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RESEARCH

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The effect of sulfadiazine in manure on accumulation of sulfonamide resistance genes in freshly consumable plants

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Abstract

Background Antibiotic resistance genes will spread via soil fertilized with animal manure to food products. Especially plants whose harvested products can be consumed freshly are of concern. The aim of this study was to assess the impact of sulfadiazine (SDZ)-manured soil on the occurrence of sulfonamide (SA) resistance genes in freshly consumable plants.

Methods Sulfadiazine-containing manure was administered via soil to lettuce and leek plants. At harvest, the rhizosphere soil, roots and leaves were investigated on the presence of SDZ-resistant bacteria and *sul*1 and *sul*2 genes via qPCR. Further, the impact of SDZ in manure on bacterial community and antibiotic resistance gene composition via amplicon sequencing and shotgun metagenomics was investigated in rhizosphere soils.

Results Amendment of SDZ to manure resulted in an increase in *sul*2 genes in manure. However, abundances of *sul* genes in rhizosphere soils was strongly determined by plant growth and not by soil treatments with SDZ-manure, which was also the case for the bacterial community composition. Effects of SDZ at low or undetectable levels in leek rhizosphere soil became evident by bacterial association network and resistome analyses, and also in roots and leaves by SDZ-selective bacterial cultivation.

Conclusions Antibiotic residues present in animal manure can lead to an increase in antibiotic resistances in food products. Plants play an important role in selection of antibiotic resistance genes present in manured soil. Transmission of antibiotic resistances via manure to the soil–plant ecosystem must be placed into the context of soils as vast reservoirs of ARGs.

Keywords Antibiotic resistance, Freshly consumable plants, One health, Rhizosphere soil, Sulfadiazine, Sulfonamide resistance genes

Background

Bacteria carrying antibiotic resistance genes (ARGs) will spread via animal manure to agricultural production soils (Zhang et al. 2019; Zalewska et al. 2021; Billet et al. 2022). In spite of limitations on the use of antibiotics in livestock production in many countries, residues still can be measured in manured soils (Li et al. 2011; Cycoń et al. 2019). Although residue levels can be low, effects on soil bacterial populations are still measurable (Davies et al. 2006;

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Gullberg et al. 2011). Antibiotic resistance genes can persist in the absence of antibiotic selection because mobile genetic elements (MGEs) carrying ARGs will prevent segregation of sensitive populations, and if segregated, then these populations may reacquire ARGs via horizontal gene transfer (Johnsen et al. 2009). Antibiotic resistance genes were not only found in human pathogens, but also in opportunistic pathogens and commensal bacteria and these species were shown to be transferred from manure, via fertilized soils to plants (Chen et al. 2019; Hölzel et al. 2018). Non-pathogenic bacteria were demonstrated to carry ARGs in their genomes, of which some were categorized as of 'high clinical concern', according to the list published by the World Health Organization (WHO) (Zhang et al. 2021). Genetic exchange of ARGs between microbial species via horizontal gene transfer, followed by outgrowth of bacterial populations with acquired antibiotic resistances, will take place in microbial hotspots for gene transfer, i.e. the intestinal track system of livestock animals and rhizosphere soil (Heuer et al. 2011; Overbeek et al. 2014). From there, antibiotic resistant populations will reach edible parts of food crop plants that are produced in soils fertilized with manure from livestock animals (Zhang et al. 2019; Boxall et al. 2006; Marti et al. 2013).

Antibiotics have distinct physico-chemical properties and will behave differently in soils (Cycoń et al. 2019; Hamscher et al. 2005; Bailey et al. 2016; Berendsen et al. 2021). In these studies, it was shown that adsorption to soil particles determines the distribution of antibiotics in different soil compartments, and that soil type, water content and pH played important roles in this process. Overall, pH tend to decrease more proximate to plant roots and therefore adsorption properties of antibiotics will differ between bulk and rhizosphere soils (Cycoń et al. 2019). Availability of organic compounds, resulting from exudation by roots, will stimulate growth and activity of micro-organisms and this may impact the biological stability of antibiotics in rhizosphere soils. Antibiotics present in rhizosphere soil can be taken up by roots and further distributed over the different plant organs. Uptake differed between plant species, but also depended on the chemical structure of the antibiotic compound (Boxall et al. 2006). Because antibiotics are not always present in measurable quantities, it is complicated to establish causal relationships between antibiotic concentrations and ARG abundances in rhizosphere soils and different plant compartments (Cycoń et al. 2019; Kopmann et al. 2013; Man et al. 2022).

Sulfonamides (SA) is a well-studied group of antibiotics because of its widespread presence in arable soils (Zhang et al. 2021) and its potential risk on the environment (Boxall et al. 2003). Sulfadiazine (SDZ), an antibiotic compound belonging to the antibiotic class of SA, is non-persistent in organic manure and soils, and it is mainly present in the liquid fraction of manure (Bailey et al. 2016; Berendsen et al. 2021). As such, it can be introduced into arable soils, where it is commonly present at low concentrations. Sulfonamide resistance genes *sul*1, *sul*2 and *sul*3 were found in soils that had received pig manure in the UK (Byrne-Bailey et al. 2009) and in chicken and pig-manured soils in China (Wang et al. 2014). Sulfadiazine administered via manure into soils (SDZ manure-amended soils) rapidly declined, but *sul*1 and *sul*2 gene levels that were already established in manure remained, after an initial decline, stable for six months (Heuer et al. 2008). These resistance genes were commonly associated with class 1 and 2 integrons (Wang et al. 2014; Sköld 2000; Heuer and Smalla 2007) and with low GC group plasmids (Kopmann et al. 2013; Sundin and Bender 1996), demonstrating the mobile capacity of *sul* genes. Exogenous plasmid isolation from manured soils with SDZ, using an *E. coli* strain as recipient, resulted in transconjugants that, besides SA resistance genes, also contained genes conferring resistances to many other antibiotics (Heuer and Smalla 2007). Presence of SDZ residues in manure can lead to accumulation of bacterial populations carrying multiple antibiotic resistances in manured soils and most likely also near, and inside plants growing in these soils.

Antibiotic resistant bacteria can reach the human intestinal track system via consumption of food products of plant origin (Chen et al. 2019). Transition towards more sustainable agricultural practices by replacing mineral for organic fertilizers even may lead to increased accumulation of antibiotic resistant bacteria in humans via food consumption. Animal manures and other organic side streams applied in sustainable agricultural practices such as struvite, a phosphate mineral derived from wastewater, and reclaimed wastewater are responsible for the transmission of ARGs to plants (Chen et al. 2017; Camacho-Arévalo et al. 2021). Antibiotic resistant bacteria will circulate between animals, soils, plants and humans (Sundin and Bender 1996) and these ecosystems are microbiologically interconnected with each other (Zhang et al. 2019; Billet et al. 2022; Heuer et al. 2011). Research on transmission of these bacteria to food production systems will therefore require a one-health approach (Bruggen et al. 2019; Gil-Gil et al. 2021). Plants whose harvested products can be consumed non-heated, such as leafy greens and particular types of vegetables (produce), herbs and fruits, are in particular of concern (Zhang et al. 2019; Marti et al. 2013; Veldman et al. 2014; Wang et al. 2015).

The effect of antibiotic residues in manure on the accumulation of ARGs in food crops at primary production

has not been extensively studied so far. Therefore, the aim of this study was to assess the impact of SDZ, as model antibiotic amended via manure to soil, on SA resistance in soil and in different compartments of two distinct plant species that both can be consumed freshly, leek (*Allium porrum*, a monocot plant species) and lettuce (*Lactuca sativa*, a dicot plant species). Lettuce heads are commonly harvested between six and eight weeks after planting, and leeks later in the growing season in autumn or in spring time and therefore in this study sampled at two growth stages, before and after winter time. Sulfadiazine was administered to manure at two different time points, 2 and 37 days before manure application to soil and planting, to ensure differences in the accumulation of SA-resistant bacterial populations in the two manure batches. Using this approach, we expected a differentiation in plant colonization by SA-resistant bacteria. The impact of SDZ residues still present in manured soils on rhizosphere soil bacterial community structures and networks was additionally taken into consideration.

