Heliyon 9 (2023) e16052

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

The impact of antibiotic residues on resistance patterns in leek at harvest

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ARTICLE INFO

Keywords:
Antibiotic residues
Antibiotic resistance
Antibiotic resistance genes
Antibiotic resistant bacteria
Manure
Soil
Crops
Leek

ABSTRACT

When crops are cultivated on fields fertilized with animal manure, the risk exists that plants may take up antibiotic residues and may be exposed to antibiotic resistance genes and antibiotic resistant bacteria. During cultivation in a greenhouse pot experiment, leek (*Allium porrum*) was fertilized with either pig slurry or mineral fertilizer and exposed to either no antibiotics, doxycycline (10,000 μg/kg manure), sulfadiazine (1000 μg/kg manure), or lincomycin (1000 μg/kg manure). At harvest, 4.5 months later, lincomycin, sulfadiazine or doxycycline were not detected in any of the leek samples nor in their corresponding soil samples. Further, antimicrobial susceptibility testing was performed on 181 *Bacillus cereus* group isolates and 52 *Pseudomonas aeruginosa* isolates from the grown leek. For the *B. cereus* group isolates, only a small shift in MIC50 for lincomycin was observed among isolates from the lincomycin and control treatment. For *P. aeruginosa*, only in the setup with doxycycline treatment a higher MIC50 for doxycycline was observed compared to the control, specifically the isolates selected from growth media supplemented with 8 mg/L doxycycline. Nine antibiotic resistance genes (*tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *erm*(B), *erm*(F) and *sul*2) were investigated at harvest in the leek and soil samples. In the leek samples, none of the antibiotic resistance genes were detected. In the soil samples fertilized with pig slurry, the genes *erm*(B), *erm*(F), *tet*(M), *sul*2, *tet*(W) and *tet*(O) were detected in significantly higher copy numbers in the lincomycin treatment as compared to the other antibiotic treatments. This could be due to a shift in soil microbiota induced by the addition of lincomycin. The results of this study indicate that consumption of leek carries a low risk of exposure to antibiotic residues or antibiotic resistance to doxycycline, sulfadiazine or lincomycin.

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https://doi.org/10.1016/j.heliyon.2023.e16052
Received 12 September 2022; Received in revised form 7 March 2023; Accepted 3 May 2023
Available online 5 May 2023

1. Introduction

Incorporation of animal manure in soil is an important agricultural practice for a successful crop cultivation. Antibiotic residues (ABRs) and antibiotic resistance genes (ARGs) are frequently found in animal manure, however, and are also detected in agricultural soils fertilized with animal manure [1–7]. Previously, Van den Meersche et al. demonstrated the dynamics of 6 ABRs (sulfadiazine, trimethoprim, doxycycline, oxytetracycline, ceftiofur, and tylosin) and 9 ARGs (*tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *erm*(B), *erm*(F) and *sul*2) and observed that sulfadiazine and doxycycline and all investigated ARGs could be observed in soil until harvest (5–7 months after fertilization) [7]. Furthermore, flumequine, doxycycline, oxytetracycline, lincomycin and sulfadiazine were previously found to be the most abundant ABRs in soils and slurry [6]. Through this route, ABRs may even leak into ground- and surface water [8]. Consequently, this practice contributes to an accelerated antibiotic resistance selection through the circulation of ABRs, ARGs and resistant bacteria between humans, animals and environmental sources such as contaminated soils, polluted rivers, groundwater and crops [9]. When crops are cultivated on fertilized fields, the risk therefore exists that ABRs would be taken up by the plants [5,10–14]. Furthermore, accumulation and dissemination of ARGs in the environment enhances the risk of transferring ARGs to human (pathogenic) bacteria, ultimately resulting in reduced efficacy of antibiotic treatments [10,15]. One possible route is the transfer of ARGs from soil- and manure dwelling bacteria to the plant microbiota, mainly through horizontal gene transfer mechanisms [5,14]. Humans can be exposed to the plant microbiota by handling or consuming vegetables, which in turn may result in transfer of antibiotic resistance to commensals in the human gastrointestinal tract [5,14]. It is important to investigate these potential route for antibiotic resistance transfer as this is a One Health topic and it may be of great concern for public health [16,17]. Over the last 15 years, several studies have described the uptake of ABRs in different vegetable types. Among other antibiotics, the sulfonamides have been detected in lettuce, potato, tomato, cucumber, pakchoi, radish, rape, celery, coriander, cabbage, endive and spinach in varying concentrations up to 1 mg/kg dry weight [10,13,18–20]. Fluoroquinolones have been detected in carrots, pakchoi, radish, rape, celery, coriander, cabbage, endive and spinach in very low to high concentrations (0.5–450 μg/kg dry weight) [10,19–21]. Tetracycline has been detected in a wide concentration range in wheat, tomato, lettuce, cucumber, radish, rape, celery, coriander, cabbage, endive and spinach (6–532 μg/kg dry weight) [10,18,20,22]. Lincomycin has been detected in celery, coriander, radish and rape in concentrations ranging from 0.4 to 20 μg/kg dry weight [20]. In those studies, however, the exposure of crops to ABRs was often investigated after soil inoculation with much higher antibiotic concentrations than generally found in animal manure spread on fields.

In addition to antibiotic uptake, the presence of ARGs in and on vegetables has also been investigated [5,10,12,23–26]. According to Zhang et al. (2017), the resistance genes *tetX*, $bla_{\text{CTX-M}}$, *sul*1 and *sul*2 were present in the endophytic system of pakchoi and their numbers increased along with increasing antibiotic concentrations. The ratio of antibiotic-resistant endophytic bacteria to the total cultivable endophytic bacteria in pakchoi also rose significantly as antibiotics accumulated [23]. Furthermore, fertilization of soils with manure has been shown to be the main driver for the presence of ARGs in vegetables. The type of vegetable is an important determinant for the presence of ARGs [12,26,27].

The objectives of this study were to evaluate 1) the presence of doxycycline, lincomycin and sulfadiazine, 2) the presence of 9 ARGs and 3) the presence of antibiotic resistant bacteria in leek at harvest cultivated in pots containing soil fertilized with pig slurry or mineral fertilizer and artificially contaminated with either doxycycline (10,000 μg/kg slurry), lincomycin (1000 μg/kg slurry) or sulfadiazine (1000 μg/kg slurry). In this way, we studied the crop at the time point that ARGs, ABRs and (antibiotic resistant) bacteria could be transferred to the human gut through consumption of leek. Those 3 antibiotics were chosen as they belong to different antibiotic classes that have been frequently detected in relatively high concentrations in animal manure and agricultural soil [1,2,6]. Their persistence in soil depends on the soil properties, the molecular structure and physicochemical properties of the compound itself. Tetracyclines have half-lives of a few days to months in soil and are predicted to have sometimes half-lives up to 500 days in natural conditions [28,29]. In contrast, sulfonamides are mobile compounds which is reflected in lower half-lives from <1 to 53 days [29,30].

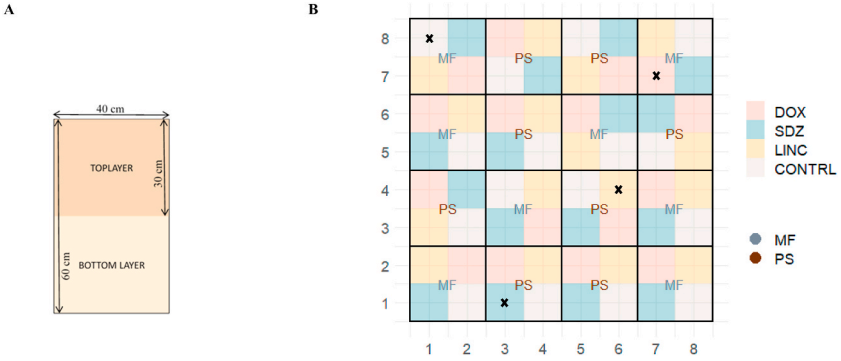

Fig. 1. A. Schematic representation of the different layers of the tub. B. Schematic representation of the experimental setup of the pot experiment in the greenhouse. The 8 different treatments are shown in color (doxycycline = DOX (red), sulfadiazine = SDZ (blue), lincomycin = LINC (yellow), no antibiotic = CONTRL (white), mineral fertilizer = MF (blue), pig slurry = PS (brown)). Each treatment is replicated 8 times and randomized in the greenhouse. Four crosses indicate the locations of the TMS4 data loggers. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

According to Berendsen et al., lincosamides dissipate relatively quickly in soil with half-lives between 1.1 and 11 days [31]. Leek was chosen as a model vegetable because it has a high nitrogen (= fertilization) demand and in practice (in contrast to other vegetables) is often fertilized with animal manure. Leek is commonly produced in Belgium (about 108,850 tons per year) and 1.74 kg per capita was used for home consumption in 2020, which makes leek the 7th most purchased vegetable. Shifts in minimum inhibitory concentrations (MICs), the lowest concentration of an antibiotic at which bacterial growth is completely inhibited, were investigated for *Bacillus cereus* group and *Pseudomonas aeruginosa* isolates from leek. Those bacteria are relevant to human health as they are foodborne or opportunistic human pathogens or human commensals. They were frequently found in and on leek during preliminary tests, specifically 16S rDNA metabarcoding and plate counts (data not published). This leek experiment was designed to mimic common agricultural practice in order to reach a realistic estimation of human exposure to ABRs and resistance via consumption of leek.

