and their bacterial hosts, and that the effects depended on the soil source. Manure enriched the antibiotic resistome of bacteria only in the soil where native bacteria had a low abundance of ARGs. Our study highlights the role of native soil bacteria in modulating the consequences of manure deposition on soil and confirms the potential of culturable *Acinetobacter* spp. and other NFGNB to accumulate AMR in pasture soil receiving fresh manure.

Keywords: antibiotic resistance; cattle manure; opportunistic pathogens; pasture soil; *Pseudomonas*; tetracycline resistance

Introduction

Antimicrobial resistance (AMR) represents a global threat to both human and animal health. The widespread use of antibiotics in human and veterinary medicine has accelerated the emergence of antibiotic-resistant bacteria (Dunlop et al. 1998), which accounted for 1.27 million deaths worldwide in 2019 (Murray et al. 2022). Studying the biological and ecological processes, as well as the routes by which AMR can spread in the environment is important to better understanding the potential risk of human activities that contribute to the dissemination of AMR (Ashbolt et al. 2013).

Manure from farms that use antibiotics is a major route through which active substances from antibiotic residues and health-risk bacteria enter the soil (Semenov et al. 2010, Black et al. 2021, Köninger et al. 2021). The widespread use of antibiotics in farms has created selective pressure for antibiotic resistance (Hart et al. 2006, Wichmann et al. 2014), which can transfer and persist in farm animals and their surroundings (Kyselková et al. 2015). Indeed, manure is one of the main sources contributing to the enrichment of soil resistome with antibiotic resistance genes (ARGs) (Kyselková et al. 2015, Lima et al. 2020, Marutescu et al. 2022). Nevertheless, manure is also a necessary part of agricultural practice (Köninger et al. 2021), including pasture management (Yang et al. 2020), and therefore, more detailed information about manure-borne microbes that may pose a potential health risk is needed.

Non-fermenting Gram-negative bacteria (NFGNB) such as *Acinetobacter* spp. have raised concern because of their role in AMR and as critical healthcare-associated pathogens (Bonomo and Szabo 2006, McGowan 2006). Although *Acinetobacter* spp. and other NFGNB are ubiquitous, particularly in soil, water, and animal gut, they frequently exhibit intrinsic resistance to a broad group of antibiotics (Gales et al. 2001). In addition, they can easily acquire further resistance to antibiotics (Bonomo and Szabo 2006, Enoch et al. 2007), which has turned them into pathogens of great interest and importance in the clinic (Mulani et al. 2019). *Acinetobacter* spp. and other NFGNB are resistant to a wide range of antibiotics, including tetracycline, which is one of the most used classes of antibiotics for human and animal treatment (McGowan 2006). Due to high usage in veterinary medicine, agriculture, and aquaculture, contamination with tetracycline resistance genes can be widespread in agricultural soils (Kyselková et al. 2015, Grossman 2016). Indeed, we have previously shown that fresh manure from tetracycline-treated cattle enriched the soil with tetracycline resistance genes that remained in the soil under experimental

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conditions for at least three months (Pérez-Valera et al. 2019). The role of *Acinetobacter* spp. in the spread of tetracycline resistance genes in soil has also been suggested (Kyselková et al. 2016, Leclercq et al. 2016, Pérez-Valera et al. 2019). The fact that *Acinetobacter* spp. can thrive in both soil and manure in the first days after application (Leclercq et al. 2016, Pérez-Valera et al. 2022) may suggest that *Acinetobacter* spp. is one of the major players in the spread of AMR and tetracycline resistance genes in agricultural soils. However, there is a lack of information on whether the increase of *Acinetobacter* spp. and other bacteria in the soil after manure application also contributes to an increase and further persistence of AMR and tetracycline resistance genes in the soil.

In this study, we investigated the role of fresh cattle manure in the spread of NFGNB and associated AMR in pasture soil by focusing on the tetracycline resistome of *Acinetobacter* spp. under laboratory conditions. We analyzed the antibiotic resistome and bacterial hosts through shotgun metagenomic sequencing of CHROMagar Acinetobacter cultures from a previously published microcosm experiment (Pérez-Valera et al. 2022), in which fresh cattle manure from animals under tetracycline prophylaxis was applied to the pasture soil from two organic farms. Together with the microcosms that contained pasture soil treated with manure, we used identical microcosms that consisted of either manure or soil alone, as controls, to specifically investigate (i) the taxonomic composition of bacterial hosts of ARGs *vs* all culturable bacteria in CHROMagar Acinetobacter from pasture soil microcosms in close contact with fresh manure, (ii) the composition and relative abundance of ARGs and tetracycline resistance genes (i.e. the resistome profile), and (iii) the origin of ARGs and tetracycline resistance genes (i.e. whether chromosomal or plasmid). We hypothesized that an increase in the relative abundance of *Acinetobacter* spp. in pasture soil microcosms treated with fresh manure would result in an enrichment of the antibiotic resistome and changes in the composition of both ARGs and tetracycline resistance genes of culturable bacteria from soil on CHROMagar Acinetobacter.

Materials and methods

Microcosm set-up and sampling of soil and manure

A microcosm experiment to simulate the natural deposition of fresh manure on pasture soil was used in our study, as described in Pérez-Valera et al. (2022). Briefly, experimental plastic pots of around 300 ml were initially filled with soil (ca. 120 g), and fresh manure was deposited on top (ca. 100 g). Soil and manure were horizontally delineated with a sterile plastic mesh (1.4 mm). In addition, we used a mesh to delineate a soil layer horizontally affected by manure (ca. 60 g, hereafter referred to as "treated soil") to ensure that the soil was sampled at a constant distance from the manure. Microcosms containing only soil or manure (representing control soil or manure, respectively) were set up similarly to compare with microorganisms naturally occurring in manure and soil and to account for differences in the bacterial composition due to differential incubation times. In contrast to the experiment described by Pérez-Valera et al. (2022), only non-gamma-irradiated soil treatments were used in this study.

The soil used for the microcosm experiments was sampled in September 2018 from two organic cattle farms (S and B) in the Czech Republic (ca. 48°North, 14°East), as described in our previous study (Pérez-Valera et al. 2022). The organic farms were selected to avoid confounding effects of antibiotic exposure through the farming process. The farms are about 500 m apart and subjected to similar grassland management, but the soils have different characteristics (Pérez-Valera et al. 2022). At each farm, using plots of 1×1 m along a linear transect (200 m), a soil sample composite of ten sub-samples (5–15 cm) was collected and transported on ice to the laboratory. The soil was stored at 4°C until setting up the experiment. Four days before starting the experiment, soil samples were pre-incubated at 20°C in the dark.