Materials and methods

Experimental field design and manure treatments

The field experiment performed in this study was integrated in the one previously described in van Overbeek et al. (2021). In short, a field trial was set up in 2018 to investigate the colonization of lettuce and leek plants by *E. coli* strain 0611, introduced via manure into field soil (Overbeek et al. 2021), and to assess the impact of SDZ on accumulation of SA resistance genes in lettuce and leek plants (this study). Although the same field soil and plant starting materials were used, the objectives of both studies were different. Manure treatments with SDZ were only applied in this study and therefore 10 mg SDZ (SigmaAldrich, Saint Louis, MO) per kg cow manure was amended to separate batches from the same stable at different time points, i.e. 35 days before mixing through soil and further denoted as 'manure with SDZ early' (MSE) treatment and two days before mixing through soil and further denoted as 'manure with SDZ late' (MSL) treatment (Table 1). Then, MSE and MSL were mixed through separate soil batches, freshly collected from the field, reaching a final manure content of 3.5% on weight basis resulting in, respectively, S-MSE and S-MSL soils. Control soil treatments were field soils that were mixed either with 3.5% untreated manure (M) from the same batch as used for MSL (S-M) or with 0.4 g per kg dry soil of inorganic fertilizer, NPK (S-NPK), and these soil treatments were the same as described before (Overbeek et al. 2021). Two days after mixing of the different manure batches and NPK through soil, boreholes (diam. 6.5 cm; depth 7 cm) in field soil -covered with plastic foil- were filled with the four soil mixtures and young plants of lettuce (*Lactuca sativa* cultivar Lollo Rosso, HortiTops seeds, Heerenveen, The Netherlands) and leek (*Allium porrum* cultivar Pluston F1, BASF Nunhems, The Netherlands) were planted in the treated soils. In total there were three plots and each plot consisted of four different soils (S-NPK, S-M, S-MSE, S-MSL), each in separate rows of 30 plants. The separate plots represented differences in plant species (lettuce versus leek plants) and in growth stages (leek only via sampling at different time points, i.e. in November 2018 and in May 2019). The field experiment started in August 2018 and lettuce plants were sampled at 39 days after planting (DAP) and this plant treatment is further denoted as 'lettuce', whereas leek plants were sampled at 90 and at 272 DAP and further denoted as, respectively, 'leek 2018' and 'leek 2019'.

Soil and plant sample processing

Samples of manure (M, MSE, MSL), bulk soil (unamended soil, S-NPK, S-M, S-MSE, S-MSL at the start of

Table 1 Soil and manure types used in this study including measured SDZ concentrations and *sul*-gene specific ΔCt values

Short name	Sample description	SDZ concentration (µg per kg)	ΔCt values:	
			*sul*1	*sul*2
M	Untreated cow manure	BD	16.8	12.6
MSE	SDZ[a] amended to M 37 days before planting	7900	13.3	3.91
MSL	SDZ[a] amended to M 2 days before planting	10,600	14.4	9.86
S	Untreated field soil	BD	12.2	10.6
S-M	Soil mixed with M (3.5% on weight basis)	BD	12.7	11.3
S-MSE	Soil mixed with MSE	67	12.7	6.84
S-MSL	Soil mixed with MSL	82	12.9	9.96
S-NPK	Soil mixed with 0.4 g NPK per kg soil	BD	12.5	10.8

BD Below detection

[a] SDZ added to M was 10 mg per kg

the experiment and taken at leek 2019 sampling), rhizosphere soil, roots and leaves, taken from lettuce, leek 2018 and leek 2019 plants over all four soils, were processed for: (i) SDZ measurements, (ii) DNA extraction for *sul*1, *sul*2 and bacterial 16S rRNA-directed qPCRs, 16S rRNA amplicon sequencing and shotgun metagenomics, and (iii) cultivation of SDZ-resistant bacteria (M, MSE and MSL-treated rhizosphere soils, roots and leaves of leek 2018 and leek 2019 plants). Therefore, manure and bulk soil samples and roots with tightly adhering soil (1 g per sample) were transferred to sterile 50 ml plastic vials containing nine ml 0.1% sodium pyrophosphate (NaPPi) solution and one g gravel and vials were rotary shaken for 10 min at room temperature. Manure and bulk soil suspensions were directly used in further downstream processing steps, whereas roots were aseptically removed from the rhizosphere soil suspensions and three times washed in sterile tap water. Leaves, taken from the central parts of lettuce heads and leeks, were also three times washed in sterile tap water and both washed root and leaf samples were aseptically transferred to BioReba bags (Bioreba AG, Reinach, Switzerland) containing 5 ml Ringers solution (SigmaAldrich). Samples were crushed with a plastic hammer and resulting homogenates, filtered from coarse materials in the BioReba bags, were further used in downstream processing steps. Manure, bulk and rhizosphere soil suspensions and root and leaf homogenates (2 ml) were transferred to sterile Eppendorf vials (in duplicate) and centrifuged at full speed for two min. Resulting supernatants, transferred to fresh vials, and remaining pellets were stored at − 80 °C for later SDZ analysis and DNA extraction, respectively.

Cultivation of SDZ-resistant bacteria from leek plants

In order to determine the long-term effect of SDZ in manured soils on accumulation of SA-resistant bacterial populations, leek 2018 and leek 2019 plants were investigated on the presence of SDZ-resistant bacteria by cultivation under SDZ selection on an agar medium, R2A (BD DIFCO™, NJ), that allows growth of a wide spectrum of bacterial species. For that purpose, rhizosphere soil suspensions and root and leaf homogenates of leek 2018 and leek 2019 samples under MSE and MSL treatments, with M treatment (absence of SDZ in manure) as control, were used. Nondiluted and tenfold serially diluted soil suspensions and plant homogenates (50 µl) were plated onto R2A medium (Oxoid, Basingstoke, UK), supplemented with 50 µg SDZ and 100 µg cycloheximide (SigmaAldrich) per ml. Plates were incubated for 72 h at 27 °C, whereafter colonies were enumerated. Resulting colony counts were converted to Log10 (CFU + 1) values and expressed as Log CFU per g dry soil or per g leaf or root, on fresh weight basis. Significance of differences between means of the three soils (S-MSE, S-MSL and S-M) at two sampling points (leek 2018 and leek 2019) were calculated by one-way analysis of variance (ANOVA), (n = 10), using Genstat 19th ed. (Hemel Hempstead, UK) and in case of significance, LSD values were calculated for pairwise comparison between mean values. Differences were considered to be significant at levels of $P \leq 0.05$.

Quantification of SDZ, DNA extraction and quantitative PCR in soil and plant samples

Sulfadiazine was measured in all frozen supernatants via a validated and ISO 17025 accredited method, using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) as described before (Berendsen et al. 2015, 2018). Significance of differences in mean SDZ concentrations, expressed in µg per kg soil, between MSE and MSL treatments in bulk soils at the start of the experiment and at the moment of leek 2019 sampling and in lettuce and leek 2018 rhizosphere soils were calculated by Students t-test (n = 10).