2. Materials and methods

2.1. Pot experiment

2.1.1. Experimental setup

Leek was grown in a pot experiment in an open greenhouse (Fig. 1). Three antibiotics (doxycycline, lincomycin and sulfadiazine) and two fertilizers (mineral fertilizer and pig slurry) were incorporated into the upper 30 cm ("topsoil") of the soil. Doxycycline, lincomycin and sulfadiazine were selected based on their high detection rates and high concentrations in pig and cattle manure in previous research [1,2]. Mineral fertilizers serve as an appropriate reference for the introduction of ABRs only, thus without the introduction of antibiotic resistant bacteria and ARGs as they do not contain the complex microbiota. The concentrations inoculated in the present setup were a comparable order of magnitude as the maximum concentrations (with exclusion of outliers) found in pig slurry. The aim was to represent a realistic worst-case scenario [1]. Doxycycline (doxycycline hyclate, Sigma-Aldrich), lincomycin (lincomycin HCL, Sigma-Aldrich) and sulfadiazine (sulfadiazine sodium salt, Sigma-Aldrich) were administered at concentrations of 10,000 μg/kg, 1000 μg/kg and 1000 μg/kg slurry, respectively. An additional 2 treatments (fertilization with pig slurry or mineral fertilizer without antibiotics added) were included as controls. Each of the 8 treatments was performed in 8 repetitions (64 pots in total).

The experiment was carried out in deep tubs (depth 60 cm, diameter 40 cm; Fig. 1). Soil depth of at least 60 cm is required for a good rooting of leek. Prior to planting, the tubs were filled to a height of 30 cm with a soil-compost mixture. This soil-compost mixture (loamy sand soil with 10% compost made of white cabbage residues, carrots, celeriac residues, all green feedstock material, wheat straw and poplar bark) is described in detail in Amery et al. (2021) [32]. At fertilization, the manure was incorporated into the topsoil (30 cm).

2.1.2. Soil with manure/mineral fertilizer preparation

The soil used in this experiment was collected from the topsoil (0–30 cm) of a meadow that had been organically managed for about 15 years, representing a low risk of antibiotic contamination via fertilization. The physicochemical properties of the loamy sand soil are shown in Table S1.

A physicochemical analysis of the pig slurry (Table S2) was carried out by Inagro (Rumbeke-Beitem, Belgium). Dry matter, total nitrogen, calcium, phosphorus, potassium, magnesium, sodium and ammoniacal nitrogen were determined according to BAM parts 3,4 [33]. Organic carbon and organic matter were determined according to NEN 7432 [34]. The pig slurry originated from a farm where the animals were not treated with antibiotics during the 5 years prior to sampling. The absence of 56 antibiotics (from 10 classes) including doxycycline, sulfadiazine and lincomycin in the used pig slurry was confirmed using UHPLC-MS/MS [1].

For the topsoil, soil was mixed in a concrete mixer with the amount of pig slurry corresponding to 85 kg/ha phosphate based on the Flemish fertilization standards and advice [35,36]. For the treatments with mineral fertilizer, the same concentrations of N, P, K and MgO were added as fertilization carried out with pig slurry. Ammonium nitrate (27%), Korn-Kali (40% K_2O and 6% MgO) and Triple Super Phosphate (20% P_2O_5 and 10% MgO) were used as mineral fertilizers.

Antibiotic solutions were made by dissolving 2875 μg doxycycline (doxycycline hyclate), 288 μg lincomycin (lincomycin hydrochlorate monohydrate) or 288 μg sulfadiazine (sulfadiazine sodium salt) in 60 mL sterile distilled water. After mixing the fertilizers into the topsoil, the antibiotic solutions were added and mixed using an industrial food mixer (Beba technology GmbH & Co, Essen, Germany). In this way, concentrations of 89.9 ± 1.5 μg/kg doxycycline, 9.1 ± 0.1 μg/kg lincomycin and 8.9 ± 0.4 μg/kg sulfadiazine were obtained in the topsoils, which is comparable to the dilution made with pig slurry in the topsoil by fertilization of the agricultural field [1]. After that, the layers of topsoil were added to the tubs.

2.1.3. Cultivation of leek

Five to seven days after fertilization, seven 100-day-old leek plants (*Allium porrum*) were planted in each tub at a distance of 10 cm from each other. The leek plants (Pluston variety) were obtained from Preiplanten Depraeter (Ruiselede, Belgium). After two and seven weeks of cultivation, soil samples were investigated for N content in the upper and lower 30 cm in order to calculate how much mineral fertilizer had to be supplemented to meet the N target values, if necessary (Table S1). An irrigation system with rain water was used during cultivation. The irrigation and soil moisture were monitored using 1 pluviometer and 4 TMS4 data loggers (TOMST ®, Prague, Czech Republic) placed at regular intervals across the setup. In general, 2 mm water was given every 2 days. The soil moisture and the aboveground and underground temperatures were also monitored using the TMS4 data logger (Table S3).

2.2. Sampling

2.2.1. Leek

For the quantification of doxycycline, sulfadiazine and lincomycin using UHPLC-MS/MS, the leek plants were harvested 4.5 months after planting. Two to three leek plants were harvested from each tub. As mentioned above ('Experimental setup' in the materials and methods section) 64 tubs (8 treatments x 8 repetitions) were used. Per treatment, leek from the tubs was pooled in pairs, resulting in 32 leek samples (8 treatments x 4 repetitions). The leek plants were rinsed with fresh rain water. The consumable parts (only the root and leek tops were removed) of the leek were cut and lyophilized (Martin-Christ, Osterode am Harz, Germany).

For the bacteriological part, the leek plants were harvested 3 times during 1 month (5–6 months after planting) in order to be able to perform all the analyses in the lab on fresh material. Similar to the quantification of ABRs, two to three leek plants from two tubs were pooled, resulting in 32 samples for bacteriological plating. Only the white part of the leek was used for bacteriological analysis, as this part of the plant is both in the closest contact with the soil particles during cultivation and is also most often used for human consumption [37].

2.2.2. Soil

During the preparation of the topsoil, soil samples were taken after mixing the antibiotic solutions with the soil in order to check the homogeneity. To do so, 1 of 4 topsoil samples were taken at three locations and analyzed with UHPLC-MS/MS using the method described by Huygens et al. [6]. An additional 1 of 4 topsoils were sampled for quantification of ARGs, resulting in 16 samples (64/4).

During the leek harvest, soil samples were taken from the entire depth of each tub. Similar to the leek samples, soil samples from two tubs from the same treatment were pooled in pairs, resulting in 32 soil samples for the quantification of the respective ABR using UHPLC-MS/MS and for the quantification of 9 ARGs using qPCR (see below).

2.3. Antibiotic residues in manure, soil and leek

Doxycycline, sulfadiazine and lincomycin were quantified in 32 lyophilized leek samples and 48 soil samples. For the soil samples, the same methods were used as described by Huygens et al. but with the difference that only the three spiked components were considered instead of 54 ABRs [6].

To quantify doxycycline, lincomycin and sulfadiazine in leek, two new methods were optimized and validated. Lincomycin and sulfadiazine were quantified with extraction method A and doxycycline was quantified with extraction method B. For extraction method A, 2 g of finely chopped lyophilized leek samples were added to a polypropylene (PP) tube of 50 mL. The internal standards sulfadimethoxine $^{13}C_6$ and clindamycin were added to the tubes in order to obtain a concentration of 50 μg/kg and 5 μg/kg, respectively. After an equilibration time of 20 min, 30 mL of ACN/MeOH (75/25, v/v) was added and shaken for 30 min at 250 rotations per minute (rpm).

For extraction method B, the internal standard methacycline was added to PP tubes (50 mL) filled with 2 g of finely chopped lyophilized leek to get a concentration of 100 μg/kg per sample. After an equilibration time of 20 min, 30 mL of McIlvaine-EDTA buffer was added and shaken for 15 min at 250 rotations per minute (rpm). After a centrifugation at 4000*g* for 15 min at 18 °C, the supernatant was collected and the step was repeated with 10 mL McIlvaine-EDTA buffer on the remaining pellet. The two supernatants were poured together in a PP tubes (50 mL). For both methods A and B, the PP tubes were placed in an ultrasonic bath for 15 min and centrifuged at 4000 g at 18 °C for 15 min. For method A the supernatant was transferred to a new PP tube and evaporated in a warm water bath (40 °C, under N_2) until dryness. The extracts were re-dissolved in 20 mL HPLC water (high-performance liquid chromatography (HPLC) grade, Milli-Q Gradient purification system, Millipore, Brussels, Belgium), vortexed, placed in the ultrasonic bath for 5 min and centrifuged at 4000 *g* at 18 °C for 15 min.

For method B, the supernatants were transferred in a new PP tube (50 mL) and centrifuged at 12,000 *g* for 10 min at 18 °C.