Fresh livestock excrement (hereinafter "fresh manure") was collected from a private dairy farm in the Czech Republic (ca. 48°North, 14°East), where the animals were under antibiotic prophylaxis and treatment with chlortetracycline and amoxicillin (Kyselková et al. 2016). We used only fresh manure collected directly from a private farm with the permission of the farm's owners and veterinarian, using standard procedures at the farm that meant no impact to the animals. The information about the farm management, cow gut bacterial community, and resistome has been previously described (Kyselková et al. 2015). Fresh manure from 20 adult animals (3–7 years old) was sampled aseptically, as described elsewhere (Kyselková et al. 2015, Pérez-Valera et al. 2019), and pooled into one composite sample. Fresh manure was sampled on the same day of setting up the microcosms and taken to the laboratory for immediate use.

Experimental design and microcosms sampling

Two independent experiments using fresh manure and soil from farm S (experiment S) and farm B (experiment B) were set up in September and October 2018, respectively, as described in Pérez-Valera et al. (2022) (Fig. S1). Briefly, for each experiment, three microcosm replicates per treatment (i.e. treated soil, control manure and control soil) and time point were set up (i.e. 2 experiments × 3 treatments × 3-time points × 3 replicates = 54 samples) and destructively sampled after incubation at different times. In this study, we focused specifically on samples incubated for 2, 14, and 28 days, based on the short-term dominance of *Acinetobacter* spp. in microcosms analyzed via 16S rRNA amplicon sequencing in a previous study (Pérez-Valera et al. 2022). We also considered the short-term ARG persistence in manure-treated soil, as previously reported (Leclercq et al. 2016, Pérez-Valera et al. 2019), as well as the detection of the tetracycline resistance gene *tet*(Y) in manure samples through a preliminary test (data not shown). In contrast to our previous study, in which 16S rRNA amplicons were analyzed to study bacterial community composition, this study is based entirely on shotgun metagenomic sequencing results from cultured bacteria (see below).

During sampling, treated soil was thoroughly separated from the top and bottom layers, homogenized, and subdivided into aliquots for downstream analyses. Control soil and control manure microcosms were similarly sampled. A perforated lid was used to cover the microcosms, allowing aeration. Following standard methods, microcosms were incubated at a constant 20°C in the dark. Water was not restituted throughout the experiment. The water content of the microcosms in both experiments at each sampling time is summarized in Table S1.

Cultivation of the microbial community

Acinetobacter spp. and other non-fermenting Gram-negative bacteria (NFGNB) were analyzed via cultivation on CHROMagar Acinetobacter (CHROMagar, Paris, France). Although this medium is specifically designed for the detection of *Acinetobacter* spp. in healthcare settings (Leaflet: CHROMagar Acinetobacter), it may also allow the growth of other non-fermenting Gram-negative bacterial taxa, such as *Pseudomonas* spp. and *Stenotrophomonas*

spp., that cannot utilize lactose as a carbon source, especially in environmental samples (Hrenovic et al. 2019). The inoculum was prepared from each microcosm as follows: 5 g of either treated soil, control soil or control manure were placed in flasks containing 45 ml of sterile 0.9% NaCl. Flasks were sonicated for 2 min preceded by a 30 s vortex. One hundred microliters of serial 1/10 dilutions (up to 10^{-5}) were inoculated on CHROMagar Acinetobacter agar plates (in duplicates). After incubating the plates at 28°C for 24 h, total and red colony-forming units (CFUs) were estimated by visual examination of the plates (Tables S2 and S3). The incubation temperature of plates (i.e. 28ºC) was chosen based on our previous study (Pérez-Valera et al. 2019) and is at the upper limit of the temperature optimum for mesophilic soil bacteria (Alexander 1977). Microbial biomass was harvested immediately after counting. No antibiotic was used for selection purposes.

Plates that accounted for approximately 100–5000 CFUs were chosen for harvesting. Total biomass from the solid medium of two plates (ca. 0.1 g) was harvested by suspending it in sterile 0.9% NaCl. Microbial biomass was thoroughly homogenized across plates and evenly distributed into several 1.5 ml plastic tubes. After centrifugation (12170 RCF for 5 min) and removal of the supernatant, the microbial biomass was stored at -20ºC for downstream analyses. Biomass harvesting and subsequent analyses, such as DNA extraction, were not performed in control soil cultures in experiment B on day 2 due to the slow growth and insufficient bacterial biomass.

DNA extraction and shotgun metagenomic sequencing

Bacterial DNA from the harvested biomass was extracted using the Fast DNA Spin Kit (MP Biomedicals, Santa Ana, CA, USA), according to the manufacturer's protocol, as described in Pérez-Valera et al. (2022). The quality of DNA used for downstream PCR analyses was assessed by electrophoresis on 1% agarose gel and quantified using Qubit v3. DNA samples were sent to Novogene's sequencing facility for shotgun metagenomic sequencing. A total amount of 1.0 μg DNA per sample was used as input material for DNA sample preparations. According to the manufacturer's recommendations, the sequencing libraries were prepared using the NEBNext DNA Library Prep Kit (NEB, USA), and indexes were added to each sample. Genomic DNA was randomly fragmented by sonication to a size of 350 bp. DNA fragments were end-polished, A-tailed, and ligated using the NEBNext adapter for Illumina sequencing and further enriched by PCR with P5 and indexed P7 oligos. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Inc). The resulting libraries were analyzed for size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and quantified using real-time PCR (to meet the criteria of 3 nM). The samples were sequenced, and paired-end reads (150 bp) were generated on the instrument NovaSeq 6000. Metagenomic sequencing reads were deposited at the NCBI SRA database under BioProject accession PRJNA743290.