DNA was extracted from all frozen pellets of five randomly selected samples per plant x soil treatment using the MagAttract PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to a previously described procedure (Overbeek et al. 2021). The concentration of DNA in the extracts was measured using a Pico Green assay (Quant-IT Pico Green dsDNA Assay kit, Invitrogen) on a Tecan Infinite M200Pro microplate reader (Tecan Trading AG, Switzerland). DNA concentrations were set at 4 ng per µl by dilution in elution buffer. DNA extracts from three manure (M, MSE, MSL), 15 bulk soil (S, S-NPK, S-M, S-MSE, S-MSL, three of each treatment sampled at the start of the experiment) and 180 rhizosphere soil, root and leaf samples (from five randomly selected plants from all four soil and three plant treatments) were screened for the presence of *sul*1 and *sul*2 genes in relation to the total bacterial fraction in each sample by qPCR. Therefore primers qSUL653f, qSUL719r and probe tpSUL1, directing *sul*1 (Heuer and Smalla 2007), primers qSUL2_595f and qSUL2_654r and probe tpSUL2_614, directing *sul*2 (Heuer et al. 2008), and the broad-coverage bacterial qPCR system, BactQuant, directing the V3-V4 region of bacterial16S rRNA genes (Liu et al. 2012) were applied. Reaction mixtures, consisting of 5 µM of each primer and probe and 8 ng template DNA, in a solution containing 1 × TakaRa master mix, 1 × ROX reference dye II (TaKaRa Bio Inc., San Jose, CA) and Hypure molecular grade water to a final volume of 25 µl, were run in a QuantStudio real time PCR system (ThermoFisher Scientific, Waltham, MA) under the thermocycle conditions described for each system (Heuer et al. 2008; Heuer and Smalla 2007; Liu et al. 2012). Cycle threshold values obtained with the *sul*1 and *sul*2 qPCR

systems were normalized with the corresponding Ct values obtained with BactQuant by subtraction of the Ct value of BactQuant from those of *sul*1 and *sul*2, resulting in, respectively, Δ*sul*1 and Δ*sul*2 Ct values. Significance of differences between means of Δ*sul*1 or Δ*sul*2 Ct values under the three plant, and/ or four soil treatments were calculated by one- or two-way ANOVA (n = 5). Significance of differences between of Δ*sul*1 or Δ*sul*2 Ct values of bulk soils under MSE and MSL treatments at 272 days after manure amendment to soil (time point of leek 2019 sampling) were calculated by Student t-test (n = 5).

Bacterial amplicon sequencing and shotgun metagenomics of bulk and rhizosphere soil DNA

The impact of SDZ on the bacterial community and ARG composition in rhizosphere soils was investigated by bacterial amplicon sequencing and shotgun metagenomics. Therefore, DNA extracts of five randomly selected samples per treatment, the same as used for qPCR analysis, were used for amplicon sequencing and subsequently three of these five samples were randomly selected for shotgun metagenomics. Amplicon sequencing was directly performed on DNA extracts by making use of primers E341F and 805R, targeting the V3-V4 hypervariable regions of the bacterial 16S rRNA gene (Liu et al. 2012; Herlemann et al. 2011; Klindworth et al. 2013). Sequencing of resulting amplicons, bio-informatics processing of reads and annotation to amplicon sequence variants (ASV) was performed as described before (Overbeek et al. 2021). For shotgun metagenomics, DNA was sheared before short read sequencing (Overbeek et al. 2021) and resulting metagenome reads were first taxonomically classified at protein level using Kaiju software, after which translated sequences were matched in six reading frames with annotated proteins from microbial reference genomes in databases (Menzel et al. 2016). Resulting output, expressed in maximal exact matches (MEMs) with reference genomes, were used as seeds for taxonomical identification at strain or species level and in case of ambiguity to next higher taxonomic levels. Statistical differences between mean Shannon diversity index values (H') of bulk and rhizosphere soil samples, determined either via bacterial amplicon sequencing or via shotgun metagenomics (alpha diversity), were calculated by one and two-way ANOVA. Differences in bacterial composition between samples (beta diversity) were calculated by analysis of similarities (ANOSIM), based on dissimilarity indices computed in *vegdist* using VEGAN package in R (Oksanen et al. 2015), and distances between samples were visualized in 2D ordination plots by non-metric multi-dimensional scaling (NMDS). Further, metagenome reads were classified on the basis of 100% nucleotide identity with ARGs in the ResFinder database (Florensa et al. 2022) using BBMap software (downloaded on 27.01.2020, PMID: 32780112). Statistical comparisons were made between normalized ARG reads per kilobase million (RPKM) values of S-NPK, S-M and S-MS (S-MSE and S-MSL together) rhizosphere soils for each plant treatment separately using the chi-squared Kruskal–Wallis test. Data were visualized in R environment using PHYLOSEQ package (McMurdie and Holmes 2013).

Network analysis was performed on the 200 most abundant ASVs in libraries from M, MSE and MSL-treated rhizosphere soils of leek 2018 and leek 2019. Comparison was made between rhizosphere soils that had received SDZ (S-MS) versus the one that did not have (S-M). Amplicon sequence variant abundances in the datasets were normalized by the centred log ratio transformation (clr) for SpiecEasi network analysis using *netConstruct* from NETCOMI R package (Peschel et al. 2021). The Jaccard index, adjusted rank index and network centrality measures were calculated with the function *netCompare* from NETCOMI R package. Network plots were made with IGRAPH R package v.1.3.0 2022 (Csardi and Nepusz 2006).

All sequence data, submitted to the NCBI database, is made available under the BioProject numbers PRJNA898790 (https://www.ncbi.nlm.nih.gov/bioproject/898790) (PCR amplified 16S rRNA gene sequences) and PRJNA608274 (metagenomic reads) (https://www.ncbi.nlm.nih.gov/bioproject/608274).

Results

Presence of SDZ in soils and plants

Sulfadiazine concentrations were measured in manure, soil and plants in order to determine the extent of SA selective pressure in these ecosystems. The concentration SDZ in non-treated cow manure (M) was below the limit of detection (0.25 µg per kg manure). Shortly before application to soil, SDZ in manure reached concentrations of 10,600 and 7900 µg per kg manure for, respectively, MSL and MSE (Table 1). Three hours after application of all three manures to separate field soil batches, the SDZ concentration was, as expected, below detection (limit of detection was 0.25 µg per kg soil) for S-M, but detectable for S-MSE and S-MSL at levels of, respectively, 66.6 and 82.2 µg per kg soil. When planted to lettuce, SDZ concentrations in rhizosphere soil were also measurable at 0.89 and 1.45 µg per kg soil, for, respectively, S-MSE and S-MSL, and difference of means was significant at the level of $P < 0.001$ (two-tailed t-test, n = 10) (Fig. 1). Sulfadiazine concentrations in rhizosphere soils of lettuce plants grown in S-M and S-NPK were all below detection. In root samples under all four soil treatments, SDZ concentrations were below

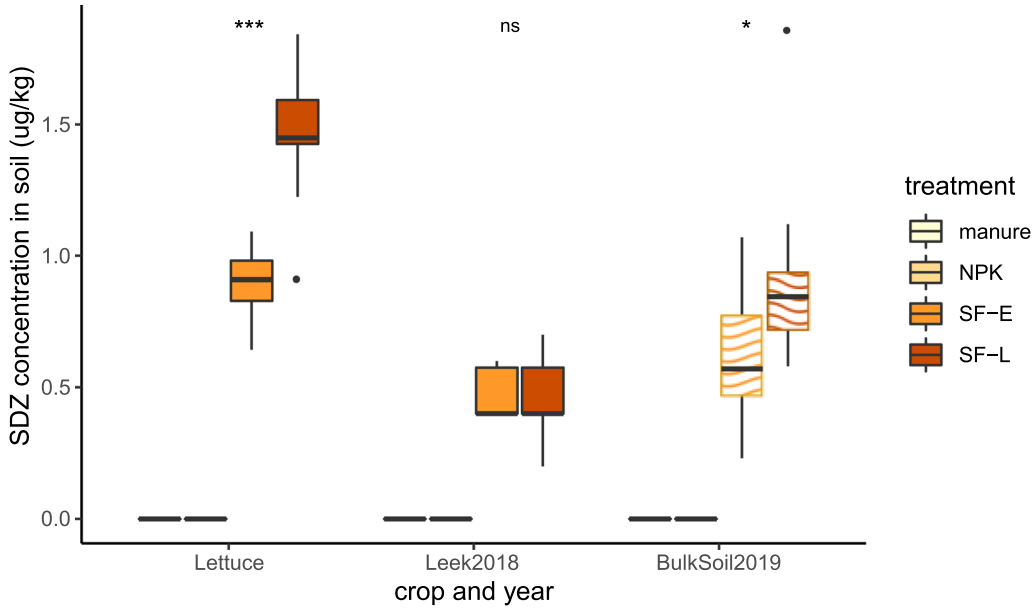

Fig. 1 SDZ concentrations (µg per kg soil) in M, NPK, MSE and MSL-treated lettuce and leek 2018 rhizosphere, and leek 2019 bulk soils. Sulfadiazine concentrations were below detection in NPK and M-treated lettuce and leek 2018 rhizosphere soils and also in all leek 2019 rhizosphere soils