For both methods A and B, the extracts were purified using hydrophilic-lipophilic balance (HLB) 6-cc columns (Waters N.V., Milford, MA). The columns were conditioned with consecutively 5 mL methanol and 5 mL HPLC water. The extracts were loaded onto the columns and subsequently washed with 5 mL of HPLC +5% MeOH. The columns were then eluted twice with 5 mL MeOH. The eluates were evaporated in a warm water bath (40 °C, under N_2) until dryness. The extract was re-dissolved in 1 mL mobile phase (H_2O/MeCN/MeOH (50/25/25) + 0.05% acetic acid (AA)) and consecutively vortexed, placed in an ultrasonic bath for 5 min, filtered through a 0.22 μm filter, transferred to a vial with insert and injected into the UHPLC-MS/MS system (Acquity UHPLC, column: BEH C18 (100 mm × 2.1 mm i.d., 1.7 μm, solvent A: water + 0.05% AA, solvent B: ACN/MeOH (50/50) + 0.05% AA), Xevo TQ-XS2 mass spectrometer (Waters Corporation)).

Matrix matched calibration curves with finely chopped lyophilized leek samples free of antibiotics were used for the quantification. The calibration curve for lincomycin and sulfadiazine ranged from 0–5–10–15–20–25–30 μg/kg and for doxycycline from 0–0.25–0.5–1–2–3–4 μg/kg, respectively.

The limit of detection (LOD), the limit of quantification (LOQ) and linearity of both methods were determined using three sets of matrix-matched calibration curves of 7 points, with the response plotted as function of the concentration. The LOD was calculated as 3 times the standard error of the y-intercept of the regression line divided by the slope. The LOQ was calculated as 10 times the standard error of the y-intercept of the regression line divided by the slope. The recovery, repeatability (RSD_r) and intra-laboratory reproducibility (RSD_R) were measured using 3 series of 8 repetition points at 1 concentration (Table S4).

Furthermore stability of doxycycline, sulfadiazine and lincomycin during the lyophilization process was tested by spiking blank fresh leek with 100 μg/kg of each antibiotic followed by the lyophilization process.

2.4. Antibiotic resistance genes in manure, soil and leek

The DNeasy Plant Mini kit (QIAGEN) was used to isolate DNA from 0.020 g of each of the 32 lyophilized leek samples. Before the extraction, the lyophilized leek was pulverized with liquid nitrogen, a pestle and mortar in order to obtain a homogeneous sample. The PowerSoil DNA Isolation Kit (QIAGEN) was used for the DNA isolation from 0.25 g of each of the 48 soil samples and the one slurry sample. The DNA yield was measured using a NanoPhotometer (Implen, München, Germany) and Quantus™ fluorometer (Promega, Madison, WI, USA) in order to determine the DNA quality and quantity. Subsequently *tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *sul*2, *erm*(B) and *erm*(F) were quantified in 32 lyophilized leek samples, 48 soil samples and 1 slurry sample by qPCR. Furthermore, the abundance of the ARGs in each soil and slurry sample was normalized by the quantification of the 16S rRNA gene. The same methods were used as described in Huygens et al., 2022 [6]. Before analysis, possible inhibition of the qPCRs by matrix components was tested in blank lyophilized leek samples by spiking DNA extracts with 10^3 gene copy numbers (GCN) of the gBlock gene fragment containing the respective ARG, analyzing dilutions of the extract (undiluted – 10 times diluted – 100 times diluted) with qPCR, and comparing Cp values between the dilutions. Based on these tests, the PCR reactions of *tet*(B) and *erm*(B) were performed on a 10-fold diluted DNA extract and of other ARGs on the undiluted DNA extract.

2.5. Isolation of Pseudomonas aeruginosa and Bacillus cereus group

2.5.1. Plating and counting

The white parts of the leek plants were rinsed with sterile water until visually clean. Longitudinal sections were taken from each leek plant and collected in order to obtain a representative 15 g sample per leek plant. Subsequently the samples were diluted 10 times using buffered peptone water (Oxoid ltd, Basingstoke, UK) and then homogenized using a Homex 6 machine (Bioreba, Reinach, Switzerland). The leek extracts were plated on Pseudomonas agar base + C–F–C supplement (PAB, Oxoid ltd) and Mannitol egg yolk polymyxin agar + Polymyxin B supplement + Egg yolk emulsion (MYP, Oxoid ltd) with or without additional antibiotics to count total and antibiotic resistant *Pseudomonas* and *B. cereus* group isolates, respectively. These two bacteria with human relevance in leek were chosen as they were the most abundant during a preliminary experiment (data not shown). Sulfadiazine or doxycycline were added to PAB agar in concentrations based on MIC values for *P. aeruginosa* from the literature as no ECOFF values were available on EUCAST [38]. Specifically 8 mg/mL doxycycline and 128 mg/L sulfadiazine were added to the PAB agar to select the presumable resistant population [39–48]. Lincomycin was not added to PAB agar as most Gram-negative bacteria have a natural resistance against the lincosamides [49]. None of the antibiotics were added to the MYP agar as no unambiguous MIC values were found for *B. cereus* in literature [49–51]. Table S5 shows on which growth media the samples from the different treatments were plated. Appropriate dilutions were made in order to count bacteria suspicious for *Pseudomonas* and *B. cereus group* (Table S5). The PAB agar plates were incubated for 24–48 h at 30 °C. The MYP agar plates were incubated for 48 h at 30 °C. Subsequently, all the different colony types indicative of the *Pseudomonas* and *B. cereus* group were counted and 3 colonies from each colony type were picked up from the smallest dilution plate, purified on Mueller Hinton Agar 2 (Sigma-Aldrich, Diegem, Belgium) (with or without antibiotic supplement) and stored in Brain Heart Infusion (Oxoid ltd) + 15% glycerol (Merck KGaA, Darmstadt, Germany) at −20 °C. Finally, an irrigation water sample and 2 soil samples (1 with mineral fertilizer and 1 fertilized with pig slurry) were also plated in an analogous manner on antibiotic-free MYP and PAB plates in order to check the background occurrence of *Pseudomonas* and *B. cereus* group.

2.5.2. Characterization and identification of isolates

The isolates suspicious for *Pseudomonas* (327 isolates) were lysed by suspending a colony in 100 μL of sterile DNA-free ultrapure water, briefly vortexing the suspension, and incubating it at 95 °C for 15 min. Isolates suspicious for the *Bacillus cereus* group (190 isolates) were lysed in 100 μL of a 0.1 M NaOH + 0.25% SDS (1/1) at 100 °C for 10 min. To cluster isolates, BOX-PCR was performed on the *Pseudomonas* lysates and $(GTG)_5$-PCR was performed on *B. cereus* group lysates as described by Versalovic et al., 1994 [52]. The PCR reactions were performed in a GenAmp® PCR System 9700 (Applied Biosystems, Waltham, USA). The PCR products were further analyzed as described by Maes et al. [53]. From each cluster, at least one suspected *Pseudomonas* isolate was identified at species level via 16S rRNA [54,55]. For the *B. cereus* group, species-specific PCRs (based on Gyrase B coding gene) were performed on all suspected isolates to differentiate *B. thuringiensis*, *B. anthracis* and *B. cereus s.s.* [56]. Based on the *B. mycoides* ATCC 6462 strain described by Yamada et al. (1999), forward primer BM1 (5′-ATA GGT GAA ACT GAT CGT ACA-3′, previously developed at ILVO) and reverse primer BT2R were used to amplify a 368-bp fragment specific to *B. mycoides* [56]. Reaction volumes of 25 μL contained 1 μL lysate, 0.1 μL Amplitaq polymerase (5U/μL), 2.5 μL dNTPs (2 mM), primers (2 μM) at 1 μL each, 1.5 μL $MgCl_2$ (25 mM), 2.5 μL reaction buffer (10×) and 15.4 μL sterile milliQ water. The PCR reactions were performed in a GenAmp® PCR System 9700 under conditions previously described [56].

2.5.3. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for doxycycline, sulfadiazine and lincomycin were determined by broth microdilution methods described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) [38,57]. The MICs were determined on the identified isolates from the pot experiment in order to investigate shifts in MIC values between isolates from the different treatments of the pot experiment. Fresh stock solutions were prepared in sterile milliQ water: 1000 mg/L doxycycline, 3000 mg/L sulfadiazine and 1000 mg/L lincomycin. For each antibiotic, 2-fold dilution series were prepared in order to test MIC values for lincomycin and doxycycline in a range from 0.002 mg/L to 256 mg/L and for sulfadiazine in a range from 0.002 mg/L to 1024 mg/L. The MIC determinations were determined according to the guidelines described by CLSI and

EUCAST [57,58]. The microtiter plates were incubated for 18–20 h at 35 ± 2 °C. Reference strains *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC® 29,212 and/or *Escherichia coli* ATCC 25922 were taken along within each batch of antimicrobial susceptibility testing as internal quality control. The plates were visually interpreted.

2.6. Statistical analyses

Differences in counts (log CFU/g leek) of the bacteria (*P. aeruginosa*, *B. cereus group*) between the antibiotic treatments were tested with ANOVA.

The ANOVA model was fitted with the lm function of R and the emmeans package was used to test post-hoc for statistical differences between the mean counts [59]. Compact letter display (CLD) was used to synthesize the hypothesis testing between the means [60].