Sequence processing and statistical analysis

All DNA sequences were processed using free open-source software, custom scripts, and locally under Ubuntu Linux-operated computers. SingleM 1.0 (Woodcroft 2023) was used for the overall taxonomic assignment from raw metagenomic sequences using 59 single-copy marker genes that are also resolutive at the species level. Sequences were then processed to trim Illumina adapters and remove low-quality reads and contaminants (masked against contaminant references such as human DNA, PhiX and p-Fosil2) using BBMap and BBduk (https://sourceforge.net/projects/bbmap/). Decontaminated and trimmed sequences were assembled with metaSPAdes 3.14.1 (Nurk et al. 2017). Read assembly was performed separately for each treatment and experiment after merging all time points. Later, contigs from the six assemblies were merged into a single co-assembly using SqueezeMeta pipeline v1.3.0 (Tamames and Puente-Sánchez 2019). This resulted in a total of 323602 contigs with N50=10832 base pairs. SqueezeMeta was also used for further steps, including taxonomic assignment of the functional genes, i.e. the predicted hosts of ARGs, using the Last Common Ancestor (LCA) algorithm by DIAMOND (version 0.9.24) homology searches (Buchfink et al. 2015) against the GeneBank non-redundant protein database (Tamames and Puente-Sánchez 2019). Along with implementing the CARD database 3.1.0 (Alcock et al. 2020) in SqueezeMeta, DeepARG 1.0.2 (Arango-Argoty et al. 2018) and Resistance Gene Identifier (RGI) 5.1.1 (Alcock et al. 2020) were also used independently to annotate ARGs in the metagenome. The annotation results of individual antibiotic resistance genes from CARD, DeepARG, and RGI were merged into a single comprehensive collection of tetracycline resistance genes with a cut-off of 50% identity and e-value < 10^{-3}. In all the cases, 50% identity was used for the primary cut-off criteria for the tetracycline resistance genes as none of the predicted gene's e-value was above 10^{-3}. The antibiotic families were annotated according to the CARD database nomenclature. We used PlasFlow 1.1 (Krawczyk et al. 2018) to predict the plasmid or chromosomal origin of ARGs in our metagenome. We used TPM values (transcript per million, as originally described) as normalized gene abundances (Wagner et al. 2012). TPMs indicate the number of times we would find a gene when randomly sampling one million genes in the metagenome, thus accounting for gene length and sequencing depth (Puente-Sánchez et al. 2020). The number of predicted genes for individual tetracycline resistance genes in each resistance taxa were imported into Cytoscape 3.8.2 (Shannon et al. 2003) to build the interconnection and association map. Based on the relative abundance, an alluvial diagram was used to show the association among different treatment conditions with the tetracycline resistance genes using the R package ggalluvial (Rosvall and Bergstrom 2010, Wickham 2010). Relative abundance of ARGs accounting for a minimum 0.5% of total abundance were used for heatmap representation using hclust2 (https://github.com/SegataLab/hclust2). Abundance values were log transformed to make the color gradients in the heatmaps. The R package ggplot2 (Villanueva and Chen 2019) was used to represent the box, bar, and PCA plots. All statistical analyses were conducted using R software version 3.5.2 (R Core Team 2019). Box plots for the analysis of relative abundance of *otr*(C) were done in GraphPad Prism v.8.4.2 (GraphPad Software, LLC.).

Results

Taxonomic composition of all cultured taxa and hosts of antimicrobial resistance

The genus *Acinetobacter* had the highest taxonomic abundance across the entire cultured metagenome, followed by *Stenotrophomonas* and *Pseudomonas*. While the genus *Acinetobacter* was more abundant on day 2 under all experimental conditions, *Pseudomonas* tended to be more abundant on day 14 in control manure and treated soil in experiment B (Fig. 1A). At the species level, *A. calcoaceticus* dominated in treated soil on day 2 in both experiments, and in control soil in experiment S, while *A. gandensis*

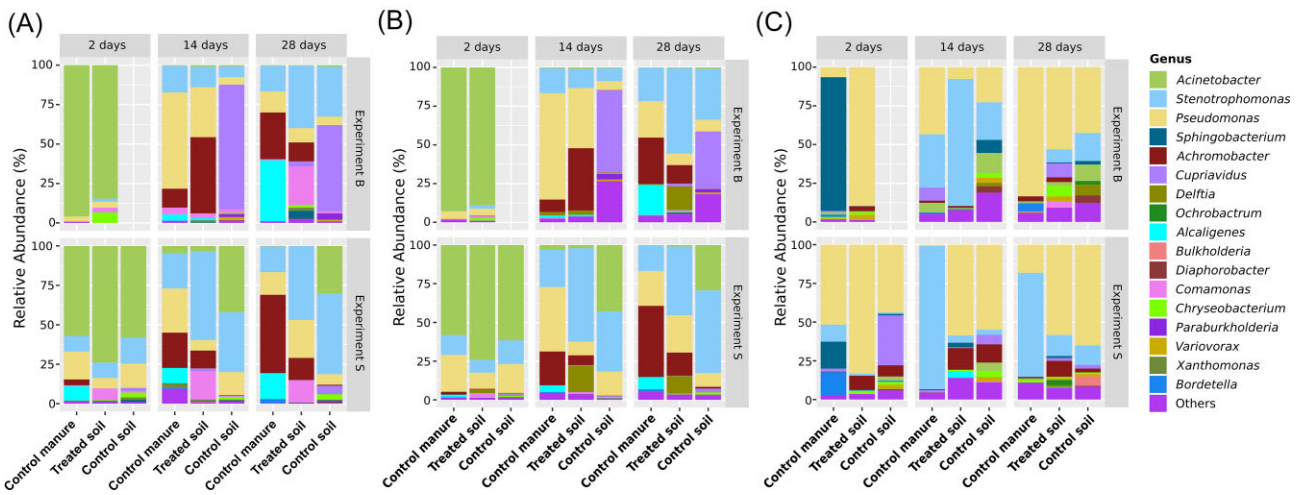

Figure 1. Relative abundance of (A) bacterial genera in the entire metagenome, (B) predicted hosts from all identified ARGs, and (C) predicted hosts from tetracycline resistance genes in the cultured metagenome. The taxonomy of genera in the entire metagenome was identified from raw metagenomic sequences using 59 single-copy marker genes through singleM (Woodcroft 2023). Host taxonomy of ARGs and tetracycline resistance genes was predicted using SqueezeMeta with the Last Common Ancestor (LCA) algorithm by DIAMOND (Buchfink, Xie and Huson 2015). Relative abundances of genera with > 0.1% of total abundance are shown in the figure. All the unclassified genera and those having < 0.1% of total abundance are grouped within "Others". The genus abundances are in descending order.

and *A. johnsonii* dominated in control manure, especially on day 2 (Fig. S2).