detection (0.1 µg per kg plant). However, concentrations in lettuce leaves under MSE and MSL treatments were at the limit of detection for, respectively, one and three out of 10 plants, whereas in leaves under NPK and M treatments below detection in both cases. In leek 2018 rhizosphere soil, SDZ concentrations were measurable at a level of 0.47 µg per kg soil for both MSE and MSL treatments (Fig. 1) and below detection for NPK and M treatments. Sulfadiazine concentrations in roots were below detection under all four soil treatments and in leaves at the limit of detection for seven and eight samples under, respectively, MSE and MSL treatments but below detection in leaves under NPK and M treatments. At 272 DAP, SDZ was measurable in bulk soil samples at levels of 0.63 and 0.92 µg per kg soil under treatments of, respectively, MSE and MSL but undetectable in NPK and M treated bulk soils. No SDZ was measured in all rhizosphere soils, roots and leaves under the four soil treatments.

Cultivation of SDZ-resistant bacteria from leek plants

Because measurable quantities of SDZ were still present in bulk soil up to 272 DAP, bacterial cultivation was applied to determine eventual selection of SDZ-resistant bacteria in and near leek plants. In leek 2018 rhizosphere soil, significant (one-way ANOVA, $n=10$, $P<0.001$) higher mean Log SDZ-resistant CFU numbers were present under MSE and MSL (both 7.07) than under M (5.79) treatments and in leek 2019 rhizosphere soil, mean numbers were significantly (one-way ANOVA, $n=10$, $P=0.009$) higher under MSE (7.11) than under M (6.63) and MSL (6.85) treatments (Fig. 2). In leek 2018 and leek 2019 roots, mean Log SDZ-resistant CFU numbers significantly (one-way ANOVA, $n=10$, $P<0.001$ in both cases) differed between soil treatments with respective highest mean values for MSE (6.04 and 6.37), followed by MSL (5.69 and 5.63) and lowest for M (4.64 and 5.11). In leaves, mean Log SDZ-resistant CFU numbers significantly (one-way ANOVA, $n=10$, $P=0.011$) differed between soil treatments in leek 2019 only and numbers were lower under M (2.76) than under MSL and MSE treatments (respectively, 4.10 and 4.32 and both mean values were statistically indistinguishable from each other) (Fig. 2).

Detection of *sul*1 and *sul*2 genes in soils and plants

The selective effect of SDZ measured in bulk, and in lettuce and leek rhizosphere soils was further investigated on two SA-resistance genes, *sul*1 and *sul*2. Quantitative PCR, using primers directing *sul*1 and *sul*2 genes (*sul*1 and *sul*2 qPCR systems) and broad-coverage bacterial 16S rRNA V3-V4 gene region (BactQuant system), used for correction in bacterial fluctuations, revealed presence of *sul*1, *sul*2 and bacterial 16S rRNA genes in untreated manure and field soil samples at Ct values of, respectively 31.3, 27.1 and 14.5 for manure and 27.1, 25.4 and 14.9 for soil. When corrected for the variation in the bacterial fraction per sample, resulting *sul*1 and *sul*2 ΔCt values were, respectively, 16.8 and 12.6 for manure and 12.2 and 10.6 for field soil (Table 1). These values indicate that both SA genes were already present in the untreated

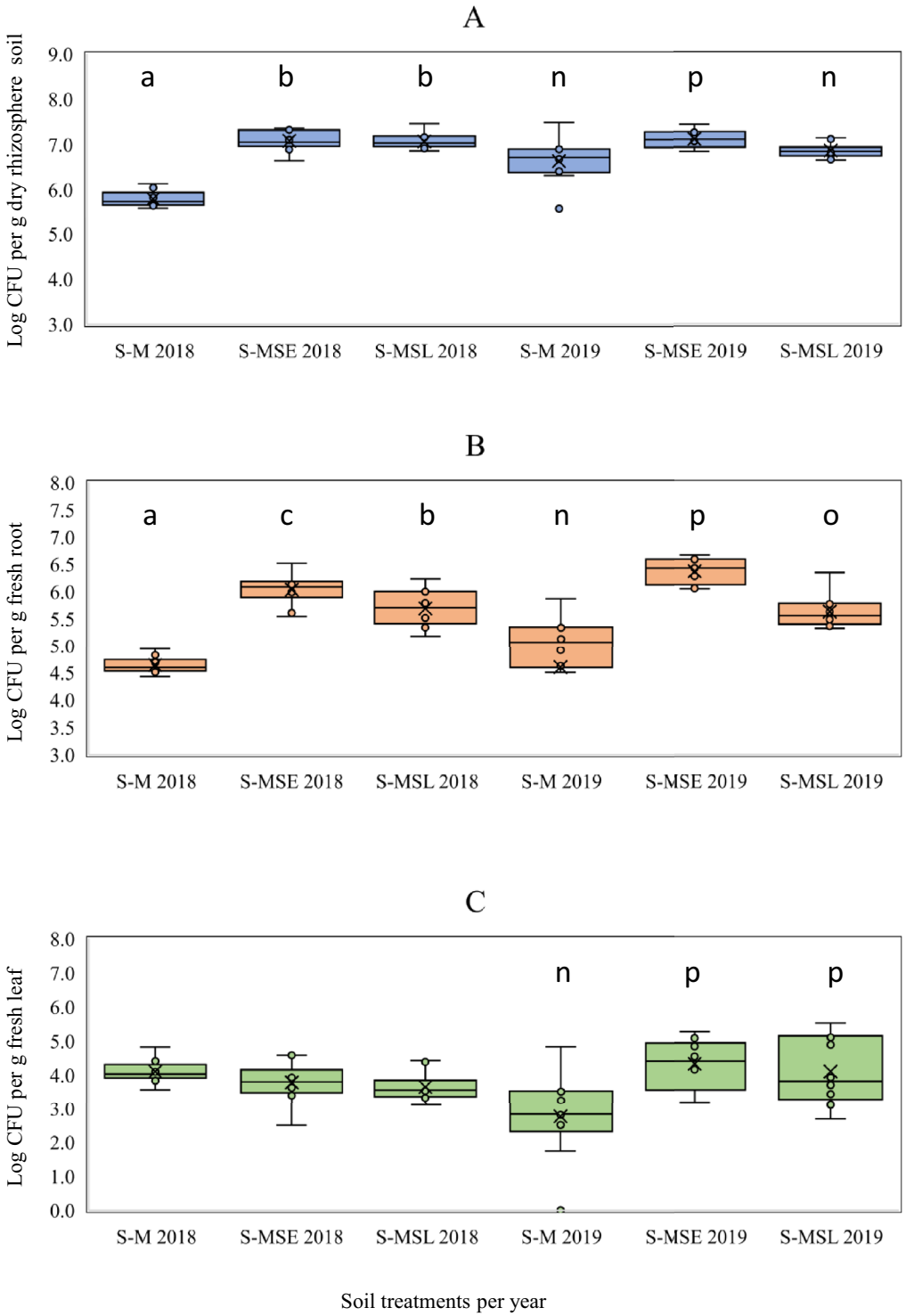

Fig. 2 Log SDZ-resistant CFU numbers in rhizosphere soil (**A**), roots (**B**) and leaves (**C**) of leek plants harvested at two time periods (leek 2018, leek 2019) and grown in M, MSE and MSL-treated soils. a, b, c (leek 2018) and n, o, p (leek 2019) indicate significant differences between mean values of soil treatments (n = 10), calculated by one-way ANOVA, where a < b < c and n < o < p. For leek 2018 rhizosphere soil, $P < 0.001$ and least significant difference (LSD) = 0.23; for leek 2019 rhizosphere soil, $P = 0.009$, LSD = 0.28; for leek 2018 roots, $P < 0.001$, LSD = 0.23; for leek 2019 roots, $P < 0.001$, LSD = 0.29; for leek 2019 leaves, $P = 0.011$, LSD = 0.79)