To test whether the distribution over the observed MIC values differs between experimental treatments, we used Pearson's chi-squared test for independence as implemented in the function chisq. test(****) in R [61,62]. As the test-statistic is only approximately distributed as a chi-square and the sample size is small, the p-value was simulated by setting the option simulate. p.value to TRUE.

Due to data constraints, only the experimental data of the setups fertilized with pig slurry were modeled. To analyze the change of the ARGs in the soil from different antibiotic treatments between fertilization and harvest, a general linear model was built with the lm-function. In this model, the response variable was the log of the normalized gene copy numbers (GCN) and the explanatory variables were the genes '*tet*(M), *tet*(O), *tet*(W), *erm*(B), *erm*(F) and *sul*2' (genes), the antibiotic treatments 'doxycycline-sulfadiazine-lincomycin-no antibiotics' (Antibiotic) and the times of sampling 'fertilization-harvest' (Time) and their interaction (Antibiotic:Time), resulting in:

$$GCN \sim genes + Antibiotic + Time + Antibiotic{:}Time$$

In essence, the linear regression model is an ANOVA with Time and Antibiotic as factors corrected for the average GCN by genes (genes is used here as a blocking factor).

With F tests we tested for the overall significance of the effects of genes, Antibiotic, Time and Antibiotic:Time and with post-hoc tests (Tukey) we tested for statistical differences between the means. Based on the model fitted, the mean concentrations (and confidence intervals) averaged over genes in soil at 2 time points and 4 antibiotic treatments were estimated with the emmeans package of R [59,63].

Statistical analysis was performed in R-4.1.3 in combination with Rstudio (2022) [61,64]. Statistical significance was considered for P-values below 0.05 and 95% confidence intervals were calculated.

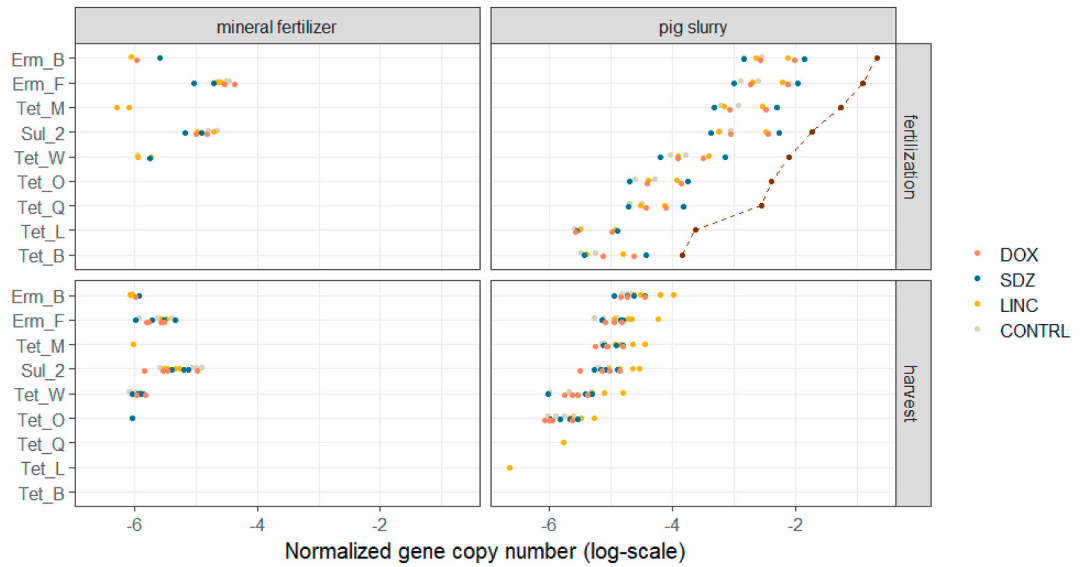

Fig. 2. The GCN (normalized to the 16S gene) for erm(B), erm(F), sul2, tet(B), tet(L), tet(M), tet(O), tet(Q) and tet(W) in manure (brown dotted line) and in soil at fertilization time and harvest time. The different colors represent the different antibiotic treatments (doxycycline = DOX (red), sulfadiazine = SDZ (blue), lincomycin = LINC (yellow), no antibiotics added = CONTRL (gray)). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Antibiotic residues in manure, soil and leek

No doxycycline, sulfadiazine or lincomycin was detected in any leek sample at harvest from the pot experiment.

At the time of fertilization, antibiotic concentrations (mean ± standard deviation) in soil during preparation of the topsoil were 95.3 ± 25.2 μg/kg for doxycycline, 8.5 ± 2.9 μg/kg for lincomycin and 0.7 ± 0.4 μg/kg for sulfadiazine, while the expected concentrations were 89.9 μg/kg ± 1.5 μg/kg, 9.1 μg/kg ± 0.1 μg/kg and 8.9 μg/kg ± 0.4 μg/kg for doxycycline, lincomycin and sulfadiazine, respectively. This indicated an acceptable homogeneity of the antibiotics in the topsoil. The concentration of sulfadiazine in the topsoil was about 10 times lower than expected, however, possibly indicating some instability of sulfadiazine. In the stability tests during the lyophilization process, doxycycline, lincomycin, and sulfadiazine had a recovery of 90%, 105% and 70% in leek, respectively, which also indicates some degree of instability of sulfadiazine.

No doxycycline, sulfadiazine or lincomycin was found in any soil sample taken at harvest time.

3.2. Antibiotic resistance genes in manure, soil and leek

Nine ARGs (*tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *erm*(B), *erm*(F) and *sul*2) were investigated in manure, soil and lyophilized leek samples from the leek experiment.

Fig. 2 shows the GCN (normalized to the 16S gene) in manure and in soil at fertilization and at harvest. All the ARGs were also detected in the manure sample. ARGs were detected in a higher GCN in soil fertilized with pig slurry compared to soil fertilized with mineral fertilizer at both fertilization and at harvest.

The results of the ANOVA analysis of the linear regression model are presented in Table 1. The results of the Estimated Marginal Means (emmeans) of the GCNs are summarized with the compact letter display in Table 2. At harvest, the GCN of all the ARGs in soil fertilized with pig slurry was significantly lower compared to the soils fertilized with pig slurry at fertilization (Table 2). Remarkably, at harvest, a significantly higher mean GCN in soil from the lincomycin treatment was observed compared to the other treatments (Table 2). This observation is confirmed by the significance of the interaction term Antibiotic:Time (Table 1).

As no relative GCN (normalized to 16S rRNA) could be performed in leek samples due to interference with probably chloroplast 16S, the expression per gram leek was calculated [65]. However, none of the ARGs were detected in the leek samples above the lowest point of the calibration curve (10 GCN/reaction).

3.3. Pseudomonas aeruginosa and Bacillus cereus group isolated from leek

The aim of the bacteriological part of this study was to investigate the effect of antibiotic treatments (doxycycline, sulfadiazine and lincomycin) in the leek pot experiment (Fig. 1) on the susceptibility of *P. aeruginosa* isolates to the respective antibiotics on two levels: 1) the abundance (log CFU/g fresh leek) of the total and the presumable resistant population and 2) the level of resistance of the total and presumable resistant population by means of the MIC distribution to the respective antibiotics. The presumable resistant population of *P. aeruginosa* was preselected on growth media supplemented with the antibiotics doxycycline or sulfadiazine) (Table S5). For the *B. cereus* group, only the total population was investigated for abundance and level of resistance because this group contains several closely related species for which resistance to the respective antibiotics could not be preselected on MYP agar (see Materials and Methods, above).

3.3.1. Isolation, identification and abundance of Pseudomonas aeruginosa and Bacillus cereus group isolates

No *P. aeruginosa* was isolated from the irrigation water or the soil samples. The distribution of the *P. aeruginosa* isolates across treatments and plating is shown in Table S6.

P. aeruginosa was present in leek ranging from 0 to 4.1 log CFU/g fresh leek (Fig. 3). As no notable differences in counts were observed between the setups treated with either pig slurry or mineral fertilizer, no distinction was made for the visualization with box plots and for the calculation of the mean.

On agar plates supplemented with 8 mg/L doxycycline, *P. aeruginosa* occurred more frequently in leek from the doxycycline treatments compared to the control treatments. Specifically *P. aeruginosa* was present in 88% and 38% of the setups, respectively. However, no significant differences in mean CFUs were observed between these treatments.

Table 1
ANOVA of the linear regression model with F-tests testing for the effects of ARG, Antibiotic treatment, Time and Antibiotic treatment:Time, and post-hoc tests for statistical differences between the means. ARG = antibiotic resistance gene.

Effects	F-values	p-values
ARG	71.6	<1e−03
Antibiotic treatment	9.1	<1e−03
Time	1481.6	<1e−03
Antibiotic treatment:Time	3.3	0.02

Table 2
Estimated marginal means (emmeans), its standard error and 95% confidence interval of the normalized GCN in soil fertilized with pig slurry at harvest and fertilization time for each treatment (lincomycin, sulfadiazine, doxycycline and control). Equal letters in a CLD indicate that the group means do not differ statistically and different letters refer to group means that are statistically different.