According to the taxonomy of the predicted ARGs hosts, the relative abundances of the main genera resembled those of the entire metagenome (Fig. 1B). That is, the taxa with the higher relative abundance were the same when comparing the abundance of all taxa and the predicted bacterial hosts of AMR. Although most of the tetracycline resistance genes were identified in both *Pseudomonas* and *Stenotrophomonas* in both experiments, genes assigned to *Pseudomonas* dominated under most experimental conditions, while those assigned to *Stenotrophomonas* were generally more abundant in experiment B on day 14 and the control manure in experiment S on days 14 and 28 (Fig. 1C). The genus *Sphingobacterium*, as predicted host of tetracycline resistance genes, was predominantly enriched in control manure in both experiments on day 2.

Relative abundance of antibiotic resistance genes

The addition of manure increased the relative abundances of predicted ARGs in cultured bacteria in treated soil, but only in experiment B, where the abundances of ARGs in control soil were significantly lower than in control manure (Fig. 2A). In experiment S, in which control soils had similar ARGs abundances to those in manure, there were no changes in treated soil (Fig. 2A). However, manure did alter the composition of ARGs, with resistance to the dominant antibiotic families differing across treatments (Fig. S3). For example, cultured bacteria in treated soil tended to show a similar antibiotic resistome profile to control manure in experiment B on day 2, with differences driven by resistance to rifamycin or macrolide, among others, at longer incubation times (Fig. S3a). In experiment S, the antibiotic resistome profile of cultured bacteria in treated soil resembled that of the control soil on day 2, with little influence of manure over time (Fig. S3b). In both experiments, a gradual decrease in the abundance of predicted ARGs in NFGNB was observed over time in control manure and treated soil (Fig. 2A).

Prevalence of tetracycline resistance genes

We did not find significant differences in the relative abundances of predicted tetracycline resistance genes' pool among the treatment conditions (i.e. treated soil and control soil and control manure on days 2, 14 and 28) in both experiments (Fig. 2B). However, cultured bacteria in treated soils tended to show differences in the tetracycline resistome profile compared to the control manure and control soil (Fig. S4). In particular, there tended to have higher relative abundances of *tet*(E), *otr*(B), *tet*A(60), and *tet*(55) in experiment B (Fig. S4A), and higher of *otr*(B), *tet*(S), *otr*(A), and *tet*(43) in experiment S (Fig. S4b).

Genes predicted as multidrug resistance were the most abundant in the cultured metagenomes (Fig. 3). In experiment B, most resistance genes were more abundant in treated soil than in control soil (ca. 78% of them), and roughly similar to control manure (ca. 48% of them) (Fig. 3A; Table S4). For example, the addition of manure significantly enriched *abeS*, *P. aeruginosa* mediated *soxR*, and *macB* in treated soil (Fig. 3A; Table S4). Interestingly, in experiment S, ca. 63% of the genes showed higher abundance in control soil than in treated soil, and ca. 58% were more abundant in treated soil than in control manure (Fig. 3B; Table S4).

otr(C), a tetracycline efflux pump gene, represented the most abundant tetracycline resistance gene in the metagenome in both experiments, followed by *otr*(B), *tet*(59), and *tcr3* in experiment B (Fig. S5a), and by *tcr3*, *tet*(X), and *tet*A(60) in experiment S (Fig. S5b). The relative abundance of *tcr3*, another efflux pump gene related to tetracycline resistance, was consistently more abundant in treated soil than in control soil and control manure (Fig. S5).

Predicted bacterial hosts of tetracycline resistance genes

In our cultured metagenome, seven out of seventeen genera were predicted by SqueezeMeta as hosts of tetracycline resistance genes and were shared across experimental conditions (Fig. 4). *Comamonas* and *Diaphorobacter* were predicted as hosts of tetracycline resistance genes only in treated soil while *Burkholderia*, *Cupriavidus*, and *Paraburkholderia* were specific to control soil. Among