manure and field soils and the question is how SDZ in treated manure and manured soils would influence their abundances in comparison with non-treated manure and manured soil. When amended with SDZ, ΔCt values for *sul*1 and *sul*2 genes in resulting MSE and MSL batches were, respectively, 13.3 and 3.91 for MSE and 14.4 and 9.86 for MSL. When MSE and MSL batches were mixed through field soil samples, resulting *sul*1 and *sul*2 ΔCt values were, respectively, 12.7 and 6.84 for S-MSE, and 12.9 and 9.96 for S-MSL. For control soils, respective *sul*1 and *sul*2 ΔCt values were 12.7 and 11.3 for S-M and 12.5 and 10.8 for S-NPK. In rhizosphere soils under the three plant and four soil treatments, ΔCt values were between 10.4 and 13.5 for *sul*1 and between 10.8 and 13.2 for *sul*2 and in roots, ΔCt values were between 7.76 and 15.8 for *sul*1 and between 8.24 and 15.0 for *sul*2 (Fig. 3). In leaves, ΔCt values could not be calculated because *sul*1 and *sul*2 Ct values were ≥ 37 (corresponding to < 1 target molecule in the qPCR reaction mixture) in all cases, whereas 16S rRNA Ct values were between 11.6 and 25.7.

Variation in *sul*1 and *sul*2 ΔCt values in rhizosphere soils could not be explained by soil treatments in all cases (Fig. 3). Effects of soil treatments on mean *sul*1 and *sul*2 ΔCt values were only found in leek 2018 rhizosphere soil samples with significantly lower mean *sul*1 ΔCt values for NPK, 11.6, than for the other three treatments, between 12.1 and 12.5 (one-way ANOVA, n = 5, P = 0.002), and significant higher mean *sul*2 ΔCt values for MSE, 12.4, than for the other three soil treatments, between 11.4 and 11.8 (P = 0.01). No significant effects of soil treatment on *sul*1 and *sul*2 ΔCt values were found among all lettuce and leek 2019 rhizosphere soil and root samples. However, significant differences between mean *sul*1 and *sul*2 ΔCt values in rhizosphere soils were found between different plant types across all four soil treatments, but differences between means of both ΔCt values were not consistent over the three plant treatments. Namely, the mean ΔCt value of *sul*1, 12.7, was significantly higher (two-way ANOVA, n = 5, P < 0.001) than of *sul*2, 11.5, in lettuce rhizosphere soil, which was the same for leek 2018 rhizosphere soil (12.2 and 11.8 for, respectively, *sul*1 and *sul*2; P = 0.002). This to the contrary for leek 2019 rhizosphere soils where the mean ΔCt value of *sul*1, 11.8, was significantly lower (P = 0.046) than of *sul*2, 12.3. About the same picture became clear for root samples, namely no soil treatment effect on mean *sul*1 or *sul*2 ΔCt values were found across all plant treatments, whereas significant differences between mean *sul*1 and *sul*2 ΔCt values were found between different plant types across all soil treatments. In lettuce roots the mean ΔCt value of *sul*1, 14.2, was significantly higher (P < 0.001) than of *sul*2, 13.1 (Fig. 3). In leek 2018 roots, however, the difference in means between *sul*1, 10.7, and *sul*2, 10.3, ΔCt values were just above the arbitrary level of significance (P = 0.07), whereas in leek 2019 roots, the ΔCt value for *sul*1, 9.54, was significantly lower (P = 0.003) than for *sul*2, 10.4.

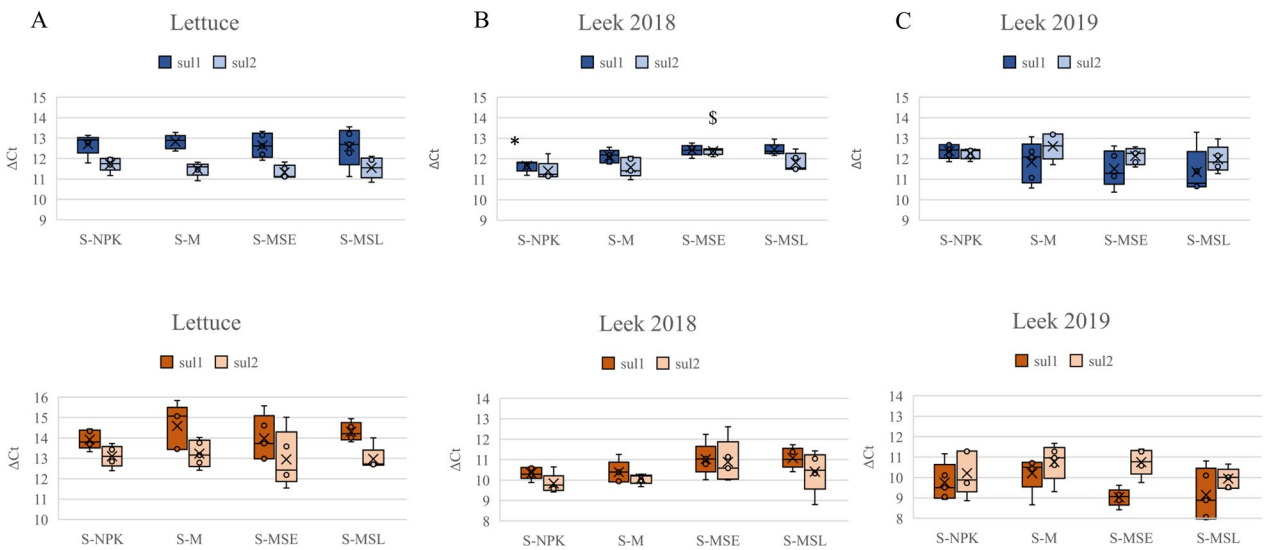

Fig. 3 Presence of *sul*1 and *sul*2 genes, normalized to the total 16S rRNA genes and expressed in ΔCt values, in rhizosphere soils (respectively, dark and light blue) and roots (respectively, dark and light brown) of lettuce (**A**), leek 2018 (**B**) and leek 2019 (**C**). *, indicate significantly lower than *sul*1 ΔCt values (P = 0.002) of the other soil treatments; $, indicate significantly higher than *sul*2 ΔCt values of the other soil treatments (P = 0.01). Significance of difference between mean *sul*1 and *sul*2 ΔCt values, over all soil treatments were: P < 0.001 (*sul*1 > *sul*2, LSD = 0.33) for lettuce rhizosphere soil; P = 0.002 (*sul*1 > *sul*2, LSD = 0.22) for leek 2018 rhizosphere soil; P = 0.046 (*sul*1 < *sul*2, LSD = 0.45) for leek 2019 rhizosphere soil; P < 0.001 (*sul*1 > *sul*2, LSD = 0.53) for lettuce roots; P = 0.07 (*sul*1 = *sul*2), for leek 2018 roots; P = 0.003 (*sul*1 < *sul*2, LSD = 0.54) for leek 2019 roots