Antibiotic treatment	Time	Emmean of normalized GCN	SE	Lower.CL	Upper.CL	Group
Control	Harvest	−5.25	0.0602	−5.37	−5.13	a
Doxycycline	Harvest	−5.23	0.0602	−5.35	−5.11	a
Sulfadiazine	Harvest	−5.17	0.0602	−5.29	−5.05	a
Lincomycin	Harvest	−4.85	0.0602	−4.97	−4.73	b
Control	Fertilization	−3.34	0.0851	−3.51	−3.17	c
Lincomycin	Fertilization	−3.07	0.0851	−3.23	−2.9	c
Sulfadiazine	Fertilization	−3.06	0.0851	−3.23	−2.89	c
Doxycycline	Fertilization	−3.02	0.0851	−3.19	−2.85	c

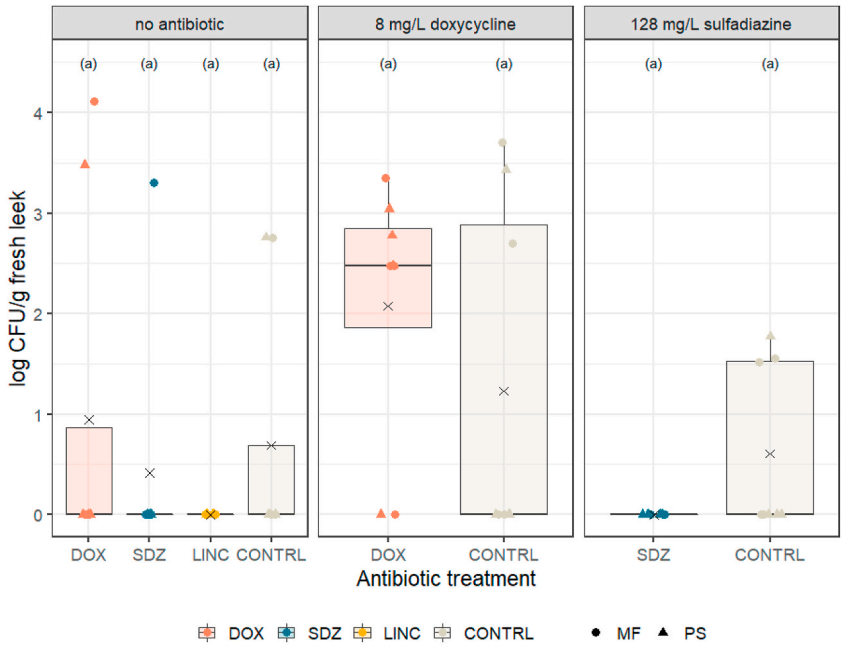

Fig. 3. Colony forming units (log CFU/g fresh leek) for *Pseudomonas aeruginosa* in leek from different antibiotic treatments (doxycycline = DOX, sulfadiazine = SDZ, lincomycin = LINC, no antibiotics added = CONTRL) and fertilization types (mineral fertilizer and pig slurry) on different agar plates (no antibiotic, 8 mg/L doxycycline, 128 mg/L sulfadiazine) represented with boxplots and mean (cross). The colors represent the different treatments (doxycycline (red), sulfadiazine (blue), lincomycin (yellow), no antibiotic added (gray)). Dots (mineral fertilizer) and triangles (pig slurry) specify the fertilizer type. Per plate type, equal letters in a CLD indicate that the mean CFU's do not differ statistically and different letters refer to mean CFU's that are statistically different. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Additionally, on agar plates without antibiotic supplementation and with a supplementation of 128 mg/L sulfadiazine, no significant differences in mean CFU between the antibiotic treatments were observed for *P. aeruginosa*.

In leek from the sulfadiazine treatments, *P. aeruginosa* was observed in only one setup with a colony count of 3.3 log CFU/g leek, counted on an agar plate without antibiotic supplementation (Fig. 3). Last, no *P. aeruginosa* was detected in leek from the lincomycin treatment.

As no different colony morphology was observed on MYP growth media, plate counts were performed on the *B. cereus* group without subdivision in species. Fig. 4 shows the CFU in leek for the *B. cereus* group. In general, the *B. cereus* group was present in leek ranging from 1 to 2.4 CFU/g. Again, no distinction was made between the setups treated with either pig slurry or mineral fertilizer for the visualization of the colony counts in box plots and for the calculation of the mean. The lowest counts of the *B. cereus* group were observed for lincomycin, but only the mean CFU from the doxycycline and lincomycin treatment were significantly different.

Using GTG_5-PCR and species-specific PCR, 40 *B. cereus*, 26 *B. thuringiensis* and 124 *B. mycoides* isolates were obtained (Table S7). The distribution of the *B. cereus* group isolates across treatments is shown in Table S8.

B. mycoides and *B. cereus* were present in the water in counts >2.5 log/100 mL (uncountable plates) and *B. mycoides* was present in soil in 5.3 log CFU/g. None of the isolates originated from water or soil clustered in $(GTG)_5$-PCR with isolates originated from leek.

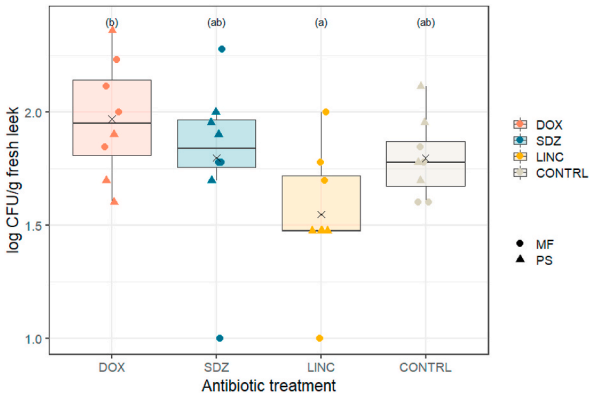

Fig. 4. Colony forming units (log CFU/g fresh leek) for Bacillus cereus group in leek from different antibiotic treatments (doxycycline = DOX, sulfadiazine = SDZ, lincomycin = LINC, no antibiotics added = CONTRL) and fertilization types (mineral fertilizer and pig slurry) represented with boxplots and mean (cross). The colors represent the different treatments (doxycycline (red), sulfadiazine (blue), lincomycin (yellow), no antibiotics added (gray)). Dots (mineral fertilizer) and triangles (pig slurry) specify the fertilizer type. Equal letters in a CLD indicate that the mean CFU's do not differ statistically and different letters refer to mean CFU's that are statistically different. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3.2. *Antimicrobial susceptibility testing*

Figs. 5 and 6 show the susceptibility (expressed as MIC distribution) to doxycycline and sulfadiazine, respectively, for the total and presumable resistant *P. aeruginosa* population isolated from leek from the control, doxycycline and sulfadiazine treatment. In this way, we investigate if antibiotic treatments in the pot experiment impacts the susceptibility of *P. aeruginosa* to doxycycline and sulfadiazine. The lincomycin treatment is not represented in the figures as *Pseudomonas aeruginosa* was not detected in that treatment.