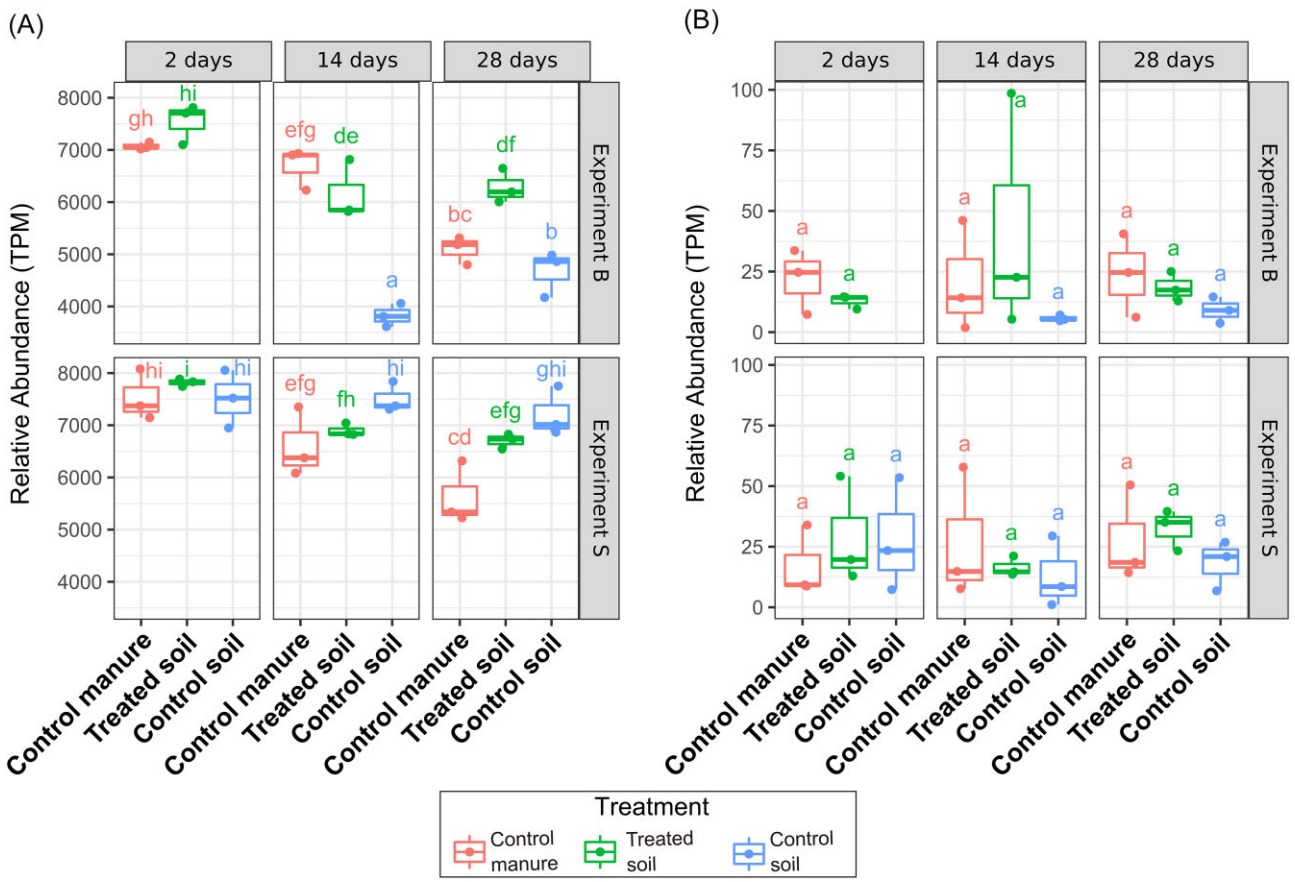

Figure 2. Box-whisker plots showing the differences in relative abundance (TPM, indicating the number of times a gene would be found when randomly sampling one million genes in the metagenome) of (A) all predicted ARGs and (B) specifically tetracycline resistance in different treatment conditions. The alphabetical letter coding has been assigned after the pairwise comparison among treatment conditions followed by the Tukey test and FDR correction. Boxes with different letters represent a significant difference.

the predicted hosts, the genus *Pseudomonas* exhibited the most diversified range of tetracycline resistance genes, particularly in treated and control soil, comprising a total of 14 different tetracycline resistance genes (Fig. 4). Among the 14 tetracycline resistance genes affiliated with the genus *Pseudomonas*, five genes (i.e. *tetA*(60), *tet*(H), *tet*(E), *tet*(43), and *otr*(B)) were unique to it (Fig. 4). The genera *Stenotrophomonas* and *Acinetobacter* were predicted to have seven and six different types of tetracycline resistance genes, respectively. Among these, four tetracycline resistance genes (i.e. *tet*(44), *tet*(45), *tet*(48), and *tet*(59) were unique to *Stenotrophomonas*, and *tet*(52) along with *tet*(Y) were unique to the genus *Acinetobacter* in control manure and treated soil. Interestingly, *tet*(Y) was found only in control manure and treated soil but not in control soil. A total of six genera including *Alcaligenes*, *Comamonas*, *Paraburkholderia, Ochrobactrum, Diaphorobacter*, and *Mitsuaria* were predicted to carry only one type of tetracycline resistance gene. The predicted hosts of other tetracycline resistance genes such as *tet*(S), *tet*(Q), *tet*(57), and *tet*(42) remained unknown.

Since *otr*(C) was the major enriched tetracycline resistance gene in our study, we compared its relative abundance in control soil and control manure with treated soil (Fig. S6). A *t-test* revealed significant differences in *otr*(C) gene abundances between *Stenotrophomonas* and *Pseudomonas* in experiment S. The relative abundance of *otr*(C) was predicted to be significantly higher in the genus *Stenotrophomonas* in control manure than in treated soil (Fig. S6a). *otr*(C) potentially carried by *Pseudomonas* was enriched in treated soil compared to control soil and control manure (Fig. S6b).

Plasmid or chromosomal origin of predicted antibiotic resistance genes

The most abundant ARGs in the NFGNB metagenome were of chromosomal origin (ca. 2 000–8 000 TPMs of chromosomal origin vs ca. 100–600 TPMs of plasmid origin) (Fig. 5A). Indeed, the abundances of ARGs of chromosomal origin resembled those of all ARGs among treatments (Fig. 5A). Overall, predicted ARGs of plasmid origin tended to be more abundant in control soils in both experiments. In the case of tetracycline resistance genes, only experiment S had a significantly higher abundance of tetracycline resistance genes of plasmid origin in treated soil after 2 and 28 days compared to the control soil (Fig. 5B).

Discussion

Our results from shotgun metagenomics of CHROMagar Acinetobacter cultures, composed of *Acinetobacter* spp. and other NFGNB, showed that the addition of fresh manure to pasture soil in laboratory microcosms altered both the abundance and composition of predicted ARGs and bacterial hosts, but the effects depended on the soil and incubation time. Using 16S rRNA amplicon data, we have previously shown that the genus *Acinetobacter* dominated in both experiments, especially in the short term (day 2) (Pérez-Valera et al. 2022). In this study, our metagenomic data confirmed the short-term dominance of *Acinetobacter* and showed that it was also the most abundant genus in our study when considering the taxonomic prediction of all detected ARGs. Leclercq