Effect of SDZ on rhizosphere bacterial communities, association networks and ARG abundances

Finally, the selective effect of SDZ still present in bulk and rhizosphere soils on bacterial community composition, association networks and ARG abundances was investigated. Bacterial community composition analysis performed via amplicon sequencing, using bacterial 16S rRNA-directed primers on DNA extracts of rhizosphere soil samples under the three plant, and four soil treatments (n = 5), revealed strong distinctions in alpha and beta-diversities between plant treatments, and weak distinctions between soil treatments. The Shannon index values (H') on ASVs, determined via bacterial amplicon sequencing on all bulk and rhizosphere soil samples, were between 4.59 and 6.68. The effect of plant treatment was significant at a level of $P < 0.001$ in two-way ANOVA (n = 5) with lowest mean value for bulk soil (5.57), followed by lettuce (5.78) and highest for leek 2018 and leek 2019 (respectively, 6.37 and 6.28 and both mean values were not significantly different from each other) (Additional file 1: Fig. S1). There was no significant effect of soil treatment over all plant types, and also not in one-way ANOVA for each plant treatment separately, although for leek 2018 the effect of soil treatment was just above the arbitrary level of significance ($P = 0.067$, lowest value for treatment with NPK, 6.27, and highest for the one with M, 6.49). The Shannon index values (H') based on MEMs, determined via shotgun metagenomics, a PCR-independent approach to determine the bacterial community composition, on all bulk and rhizosphere soil samples, were between 2.88 and 3.88 and these values were overall lower than when determined via amplicon sequencing. Mean values between rhizosphere and bulk soils, across all soil treatments, were significantly different ($P = 0.005$, two-way ANOVA, n = 3) with highest value for bulk soil (3.73) versus the three rhizosphere soils (3.51–3.60). Mean values, over all soil treatments, were significantly lower in leek 2019 rhizosphere soil (3.51) than in the ones of lettuce and leek 2018, respectively, 3.60 and 3.59. Across all plant treatments, the soil treatment with NPK resulted in a lower mean H' value (3.50) than with the other three treatments (between 3.62 and 3.66) at significance level of $P = 0.047$ and there was no significant interaction between plant x soil treatments (Additional file 1: Fig. S1).

Sample diversity in ASVs based on bacterial amplicon sequencing data and visualized in the NMDS plot (Fig. 4A) significantly differed between bulk and all rhizosphere soils ($R = 0.852$; $P < 0.001$), between lettuce, leek 2018 and leek 2019 rhizosphere soils ($R = 0.821$; $P < 0.001$) and between leek 2018 and leek 2019 rhizosphere soils ($R = 0.384$; $P < 0.001$), whereas it was not significant

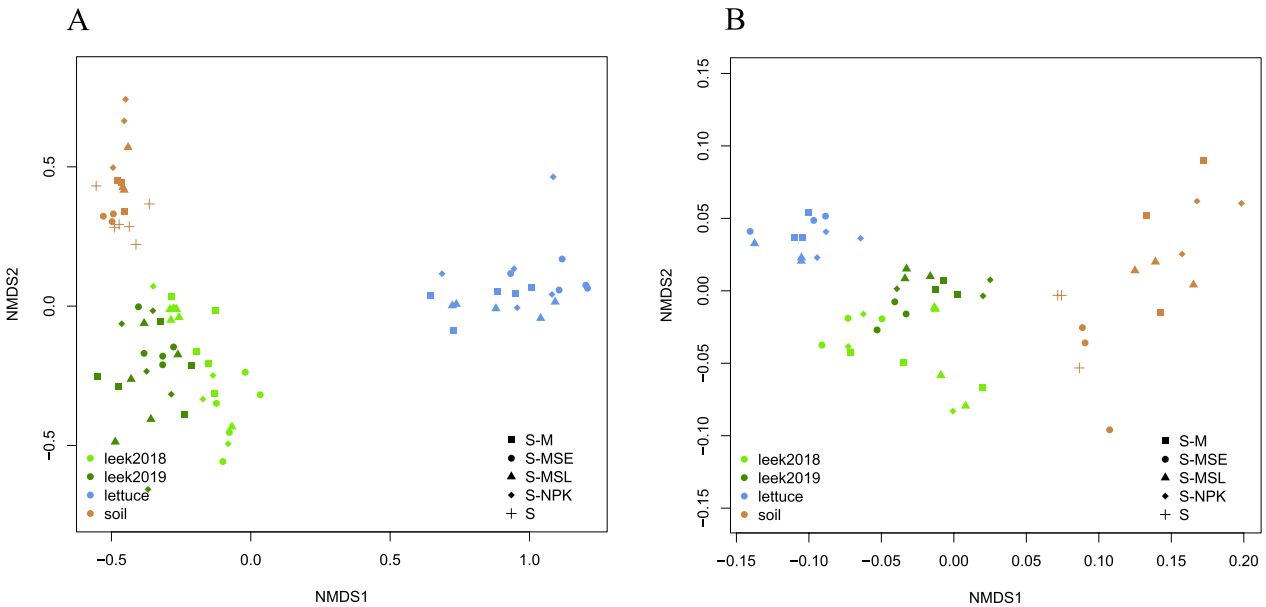

Fig. 4 Non-metric multi-dimensional scaling (NMDS) biplot showing the diversity in bacterial community composition in bulk soil and in lettuce, leek 2018 and leek 2019 rhizosphere soils under different soil treatments and determined via 16S rRNA gene amplicon sequencing (**A**, n = 5) and shotgun metagenomics (**B**, n = 3), with dimensions = 2 and stress = 0.078. For (**A**), differences between bulk and all rhizosphere soils were significant at the level of $P < 0.001$ (ANOSIM, $R = 0.852$) and between lettuce, leek 2018 and leek 2019 rhizosphere soils at the level of $P < 0.001$ ($R = 0.821$), and between leek 2018 and leek 2019 rhizosphere soils at the level of $P < 0.001$ ($R = 0.384$). For (**B**), differences between bulk and all rhizosphere soils were significant at the level of $P < 0.001$ (ANOSIM, $R = 0.4307$) and between individual rhizosphere soils at the level of $P < 0.001$ ($R = 0.3294$)

between soil treatments. However, bacterial association network comparisons focusing on interactions between individual ASV's, on the same amplicon sequence data from leek 2018 and leek 2019 samples that had received SDZ (S-MS: S-MSE and S-MSL together) versus the one that had not (S-M), revealed significant differences in four centrality parameters: degree, betweenness, closeness, and eigenvector (Additional file 1: Table S1). The interactions between taxonomic groups (at genus level) were lower in manured rhizosphere soil samples with SDZ in comparison with the ones without, whereas the percentage of positive edges was significantly higher in presence (88.7%) than in absence (73.5%) of SDZ (Additional file 1: Fig. S2, Table S1). The taxonomic composition based on MEMs, determined via shotgun metagenomics on all plant and soil treatments, also did not reveal any significant effect of soil treatment on bacterial composition. However, there was a significant segregation between bulk and all rhizosphere soil samples ($R = 0.4307$; $P < 0.001$) and between rhizosphere soils of the separate plant treatments ($R = 0.3294$; $P < 0.001$) in the NMDS plot (Fig. 4B).