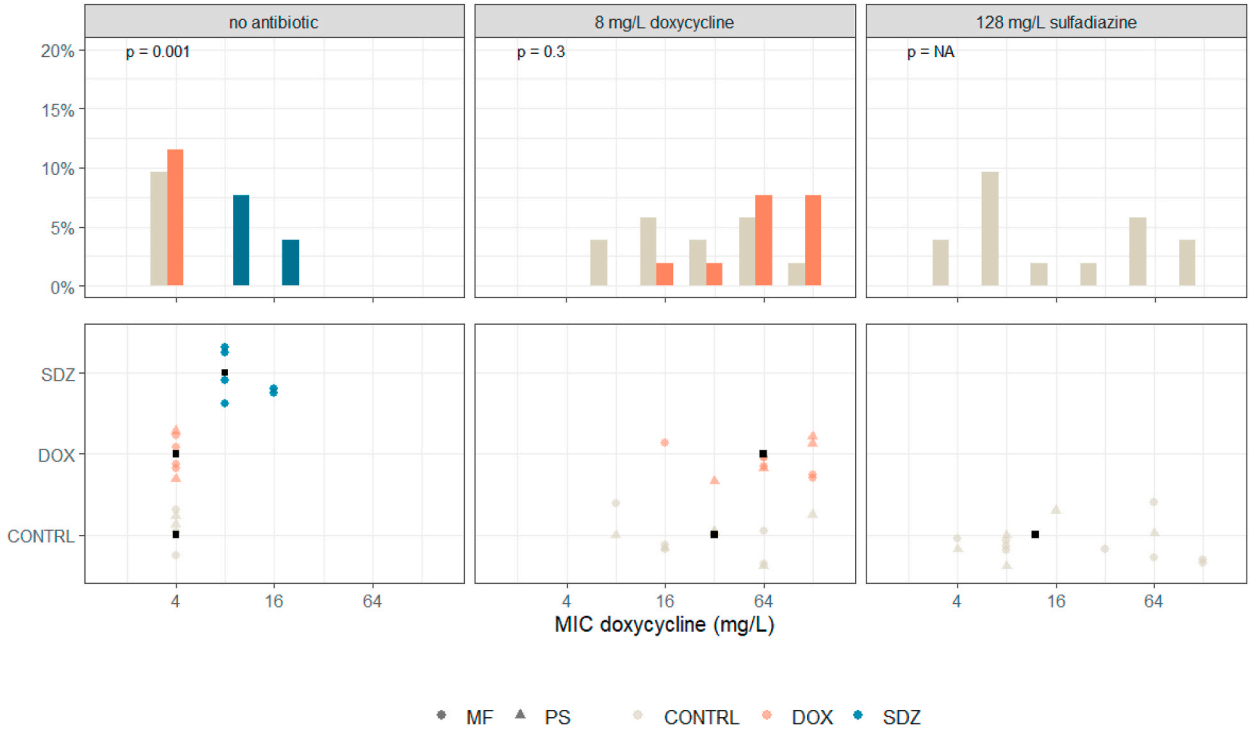

Fig. 5. The susceptibility to doxycycline for 52 *Pseudomonas aeruginosa* isolates from leek from antibiotic different treatments (doxycycline = DOX (red), sulfadiazine = SDZ (blue), no antibiotics added = CONTRL (gray)) isolated from different agar plates (no antibiotic, 8 mg/L doxycycline, 128 mg/L sulfadiazine). Above: Distribution (%) of MIC values represented in a bar plot. Below: dot plot. Dots (mineral fertilizer) and triangles (pig slurry) specify the fertilizer type. MIC50 is represented by '■'. Simulated p-values indicate the significance of differences between MIC distributions of the different treatments within plate type. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

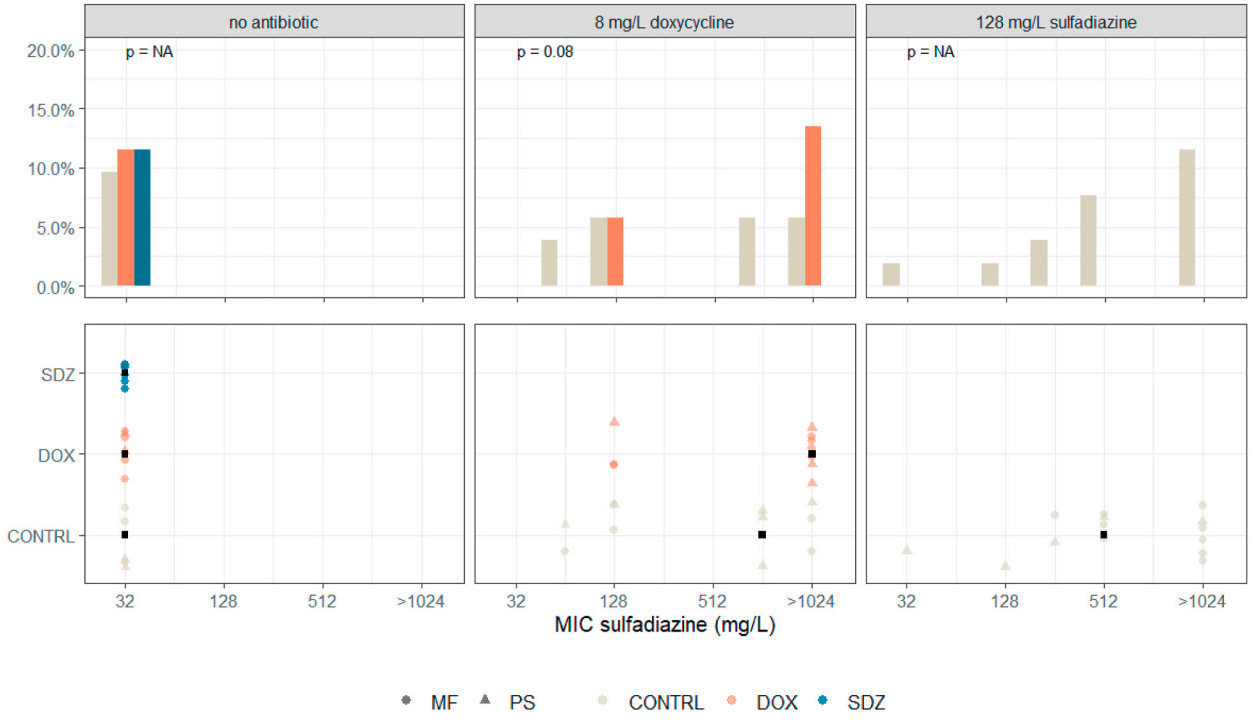

Fig. 6. The susceptibility to sulfadiazine for 52 *Pseudomonas aeruginosa* from leek from different antibiotic treatments (doxycycline = DOX (red), sulfadiazine = SDZ (blue), no antibiotics added = CONTRL (gray)) isolated from different agar plates (no antibiotics, 8 mg/L doxycycline, 128 mg/L sulfadiazine). Above: Distribution (%) of MIC value represented in a bar plot. Below: dot plot. Dots (mineral fertilizer) and triangles (pig slurry) specify the fertilizer type. MIC50 is represented by '■'. Simulated p-values indicate the significance of differences between MIC distributions of the different treatments within plate type. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

For the total *P. aeruginosa* population (from plates without antibiotics added), a significant different MIC distribution of doxycycline was observed in the sulfadiazine treatment compared to the other treatments with a p-value of 0.001 (Fig. 5, Table S9). Furthermore, the total *Pseudomonas aeruginosa* population had a higher MIC50 for doxycycline in the sulfadiazine treatment (MIC50 = 8 mg/L) compared to the control and doxycycline treatment (MIC50 = 4 mg/L).

For the *P. aeruginosa* population presumably resistant to doxycycline (from plates supplemented with 8 mg/L), no significant different MIC distributions of doxycycline were observed between the control treatment and doxycycline treatment (Fig. 5, Table S9). However the *P. aeruginosa* population presumably resistant to doxycycline showed a higher MIC50 value for doxycycline in the doxycycline treatment compared to the control treatment (Fig. 5). Specifically the MIC50 was 64 for isolates (n = 10) from the doxycycline treatment and 32 for isolates (n = 11) from the control treatment (Table S6).

When focusing on the MIC values of doxycycline in the control treatment, higher MIC50 values were observed in the *P. aeruginosa* population presumably resistant to sulfadiazine compared to the total *P. aeruginosa* population. Logically, higher MIC50 values can be seen for the *P. aeruginosa* population presumably resistant to doxycycline.

Fig. 6 represents the susceptibility to sulfadiazine of *P. aeruginosa* isolated from leek. MIC values of sulfadiazine for the total *P. aeruginosa* population (n = 17) were all 32 mg/L, so no shift in MIC50 were observed between the treatments.

A small difference in MIC distribution of sulfadiazine of the *P. aeruginosa* population presumably resistant to doxycycline was observed between the control and doxycycline treatment, which is reflected by a p-value of 0.08 (Fig. 6, Table S9). Also a small shift in MIC50 was observed between the isolates from the doxycycline treatment (n = 11, MIC50 = >1024 mg/L) and the control treatment (n = 10, MIC50 = 1024 mg/L).

When focusing on the MIC values of sulfadiazine in the control treatment, higher MIC50 values were observed in the *P. aeruginosa* population presumably resistant to doxycycline compared to the total *P. aeruginosa* population. Logically, higher MIC50 values can be seen for the *P. aeruginosa* population presumably resistant to sulfadiazine (Fig. 6).

Fig. 7 represents the susceptibility of the *B. cereus* group to doxycycline, sulfadiazine and lincomycin. For the MIC distributions of sulfadiazine, a small difference in distribution is observed between the control and sulfadiazine treatment, which is reflected by a p-value of 0.06 (Fig. 7, Table S9). Meanwhile, no notable shifts in MIC50 of sulfadiazine were observed between the different antibiotic treatments. Furthermore no significant differences in MIC distributions for lincomycin and doxycycline were observed between the different treatments. However, the MIC50 of lincomycin of isolates from the lincomycin treatment (n = 33, 16 mg/L) was a little higher compared to the control treatment (n = 38, 12 mg/L).