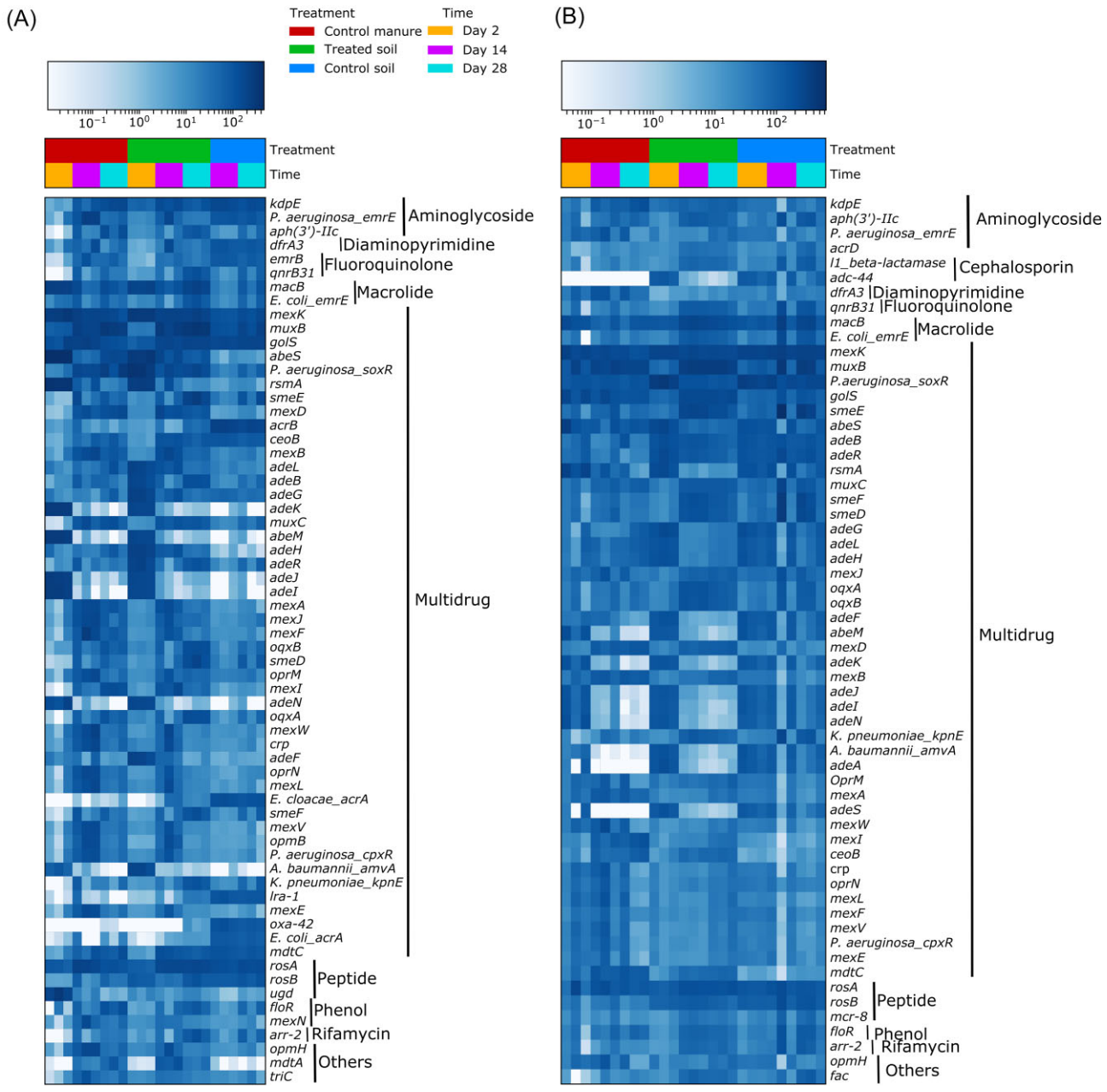

Figure 3. Heatmaps of predicted ARGs in the metagenomes from (A) experiment B and (B) experiment S for different treatment conditions are shown. Predicted ARGs accounting for a minimum of 0.5% of total abundance are shown on the heatmaps after log transformation. Different AMR classes are marked with vertical black lines on the right side of the figures. The relative abundance of predicted ARGs is plotted with the different gradients of blues, complete white being the lowest and dark blue being the highest abundance. Treatment conditions (i.e., control manure, treated soil, and control soil) and time (i.e., day 2, day 14, and day 28) are shown with different colors.

et al. (2016) found a similar short-term burst of *Acinetobacter* spp. in soils amended with pig manure and linked this to higher persistence of ARGs in soils. The short-term dominance of *Acinetobacter* spp. along with the high prevalence of AMR in this group (Towner 2009) indicate that they may pose a putative pathogenicity risk, at least for some time. The dominant species of *Acinetobacter* in our experiments, that is, *A. gandensis* and *A. johnsonii* in control manure and *A. calcoaceticus* in treated soil and control soil microcosms are reported to inhabit soil and aquatic environments (Doughari et al. 2011). However, strains closely related to *A. calcoaceticus* and *A. johnsonii* have been cultured from human clinical specimens (Seifert et al. 1993, Nemec et al. 2019), confirming the pathogenicity risk that the spread of these *Acinetobacter* spp. could pose to the environment.

Fresh manure application on soil significantly increased both the number of predicted ARGs and their relative abundances in our cultured metagenome, as shown in experiment B. Several studies have already confirmed the capacity of raw cattle manure to spread ARGs in soil (Chee-Sanford et al. 2009, Chen et al. 2017, Pérez-Valera et al. 2019, Köninger et al. 2021), although a few have explicitly focused on culturable *Acinetobacter* spp. and other NFGNB. For example, Resende et al. (2014) found multidrug-resistant NFGNB in fresh dairy cattle manure, which reduced the number of viable microbial counts after anaerobic digestion. The