Metagenome reads matching ARGs and normalized over the total abundance of reads per sample (normalized ARG RPKM) revealed differences in ARG abundance among soil treatments for lettuce and leek 2019 (Fig. 5A). Under these plant treatments, abundances in ARGs were higher in S-MS than in S-M and S-NPK for leek 2019 and lettuce at significance levels of, respectively, $P = 0.06$ and $P = 0.02$. Further, qualitative differences in ARG composition between rhizosphere soils of leek 2018 and of leek 2019 versus the one of lettuce plants were present (Fig. 5B). Although SA resistance genes dominated in all samples, the leek 2018 and leek 2019 samples additionally contained aminoglycoside and tetracycline resistance genes that were lower (aminoglycoside resistance genes) or that were virtually absent (tetracycline resistance genes) in all lettuce rhizosphere soil samples.

Discussion

The overall effects of SDZ in manure on bacterial resistances in manured soil and plants was investigated and it revealed that amendment of SDZ has led to a drastic effect in the *sul*1: *sul*2 gene ratio in cow manure and in resulting manured soils. In the presence of SDZ, *sul*2 genes were clearly more stimulated than *sul*1 genes and this effect even became more apparent when SDZ was longer present in manure as demonstrated by higher *sul*2 gene abundance in MSE than in MSL. The over 400-fold higher *sul*2 gene abundance in MSE to M demonstrated that SDZ was a strong and specific selector for bacterial populations carrying *sul*2 genes in manure. Because *sul*1 and *sul*2 genes were more often found to be associated with different classes of MGEs, the difference in selection of both genes may be representative for bacterial populations carrying different MGEs. The question that further needed to be addressed was if these bacterial populations or their MGEs, pre-enriched in manure and carrying *sul*2 genes, would be transferred via manured soil to edible plants growing in these soils.

Effects of manure application on the soil bacterial community and resistome compositions were shown to be transient (Macedo et al. 2021). The estimated time of recovery of a soil to the ARG level before manure application was estimated between 29 and 42 days (Macedo et al. 2020), which coincided with time of sampling of the lettuce plants (39 days), but which was shorter than the time points for sampling of the leek plants (90 and 272 days). Bacterial invasion from manure to soil was also observed before (Billet et al. 2022) and in another study (Overbeek et al. 2021) it was demonstrated that bacterial species typical for the cow gut system, i.e. *Clostridium* and *Romboutsia* species, were present in manure-treated rhizosphere soil of lettuce and leek plants, but not in the ones treated with mineral fertilizer. However, human pathogens may be selected under SDZ pressure (Ding et al. 2014) and some opportunistic human pathogens were indeed shown to carry *sul*2 genes, as was the case for *Stenotrophomonas maltophilia* (Toleman et al. 2007) and monophasic *Salmonella* Typhimurium ST34 strains (Branchu et al. 2019). Because of the association of *sul*2 genes with plasmids, selection for SA resistance can lead to co-selection of other antibiotic and metal resistance genes, of which the coding regions are located on the same MGE (Branchu et al. 2019; Heuer and Smalla 2007; Miller et al. 2022). The presence of aminoglycoside and tetracycline resistance genes in the leek rhizosphere soil metagenome may indicate that co-selection for these genes took place under SDZ selection.

The effect of SDZ amendment on the bacterial community structure in bulk soil revealed major shifts in bacterial taxa and abundance of particular genes involved in N cycling in farmland soils, but also in rhizosphere soils of maize and clover (Ding et al. 2014; Hou et al. 2023;

(See figure on next page.)

Fig. 5 Normalized total ARG read numbers (**A**) and the normalized read numbers specific per ARG class (**B**) in rhizosphere soils of lettuce, leek 2018 and leek 2019. The group of SA resistance genes is classified under 'folate pathway antagonist' in the legend of **B**. Sulfonamides are the only inhibitors of the dihydropteroate metabolic pathway in bacteria

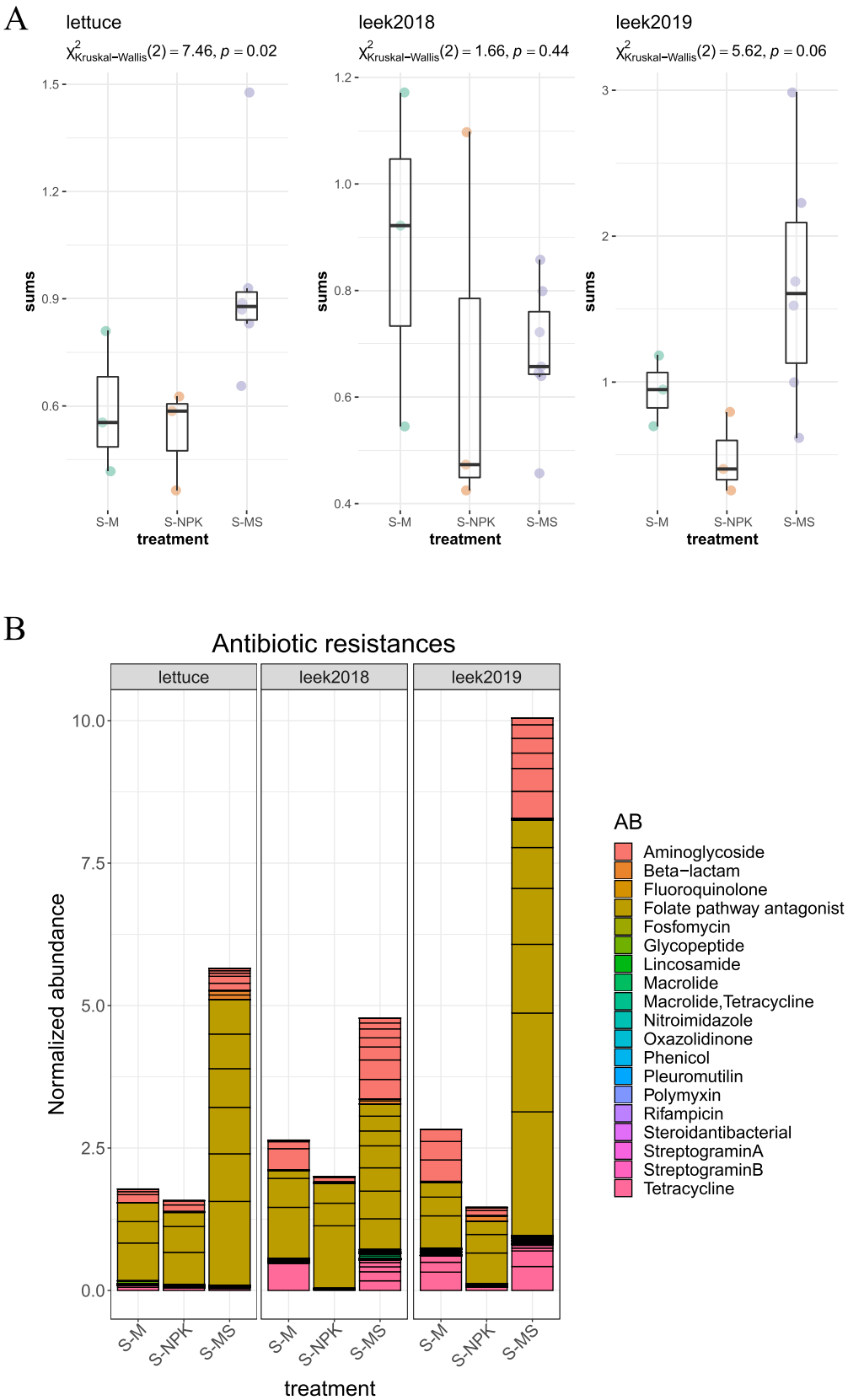

Fig. 5 (See legend on previous page.)