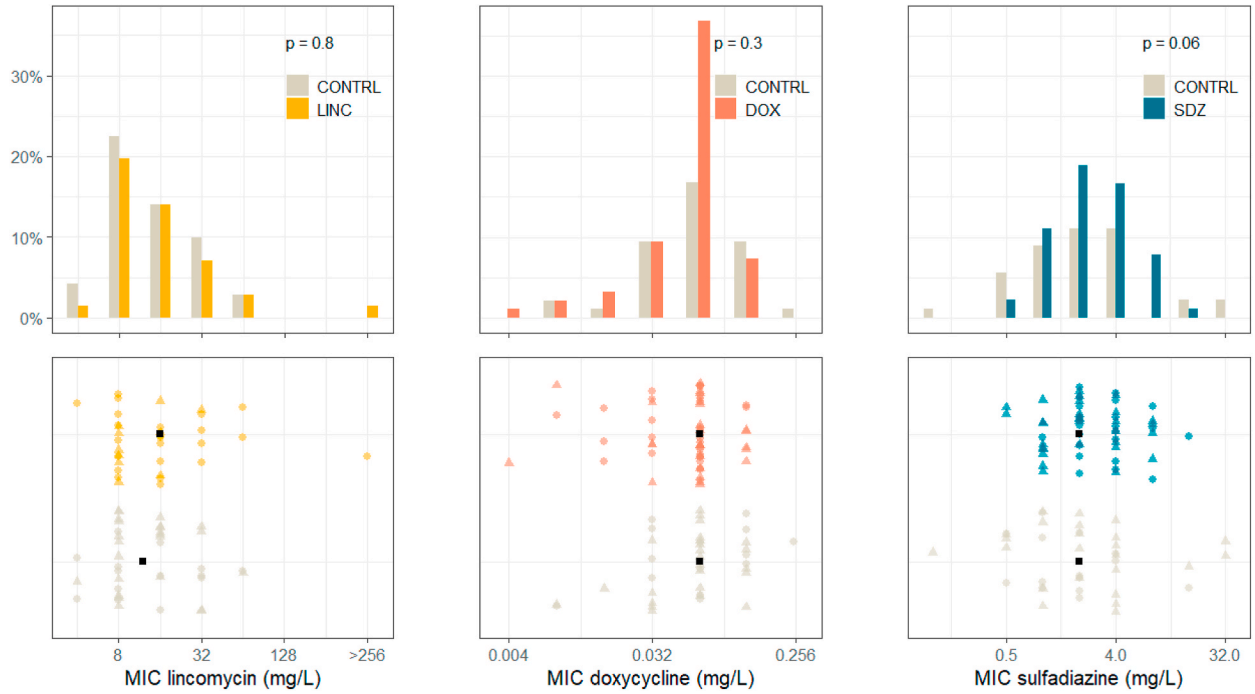

Fig. 7. The susceptibility to lincomycin, doxycycline and sulfadiazine for 181 Bacillus cereus group isolates from leek from different antibiotic treatments (doxycycline = DOX (red), sulfadiazine = SDZ (blue), lincomycin = LINC (yellow), no antibiotics added = CONTRL (gray)). Above: Distribution (%) of MIC values represented in a bar plot. Below: dot plot. Dots (mineral fertilizer) and triangles (pig slurry) specify the fertilizer type. MIC50 is represented by '■'. Simulated p-values indicate the significance of differences between MIC distributions of the different treatments within plate type. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Cultivation of leek in the pot experiment

Since this study concerns the transfer of ABRs, ARGs and resistant bacteria from animal slurry to vegetable, leek was chosen as model vegetable for the abovementioned reasons. First, leek is fertilized on agricultural fields with considerable amounts of animal slurry. This study was conducted in Belgium, one of the most important leek producers in Europe [66]. The 3960 ha of leek cultivation rank 3rd in Europe, preceded only by much larger countries. Second, the practice of topping the leeks on the field during harvest creates an extra risk for contaminating the leek with environmental bacteria, thus increasing the chance that bacteria will be transferred from soil to vegetable.

In the data processing of ABRs, ARGs and antibiotic resistant bacteria in leek at harvest, no notable differences were observed between the treatments fertilized with pig slurry and with mineral fertilizer. This indicates that the microbiota present in slurry does not have a notable impact on the selection of antibiotic resistant bacteria that were studied in leek (*P. aeruginosa* and *B. cereus* group). However, a distinct difference in the presence of ARG was observed in soil treated with these fertilizer types, indicating the introduction of ARGs in soil by fertilization with animal manure, which is confirmed by previous studies [6,7].

Note that fertilization in this experiment is slightly different compared to fertilization with manure on the field. In this study, antibiotics were mixed with soil and slurry just before fertilization. On the field, ABRs may be already present in soil for several years due to previous fertilizations. Furthermore, ABRs may be already present in slurry for several months during manure storage. In practice, therefore, selection for antibiotic resistance may have occurred long before fertilizer is applied to agricultural soils. This long-term aspect is estimated to be an important factor in antibiotic resistance selection, as reported in literature [6,7,67]. In future research the impact of repeated fertilization in the environment should be studied. Moreover, ABRs and resistance patterns in other vegetable types such as carrots and cabbage should be investigated. For instance, carrots are relevant vegetables to study, as carrots are also fertilized with animal slurry (however in low quantities) and have a high contact surface with the soil during cultivation on the field. Furthermore, a study in which soil and vegetables are sampled at multiple time points during cultivation would give valuable knowledge about the fate and kinetics of ABRs, ARGs and antibiotic resistant bacteria in vegetables. Specifically, it could be verified whether ARGs were transferred to immature crops or ABRs were taken up by immature crops in concentrations that could exert a selective effect for antibiotic resistant bacteria at different time points. This might provide insight into the potential effect of cultivation time on the presence of ABRs, ARGs and antibiotic resistant bacteria in crops.

4.2. Antibiotic residues in leek

The novelty of the present experiment lies in the exposure of leek to realistic concentrations of ABRs in a pot experiment. In many other studies, high concentrations of antibiotics were used to study the uptake by plants [10,13,18–22,68]. Such studies reveal the capacity of plants to take up ABRs, but do not realistically portray the risk of human exposure to these residues in plants.

Doxycycline, sulfadiazine and lincomycin were not detected in leek after 4.5 months of cultivation in pots. Although no residues were found at harvest, there is a possibility that residues were taken up during cultivation, had already degraded in the meantime, and had thus affected the microbiota. Moreover, antibiotic resistance selection may have already occurred in previous steps, which may also affect the bacterial flora of vegetables and consequently the intestinal microbiota of humans.

Conditions during our controlled experiment could be different than during cultivation on agricultural fields. This may have an impact on the degradation and bio-availability of ABRs in soil. The bio-availability in soil can be defined as the degree to which chemicals are either available for interaction with biological systems or may be absorbed and metabolized by ecological receptors [69]. Degradation of ABRs is influenced by soil properties, i.e., organic matter content, pH, moisture, temperature, oxygen status, and soil texture and thus climate and weather conditions [29]. But also biotic processes like microbial degradation of antibiotics can play a role [29]. Furthermore, the bio-availability in soil of ABRs also strongly depends on soil properties (pH and organic matter content) [69]. The uptake of ABRs also depends on the physicochemical properties of the residue itself as well as on the vegetable type. Future research should screen for different ABRs in leek and other vegetables for consumption as in this study only 3 ABRs were investigated in one vegetable type.

In our study, some indications showed that sulfadiazine is unstable in both soil and leek. In other studies, the half-life in soil of sulfadiazine under different experimental conditions ranged from <1 day to 10 days, which makes this antibiotic 'very non-persistent' [29,31]. This fast degradation may explain the low concentration of sulfadiazine in the fertilized soil at the start of the experiment. For practical reasons, in the present experiment the soil samples were stored at −80 °C until analysis as described by Berendsen et al. [31]. To be sure that the degradation of sulfadiazine in soil happened at the start of the pot experiment and not during storage at −80 °C, stability tests of sulfadiazine in a frozen soil matrix are recommended.

To study the presence of ABRs in vegetables, lyophilization is a common practice for sample preparation as it has advantages for storage and homogenization [10,18,20,22,23]. It is therefore important to mention that our stability data indicate that sulfadiazine appeared to be unstable after the lyophilization process. It remains unclear whether the instability is solely due to the storage time associated with the process or also due to temperature fluctuations during the lyophilization process itself. The impact of lyophilization on the stability of sulfonamides should be further investigated, as data on this is not found in literature.

4.3. Antibiotic resistance genes in manure, soil and leek

ARGs were detected in manure in relative GCN between −1 log and −4 log and in soil between −2 log and −6 log, which is comparable with previous studies [6,7,20].

Tet(M), *tet*(O), *tet*(W), *erm*(B), *erm*(F) and *sul*2 were more abundant in soil at harvest in the lincomycin treatment compared to the other treatments. Lincomycin is a narrow spectrum antibiotic active against Gram-positive bacteria, while sulfadiazine and doxycycline are active against a broader spectrum, i.e. both Gram-positive and Gram-negative bacteria [70,71]. Hence, the lincomycin treatment could possibly result in a different microbiota being induced in the soil. It has been previously described that lincomycin may change the bacterial diversity and antibiotic resistance in soil communities by increasing the selection pressure on resistance genes in the soil [72,73]. Another possible explanation is a co-resistance effect induced by lincomycin for which resistance is encoded by *erm* genes and which can be located on the same genetic element as other resistance genes (e.g. *tet*(Q) and *tet*(M)) [74].

The investigated ARGs (*tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *sul*2, *erm*(B) and *erm*(F)) were not present in leek at harvest above the lowest point of the calibration curve (10 GCN/reaction). This is consistent with Tien et al. (2017) [75] who also detected no *erm*(B) and *erm*(F) in vegetables despite detection in corresponding soil samples. In another study, *sul*2 was found in pakchoi. In that experiment, during cultivation the plant was exposed to sulfamethoxazole concentrations higher than practical occurrence in agro-ecosystems (38 and 76 mg/L) [23], while in our study realistic concentrations for doxycycline, lincomycin and sulfadiazine in topsoil were used [6]. In other studies, ARGs (including *erm*(B), *erm*(F), *tet*-genes and *sul*2) were quantified in tomato, carrot, lettuce, cucumber, pepper and beans [25,26]. However, comparisons of data between different studies are difficult due to different exposure parameters (e.g., exposure of different antibiotics in various concentrations, different time periods of cultivation, field experiments vs. greenhouse pot experiments) and different vegetable types [25,26,75].