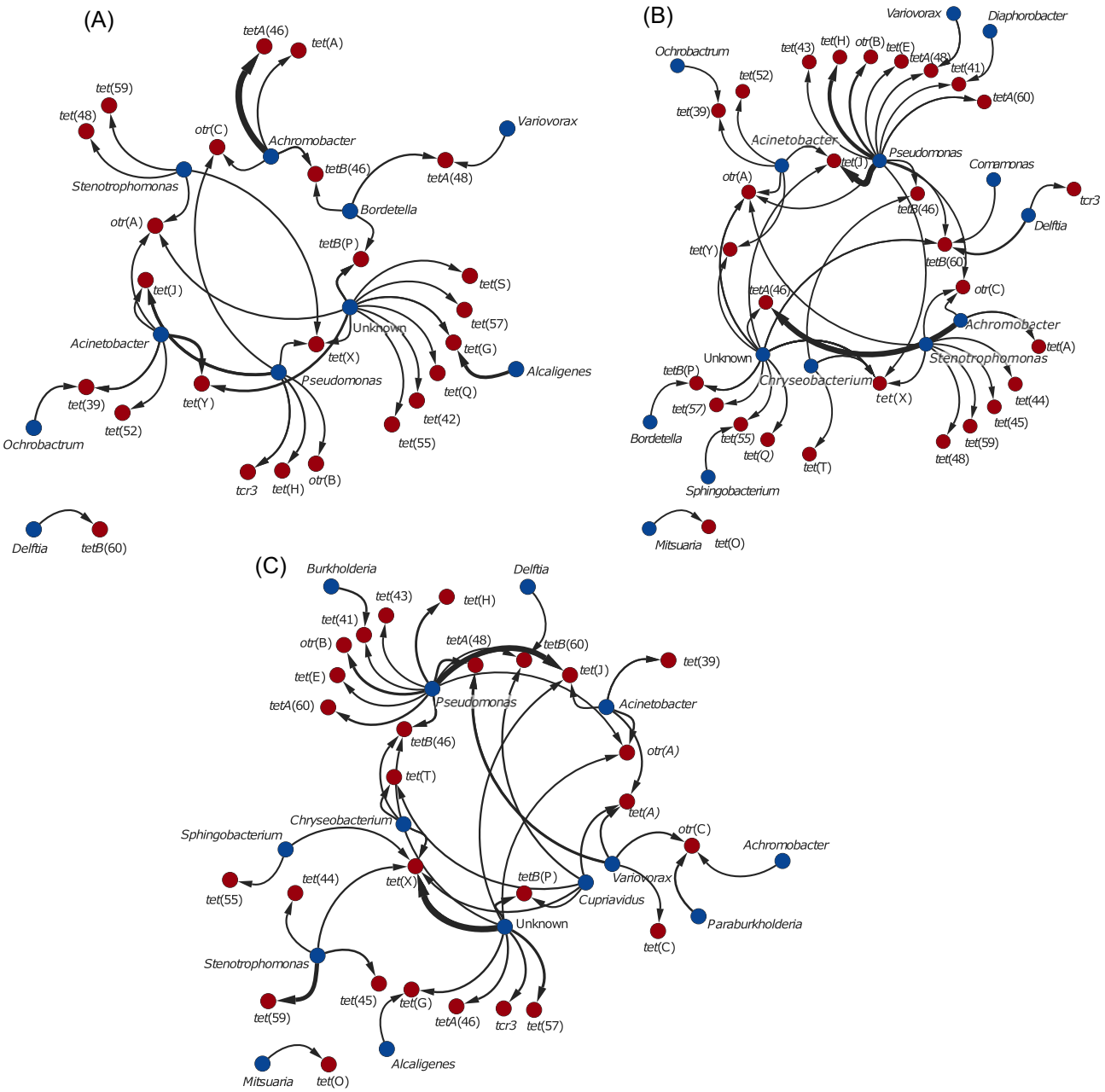

Figure 4. Association maps to describe the interconnections between tetracycline resistance genes and their bacterial predicted hosts in (A) control manure, (B) treated soil, and (C) control soil. Bacterial genera and tetracycline resistance genes are shown in blue and red spheres, respectively. The thickness of the connecting arrows is proportional to the relative abundance (TPM) of tetracycline resistance genes in the host taxa.

ways ARGs can enter the soil from manure are numerous and include the spread of antibiotic-resistant bacteria and the transfer of ARGs to native bacteria via horizontal gene transfer (Unc and Goss 2004, Chee-Sanford et al. 2009). In experiment B, the similarities in the dominant NFGNB taxa between treated soil and control manure on days 2 and 14 (i.e. mainly the genera *Acinetobacter*, *Pseudomonas* and *Stenotrophomonas*), in contrast to control soil (mainly dominated by the genus *Cupriavidus*), provide evidence that bacteria from manure are reaching the soil, and are likely associated with increased AMR. This possibility was also supported by i) the fact that most ARGs were predicted to be of chromosomal origin and ii) the low incidence of *Acinetobacter* spp. and other NFGNB in the control soil, which was alleviated in treated soil over time. Overall, these results support the role of manure in spreading bacteria carrying genes that could relate to AMR in soil. In addition, the results also indicate that the source soil in experiment B had low levels of *Acinetobacter* spp. and other NFGNB or that they were less competitive (e.g. slow growth) than those from manure, being rapidly displaced.

A different pattern was detected in experiment S, in which similar AMR values were found in all treatments despite their differences in taxonomic composition. This suggests that different taxa carried comparable levels of predicted ARGs. However, manure may have induced changes in the ARG composition in treated soil, with a resistome profile slightly differing from that in control soil. For example, *tet*(Y) assigned to the genus *Acinetobacter* was found in both control manure and treated soil, but not in control soil, suggesting that *Acinetobacter* was introduced into the soil by