Ollivier et al. 2010). These studies demonstrate that effects of SDZ on bacterial populations in bulk and rhizosphere soils can be present and even may lead to changes in the functionality of soils with respect to cultivation of plants. A higher abundance of *sul*2 over *sul*1 genes was present in rhizosphere soils and roots of lettuce and leek 2018 plants. However, this effect was overall and not specific for the treatments with MSE and MSL, where higher abundances of *sul*2 over *sul*1 genes would be expected in comparison with M and NPK treatments. Admittedly, there was a significant effect of MSE over the other three treatments among leek 2018 plants, but the higher ΔCt value for *sul*2 genes -meaning a lower *sul*2 gene level- in S-MSE versus the other three soils was opposite to what was expected based on the low *sul*1: *sul*2 gene ratio already present in MSE. This indicates that plant growth exerted a stronger effect than SDZ on the *sul*1: *sul*2 gene ratio in soil. This effect even became more apparent in leek 2019 rhizosphere soils and roots, where a higher abundance of *sul*1 over *sul*2 genes was present, indicating that plant species type and growth stage are factors determining the abundance of both genes. Overall effects of plant growth on *sul* gene abundance in rhizosphere soil has been demonstrated before (Kopmann et al. 2013; Jechalke et al. 2013; Man et al. 2022; Wang et al. 2015). A linear increase in relative *sul*1 and *sul*2 gene numbers in rhizosphere soils was observed in the time span after SDZ manure application to soil planted with maize and grass, demonstrating that SA-resistant populations were building up over time (Jechalke et al. 2013). This to the contrary to unplanted soils in the same study, where relative *sul*1 and *sul*2 gene numbers remained at the same level after SDZ manure application, which was most likely due to the lower metabolic activity of bacterial cells in bulk, than in rhizosphere soils.

Plant species type and growth stage strongly influenced the bacterial community composition in rhizosphere soil. This is not surprising as these effects on bacterial communities in rhizosphere soil were shown before in potato plants (Overbeek et al. 2008). However, it does not imply that there was no effect of SDZ at all on selection for antibiotic resistances in rhizosphere soil, but the effects of SDZ were concealed by plant growth. Via network association analysis, it revealed that particular taxonomic groups were co-selected in rhizosphere soils that had received SDZ via MSE or MSL. Some of these groups still may have originated from SDZ-amended manure and proliferated in rhizosphere soil, but it is also possible that SA-resistance genes were horizontally transferred from bacterial populations intrinsic in manure to the ones intrinsic in the soil–plant environment.

Sulfadiazine was also measured in low quantities in leek 2018 leaf samples, but not in root samples. The amount of available root material used for measurements was most likely too low for detection of SDZ. However, SDZ can be taken up by roots (Camacho-Arévalo et al. 2021), after which it systemically spreads throughout the plant. Sulfadiazine was measured in bulk soil at leek 2019 sampling, but was not present in measurable quantities anymore in rhizosphere soils, roots and leaves, although it cannot be concluded that SDZ was totally absent in these samples. Effects of antibiotics on bacterial communities, even at low levels, still exist (Davies et al. 2006; Gullberg et al. 2011). Therefore, it is plausible that SDZ selection pressure took place in all lettuce and leek leaves, explaining the observation that higher Log SDZ-resistant CFU numbers were found under MSE or MSL, than under M treatments in leek 2019 leaf samples. However, antibiotic resistant phenotypes may also result from intrinsic resistances in bacteria and thus not only from acquired genes.

Presented research fits in the holistic view on microbiome interconnectedness between animal, soil, plant and human ecosystems (Sessitsch et al. 2023). Within this context, soil and plant microbiomes are explicitly included in the one health concept, whereas soils must be regarded as vast reservoirs of ARGs (Banerjee and Heijden 2023; Singh et al. 2023). Microbial communities from different sources coalesce in plant production soils, this taking into account that plant production systems often are irrigated with water from surface water bodies that come into contact with effluent from wastewater treatments plants (Marutescu et al. 2023). The outcome of mixing of entire microbial communities on transmission of AMR is therefore rather unpredictable (Zhu et al. 2023) and this will have consequences for the microbial safety of our food, especially when it is consumed without prior heating steps (Miller et al. 2022). However, presence of ARGs in food products resulting from antibiotics either applied in husbandry or from clinical use should always be placed in the context of ARGs indigenously present in soils (Banerjee and Heijden 2023; Heuer et al. 2002; Overbeek et al. 2002; Nikolakopoulou et al. 2005; Obermeier et al. 2021). For this reason, ARGs can always be found in our food products (Miller et al. 2022), it are, however, the ones that are acquired via HGT and transmissible to clinically relevant bacterial species that are of particular concern (Velazquez-Meza et al. 2022).

Conclusion

Sulfadiazine present in manure has an impact on antimicrobial resistances in plants that can be consumed freshly. The microbial interactions in manured soil with plants is complex and growing plants were shown to exert strong effects on *sul* genes and on bacterial communities present in these soils. The presence of SA resistances in plants grown in manured soils can therefore not only

be explained by transmission of SDZ-resistant bacteria from manure to the soil–plant ecosystem. Transmission of antibiotic resistances must be placed into the context of soils as vast reservoirs of ARGs. Co-selection of different antibiotic and metal resistances by antibiotic residues still present in agricultural production soils is hazardous because of accumulation of ARGs in our food. Control of ARGs in crop production require a one health approach including all ecosystems relevant for plant production, such as soil, water, plants and livestock animals.

Abbreviations

ARG	Antibiotic resistance gene
DAP	Day after planting
M	Manure
MGE	Mobile genetic element
MSE	Manure SDZ application early
MSL	Manure SDZ application late
NaPPi	Sodium pyrophosphate
NPK	Nitrogen, Phosphorous, Potassium fertilizer
S	Soil
SA	Sulfonamide
SDZ	Sulfadiazine
S-MS	Soil treatments with MSE and MSL combined

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43170-023-00174-2.

Additional file 1: Figure S1. Shannon diversity index values (H') from NPK, M, MSE and MSL-treated rhizosphere soils of lettuce, leek 2018 and leek 2019, based 16S rRNA gene amplicon sequencing (A) and shotgun metagenomics (B). Differences in mean H' values between plants across soil treatments were significant at the level of $P < 0.001$ (two way ANOVA, $n = 5$) for amplicon sequencing and $P < 0.001$ (two way ANOVA, $n = 3$) for shotgun metagenomics. Differences in mean H' values between soil treatments across all plants were significant at the level of $P = 0.047$ (two way ANOVA, $n = 3$) for shotgun metagenomics only and interactions between plant x soil treatments were not significant with both methods. **Figure S2.** Bacterial association network comparisons between ASVs, at genus level, from leek 2018 and leek 2019 rhizosphere soils. Comparisons were made between samples where SDZ was amended to manure, S-MS (S-SME and S-MSL combined) versus S-M, where no SDZ was amended to manure. Jaccard index significantly differed between both networks for the following descriptors: degree ($P < 0.05$), betweenness ($P < 0.001$), closeness ($P < 0.001$) and eigenvector centralities ($P < 0.001$) (Table S1). Connections between ASVs are indicated in red (negative) and in green (positive) edges; nodes with the same colour belong to the same cluster. **Table S1.** Centrality parameter values measured by making bacterial association network comparisons, based on amplicon sequencing data from rhizosphere soils, between SDZ-containing soils (S-MS) and soil without SDZ (S-M).

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Author contributions
LvO and BB received the research grant; LvO and BAJ wrote the first version of the manuscript; LvO, BB, EN and LR designed the experiment, EN, LR, BAJ and KA performed amplicon sequencing and metagenome data analyses, BB performed SDZ measurements; SA, performed qPCR analysis.

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Availability of data and materials
The datasets used and analyzed during the current study and R scripts are available in the 4TU Research Data database (https://doi.org/10.4121/21632186). All sequence data, submitted to the NCBI database, is made available under the BioProject numbers PRJNA898790 (https://www.ncbi.nlm.nih.gov/bioproject/898790) (PCR amplified 16S rRNA gene sequences) and PRJNA608274 (metagenomic reads) (https://www.ncbi.nlm.nih.gov/bioproject/608274).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
Not applicable.

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