Although no ARGs were detected in leek at harvest, small shifts in MIC50 values were observed. This contradiction can be possibly due to the shortcomings of existing methodologies such as a difference in sensitivity. With 0.020 g lyophilized product for the DNA extraction (corresponding to approximately 0.200 mg fresh product), the chance that a resistance gene will be caught is substantially lower than in the 15 g of fresh leek used for extraction to make the bacterial cultures. Furthermore the shift in MIC50 was only seen in the presumably resistant subpopulation of *P. aeruginosa,* which corresponds to only a small part of the total microflora of leek. Another explanation is that the shifts in MIC values for DOX are possibly caused by other resistance genes than the ones investigated; from more than 50 identified resistance genes coding for tetracycline resistance, we only focused on five of them. The shift in MIC50 for lincomycin in the *B. cereus* group was also rather small.

In this study, antibiotic resistance was investigated based on the presence of ARGs and susceptibility data (MIC values). Further research could also investigate changes in gene expression and transferability of ARGs leading to physiological adaptations to antibiotic stress by investigating the relative expression levels of certain ARGs and gene copy numbers of transferable elements such as

plasmids, respectively. It would provide valuable insights into the expression, selection and transmission of antibiotic resistance under certain environmental conditions.

4.4. Pseudomonas aeruginosa and Bacillus cereus group isolated from leek

Antibiotic resistant bacteria are ubiquitous in vegetables, regardless of vegetable type, farming practices, climate conditions and origin [5]. Different bacteria (such as *Enterobacter* sp., *Citrobacter* sp., *Klebsiella* sp., *E. coli*, *C. perfringens*, *Yersinia* sp., *Campylobacter* sp., *Pseudomonas aeruginosa* and *Bacillus cereus*) resistant to different antibiotics (such as tetracyclines, macrolides, aminoglycosides, chloramphenicol) have already been studied in fruits and vegetables in the past, but different methodologies make it difficult to compare data between different studies [5,76–80].

In our study, *P. aeruginosa* and the *B. cereus* group were frequently present in leek at harvest, unlike the general indicator bacterium *E. coli*, which was not found. Both species are good indicator bacteria to study the impact of antibiotic exposure on antibiotic resistance selection during vegetable cultivation. These species are widely distributed in the environment (such as soil, water, plants) and are thus prone to resistance selection [77,81,82]. They can also be pathogenic to humans, underscoring the importance of successful antibiotic treatment. *P. aeruginosa* belongs to the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp) and its resistance is clinically important as these bacteria may act as donors of ARGs for other pathogenic species [83,84].

Previous studies have shown an antimicrobial activity of leek extracts against Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*) [85,86]. The antimicrobial properties may be due to several organosulfur and phenolic compounds, which may have an impact on the colonization of bacteria on the vegetable [81,85,87,88]. This concurs with a study where a lower average CFU/g for *Bacillus cereus* was observed in vegetables from *Alliaceae* family (<1 CFU/g) compared to a higher average in pepper, cucumber and carrot (10^2 to 7,8 $\times$ 10^3 CFU/g) [81]. In another study, *Pseudomonas* spp. was found to be less present in onion or leek (26% of the samples) than in other vegetables such as lettuce (90% of the samples) [82]. In our study, *P. aeruginosa* was either not found in some treatments or found in low concentrations.

In the present study, it is difficult to interpret whether or not the target bacteria were resistant to the tested antibiotics because of the lack of resistance breakpoints for the antibiotics [89]. Nevertheless, we defined *P. aeruginosa* isolated from plates with predefined concentrations of doxycycline or sulfadiazine as representing a population presumably resistant to the respective antibiotic. Moreover, *P. aeruginosa* is known for its resistance to many antibiotics. Their resistance is defined by intrinsic, acquired and/or adaptive resistance [90]. It has been described that intrinsic mechanisms like efflux systems such as mexAB-oprM and mexXY-oprM play a major role in resistance to tetracyclines, sulfonamides and lincosamides [49,90–94]. Importantly, regulatory changes due to subinhibitory antibiotic exposure can lead to overexpression of these genes encoding efflux pumps and thus leading to an adaptively more resistant bacteria [90]. It has also been described that for *P. aeruginosa,* many additional mutations of a particular resistome lead to a stepwise increase over time to higher resistance levels. This phenomenon, termed 'creeping baselines', can result in modest shifts in MIC values, but ultimately lead to its high-level resistance over time [90]. This is why we studied shifts in MIC distribution as a possible effect of antibiotic exposure of leek during cultivation.

Even though antibiotic resistance in the environment is largely influenced by the long-term exposure due to repeated fertilization events, a small and specific effect was observed for one single fertilization, especially for doxycycline. In the *P. aeruginosa* population that is presumably resistant to doxycycline, a small shift in MIC50 for both doxycycline and sulfadiazine was observed between the control and doxycycline treatment. This effect may be solely attributed to the addition of doxycycline to the set ups as no notable distinction between set ups fertilized with pig slurry and mineral fertilizer was observed. This indicates that particular attention should be given to the selective effect of doxycycline. However, the proportion of the presumably resistant population compared to the total *P. aeruginosa* population is not known. Lower counts of *P. aeruginosa* were observed on plates without antibiotics added compared to plates with doxycycline supplementation. So on plates without antibiotic supplementation, *P. aeruginosa* is probably outcompeted by other bacteria while *P. aeruginosa*, characterized by multi-resistance, had a growth benefit on plates with doxycycline supplementation [90]. So consequently it is hard to estimate the relevance of the observed shifts in MIC50 within this presumably resistant population.

In the total *P. aeruginosa* population, a significantly different distribution was observed in the sulfadiazine treatment compared to the doxycycline and control treatment. However, it is difficult to determine whether these results are representative for the total *P. aeruginosa* population as only 6 *P. aeruginosa* isolates were observed in the sulfadiazine treatment and they all originated from the same leek extract.

Remarkably, when preselecting for resistance to doxycycline, higher MIC values for both doxycycline and sulfadiazine were observed compared to isolates without preselection (from plates without antibiotics added). The same was observed for preselection to sulfadiazine. This phenomenon suggests co-resistance (where several genes can be co-selected), potentially related to the mexAB-oprM efflux system [95].

For the *B. cereus* group, the lower counts and the slightly higher MIC50 in isolates from the lincomycin treatment compared to the control treatment suggest a potential minor selective effect of lincomycin in the pot experiment.

5. Conclusion

At harvest, no ABRs could be detected in the leek nor in the soil. Further, no ARGs were detected in leek at harvest. In soil at harvest, *erm*(B), *erm*(F), *tet*(M), *sul2*, *tet*(W) and *tet*(O) were present in significantly higher GCN fertilized with pig manure containing lincomycin (1000 μg/kg manure). This could be due to a different microbiota induced in the soil by the administration of lincomycin and/or

a co-resistance effect induced by lincomycin.

Comparison of the MIC values of the isolates of the two target species (*B. cereus* group and *P. aeruginosa*) from leek exposed during growth to one of the 3 antibiotics and from leek without antibiotic exposure revealed no large differences between the different experimental setups. Nevertheless, for *P. aeruginosa* isolated from agar plates supplemented with 8 mg/L doxycycline, a higher MIC50 for doxycycline and sulfadiazine was observed in the doxycycline treatment compared to the control treatment, indicating a small shift toward more resistance in *P. aeruginosa*. Furthermore, in the *B. cereus* group, a smaller shift in MIC50 for lincomycin was observed in the lincomycin treatment compared to the control treatment.

On the basis of this study, consumption of leek would carry a low risk of exposure to ABRs or antibiotic resistance to doxycycline, sulfadiazine and lincomycin.

To further evaluate the risk of dissemination of antibiotic resistance through consumption of plants, more research is needed on selection of antibiotic-resistant bacteria in vegetables, namely different relevant vegetable types, other bacteria and other antibiotics.

Funding statement

The research project that yielded these results was funded by the Belgian Federal Public Service Health, Food Chain Safety and Environment via the contract RT/17 AMRESMAN.

Author contribution statement

Judith Huygens: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Geertrui Rasschaert; Els Daeseleire; Marc Heyndrickx: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Bart Cottyn: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jeroen Dewulf; Els Van Coillie; Paul Quataert: Analyzed and interpreted the data; Wrote the paper.

Koen Willekens; Ilse Becue: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

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

Acknowledgments

We are grateful to the researchers and technicians from Proefcentrum Groenten (Practical Center Research for Vegetables in Kruishoutem) (PCG), Johan Dick (ILVO), Cindy Elisabeth (ILVO) and other colleagues for helping with the realization of the pot experiment. Special thanks to the technicians from the chromatographic laboratory to help with the LC-MS/MS analyses and to Sjarlotte Willems, Caroline Poleyn, technicians from the microbiological laboratory and students for helping with the molecular analyses. We are also thankful to Johan Witters and technicians for tracing and controlling trips during the pot experiment. We also acknowledge Miriam Levenson (ILVO) for English language editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16052.

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