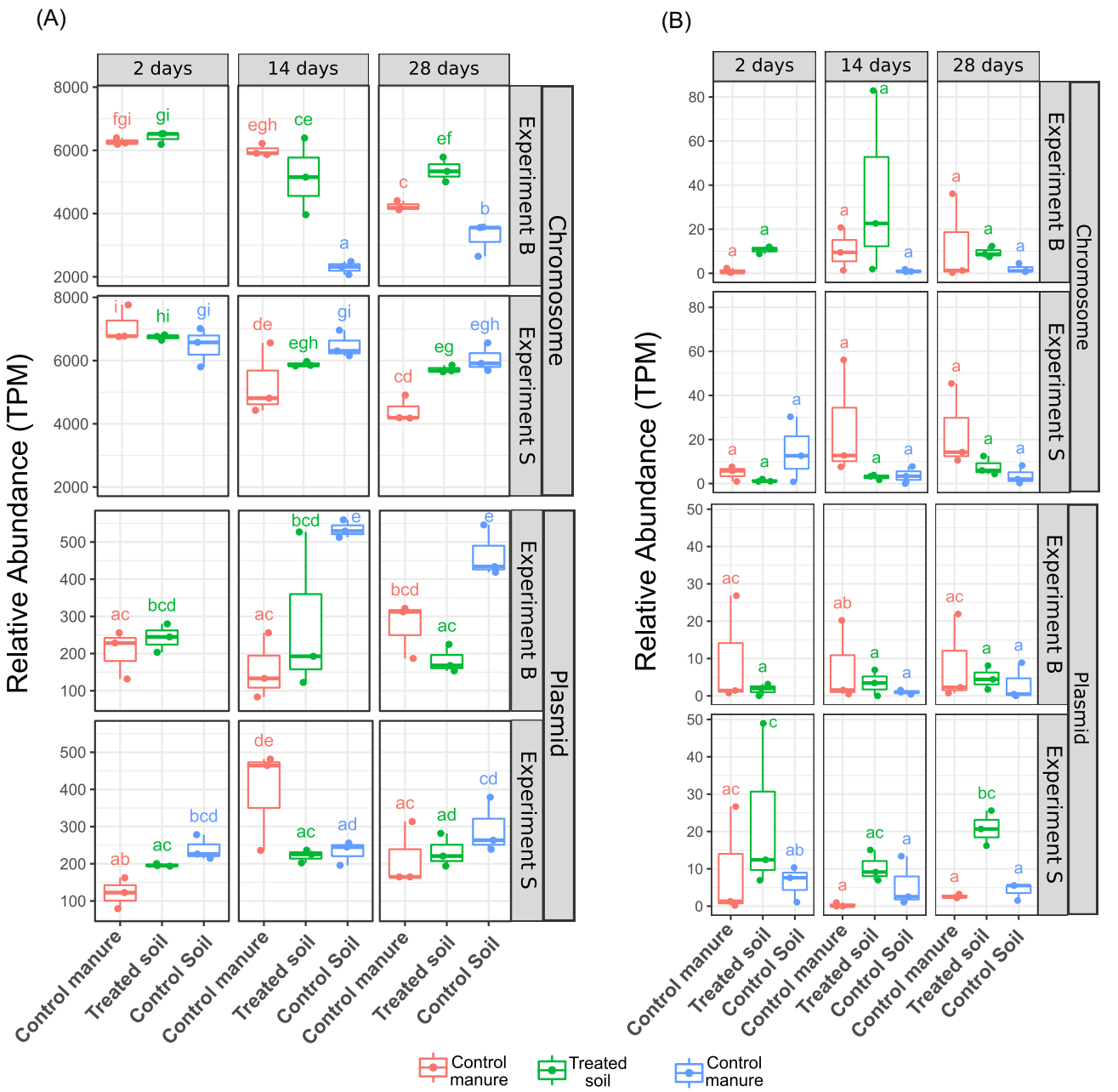

Figure 5. Box-whisker plots showing the relative abundance (TPM, indicating the number of times a gene would be found when randomly sampling one million genes in the metagenome) of (A) all predicted ARGs and (B) tetracycline resistance genes according to their origin (i.e., from chromosomes or plasmids) in different treatment conditions across the time. The alphabetical letter coding was assigned after a pairwise comparison among treatment conditions followed by the Tukey test and FDR correction. Boxes with different letters represent a significant difference.

manure deposition or the gene was transferred to soil bacteria. In addition, manure could have stimulated some native soil culturable *Acinetobacter* spp. and other NFGNB via changes in the soil characteristics, e.g. increasing soil pH or releasing labile nutrients (Unc and Goss 2004, Pérez-Valera et al. 2022).

Cultured NFGNB metagenomes showed similar relative abundances of tetracycline resistance genes under all experimental conditions. We expected culturable bacteria from manure to have a higher abundance of tetracycline resistance genes than soil because the cattle were treated prophylactically with tetracycline, and we had previously demonstrated the role of fresh manure in increasing the abundances of tetracycline resistance genes in soil microbial communities using quantitative PCR-based approaches (Kyselková et al. 2015, Pérez-Valera et al. 2019). There could be several reasons why we did not find such a pattern. First, the culture medium used did not contain tetracycline, so bacteria carrying tetracycline resistance, often found in mobile genetic elements such as plasmids, may not necessarily had growth advantage in the cultures. Jechalke et al. (2013) showed that plasmid carriage may confer a fitness disadvantage to bacteria when antibiotic selection is absent. Second, we looked for specific tetracycline resistance genes. Therefore, we cannot exclude the possibility that other genes that confer resistance to tetracycline but are not specific to it (e.g. multidrug resistance) could be overrepresented but not accounted for in our analyses. Third, culturable bacteria in soil could have high levels of tetracycline resistance genes (or at

least comparable to those in manure) if soils received manure in the past, either antibiotic-treated or untreated (Kyselková et al. 2015). Interestingly, we found that the composition of tetracycline resistance genes differed across treatments and experiments despite the similar abundances, suggesting that the taxa composition might influence the tetracycline resistome. This could also imply that soil enrichment with potentially risky bacteria and genes likely related to AMR from manure continues over time. However, the increase in the potential of NFGNB to transfer important ARGs to soil over time needs further investigation.

Pseudomonas spp. and *Acinetobacter* spp. were the AMR hosts with the highest number of tetracycline resistance genes predicted in our cultured metagenome. These two genera have been often referred to by previous studies for being resistant to a diverse range of antibiotics, including tetracycline (Lupo et al. 2018, Pachori et al. 2019). In fact, although *Pseudomonas* spp. were ubiquitously present in all the treatment conditions in our experiments, *otr*(C) genes predicted from *Pseudomonas* consistently prevailed in treated soil upon application of manure, which may be due to the supply of nutrient source from the manure (Das et al. 2017). This could be an indirect effect of manure on the NFGNB from the culture in treated soil via stimulation of soil bacteria. The use of novel conceptual frameworks, such as microbial community coalescence, could hold the key to better analyzing environmental mixing events, as it occurs in soil after manure application, accounting for changes in resources, abiotic factors and biotic interactions that contribute to better predicting the transfer of antibiotic resistance into the environment (Rillig et al. 2015).

Our data based on cultivation-dependent selection and metagenomics provided evidence that most detected and culturable NFGNB were predicted as hosts of ARGs from nearly all families currently available in antibiotic resistance databases. Furthermore, the addition of manure changed the taxonomic composition of culturable NFGNB in the soil, which in turn drove the shift in total and tetracycline resistance. However, our study is limited in that we did not use antibiotics for selection purposes or tested our cultures for antibiotic resistance, and therefore, we cannot confirm whether the bacteria were resistant to antibiotics and whether the genes predicted to be ARGs were functional and causing resistance to antibiotics. Indeed, genes predicted to be ARGs, especially multidrug efflux pump genes, may also be involved in other functions not related to antibiotic resistance (Sun et al. 2014). For example, *soxR*, which was particularly abundant in our study, has been shown to be related to stress tolerance (Palma et al. 2005), so it should be also considered that not only antibiotics but other stressors may be the trigger of such ARG. Moreover, predicted ARGs might be involved in resistance only after mutation (Alcock et al. 2020), which we could not detect with our experimental design.

The differential response of culturable bacteria in the soil to the application of manure illustrates the complexity and dynamics of the processes involved in the transfer of antibiotic resistance to soil, with both direct (i.e. soil colonization by bacteria from manure) and indirect effects (stimulation of native taxa by nutrients and micronutrients from manure) likely dependent on the native soil bacteria and the soil properties. The possibility of these events becoming increasingly important in the ecosystem requires further attention, requiring future studies using soils with different abiotic properties, microorganisms and antibiotic resistome that focus on understanding the mechanisms involved in the spread of antibiotic resistance in soil after the addition of manure.

Authorship statement

EPV and DE designed the study. EPV and DE performed the microcosm experiments. PS and EPV performed metagenomic analysis and analyzed the data. PS, DE, and EPV wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The raw metagenomic data for this study have been deposited in the NCBI Sequence Read Archive under BioProject PRJNA743290.

Author contributions

Puspendu Sardar (Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing), Dana Elhottová (Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing), and Eduardo Pérez-Valera (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing)

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Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

Conflict of interest: We declare no conflict of interest.

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