RESEARCH ARTICLE

Tracing back multidrug-resistant bacteria in fresh herb production: from chive to source through the irrigation water chain

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One sentence summary: Irrigation water quality impacts on antibiotic-resistant bacteria of chive representing raw consumed fresh produce grown under agricultural practice conditions.

Editor: Kornelia Smalla
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ABSTRACT

Environmental antibiotic-resistant bacteria (ARB) can be transferred to humans through foods. Fresh produce in particular is an ideal vector due to frequent raw consumption. A major contamination source of fresh produce is irrigation water. We hypothesized that water quality significantly affects loads of ARB and their diversity on fresh produce despite various other contamination sources present under agricultural practice conditions. Chive irrigated from an open-top reservoir or sterile-filtered water (control) was examined. Heterotrophic plate counts (HPC) and ARB were determined for water and chive with emphasis on *Escherichia coli* and *Enterococcus* spp. High HPC of freshly planted chive decreased over time and were significantly lower on control- vs. reservoir-irrigated chive at harvest (1.3 log (CFU/g) lower). Ciprofloxacin- and ceftazidime-resistant bacteria were significantly lower on control-irrigated chive at harvest and end of shelf life (up to 1.8 log (CFU/g) lower). *Escherichia coli* and *Enterococcus* spp. repeatedly isolated from water and chive proved resistant to up to six or four antibiotic classes (80% or 49% multidrug-resistant, respectively). Microbial source tracking identified *E. coli*-ST1056 along the irrigation chain and on chive. Whole-genome sequencing revealed that *E. coli*-ST1056 from both environments were clonal and carried the same transmissible multidrug-resistance plasmid, proving water as source of chive contamination. These findings emphasize the urgent need for guidelines concerning ARB in irrigation water and development of affordable water disinfection technologies to diminish ARB on irrigated produce.

INTRODUCTION

Consumer demand for fresh produce has increased in the past decades, as it is being associated with a healthy lifestyle and offers the advantage of convenient and economic meals with minimal preparation (Hu *et al.* 2014; Li *et al.* 2014; Wang *et al.* 2014b). Fresh produce consumption is actively promoted by public institutions such as the European Commission (EC) through actions like the school fruit scheme encouraging consumption by children or free distribution of produce withdrawn as remains from the market to various public service bodies (EC 2017). Such increased consumption of mostly raw or minimally processed fresh produce including fresh herbs comes with an increase in related foodborne outbreaks (Callejón *et al.* 2015). Apart from pathogenic bacteria causing such outbreaks and the naturally present harmless microbiota, it is increasingly recognized that raw or minimally processed fresh produce can deliver several antibiotic-resistant bacteria (ARB) to the consumer (Falomir, Gozalbo and Rico 2010; Pesavento *et al.* 2014; Nüesch-Inderbinen *et al.* 2015; Araújo *et al.* 2017). Antibiotic resistances, even if harbored in non-pathogenic bacteria, can potentially be spread through horizontal gene transfer to other species including opportunistic pathogens present in the environment or—upon consumption of ARB-contaminated fresh produce—the gut microbiome (Devirgiliis, Barile and Perozzi 2011) thereby contributing to the pool of antibiotic-resistance genes (ARG) in the human gut (Hu *et al.* 2013; Thanner, Drissner and Walsh 2016).

Contamination of fresh produce can occur before or after harvest through a variety of sources such as soil, irrigation water, wild or domestic animals, and manure (pre-harvest), or harvesting and processing equipment, dust, rinse water and transport vehicles (post-harvest) (Olaimat and Holley 2012). Of all these probable contamination sources, irrigation water has the potential of spreading localized contamination by directly reaching the edible plant parts, especially when applied through sprinkler irrigation (Fonseca *et al.* 2011). This is of particular interest as the microbiological quality of irrigation water can vary considerably from potable water over groundwater to various surface waters (Allende and Monaghan 2015; Uyttendaele *et al.* 2015). Irrigation water quality deserves great attention since the use of low-quality waters such as reclaimed wastewaters potentially contaminated not only with pathogenic bacteria but also with antibiotics, ARB and ARG is unavoidable (Czekalski *et al.* 2012; Fahrenfeld *et al.* 2013; Christou *et al.* 2017). This is of particular concern in parts of the world with limited access to potable water (Gemmell and Schmidt 2012) or where surface water sources such as dedicated canals are used. The presence of ARB in surface waters such as lakes, rivers or irrigation ponds has been highlighted recently (Micallef *et al.* 2013; Zurfluh *et al.* 2014a; Blaustein *et al.* 2015), and in some instances the prevalence of multidrug-resistant (MDR) bacterial strains has been found to be higher in surface waters than in wastewaters (Farkas, Bocoş and Butiuc-Keul 2016). Moreover, ARB and ARG have been detected in drinking water treatment and distribution systems (Schwartz *et al.* 2003; Xi *et al.* 2009), and the drinking water treatment process has been pinpointed as potentially increasing bacterial antibiotic resistance (Czekalski *et al.* 2012; Bai *et al.* 2015).

A direct link between irrigation water containing ARB and ARG and contaminated fresh produce has been suggested in various studies. Many of these studies have focused on indicator bacteria. *Escherichia coli* and *Enterococcus* spp. are species commonly used as indicators of fecal contamination when assessing quality of water but also of foods (Anderson, Whitlock and Harwood 2005; Jay, Loessner and Golden 2005; Pappas *et al.* 2008), and both species are also used as indicators in antibiotic resistance monitoring of foods (EFSA 2008). Moreover, they are regularly detected on fresh produce (Giraffa 2002; Johnston and Jaykus 2004; Pesavento *et al.* 2014; Faour-Klingbeil *et al.* 2016; Gekenidis *et al.* 2017). *Escherichia coli* is acknowledged to be a central player in the spread of antibiotic resistance due to its genetic flexibility and adaptability (Szmolka and Nagy 2013), and *Enterococcus* spp. have long been notorious for harboring and spreading ARG (Franz, Holzapfel and Stiles 1999; Leisibach 2004; Palmer, Kos and Gilmore 2010). To link contaminated water to fresh produce contamination, investigations have been based on isolation and characterization of antibiotic-resistant *E. coli* (Holvoet *et al.* 2013). In a very recent study, Araújo and colleagues (2017) investigated the prevalence of antibiotic-resistant *E. coli* in irrigation water and on vegetables from 16 household farms, and by typing isolates obtained from both sources using repetitive elements PCR (rep-PCR), suggested a link between water and vegetables. Other studies have also combined strain typing with characterization of antibiotic resistance for *E. coli* source tracking, however, with these methods only a potential link can be determined (Du Plessis, Duvenage and Korsten 2015; Jongman and Korsten 2016a). Concerning antibiotic-resistant *Enterococcus* spp., some studies have investigated fresh produce (Johnston and Jaykus 2004; Gomes *et al.* 2008; Pesavento *et al.* 2014) or potential irrigation water (Goldstein *et al.* 2014), but studies investigating both sources are scarce and did not establish a link between water and the irrigated produce (Abriouel *et al.* 2008; Micallef *et al.* 2013).

From a legal perspective, guidelines including critical values for indicator bacteria such as *E. coli* and *Enterococcus* spp. in irrigation water have been established in many countries. In particular, for safe use of wastewater in agriculture, the WHO has described risk assessment and management approaches taking into consideration attributable risks (WHO 2006). However, until now no guidelines addressing ARB in irrigation water exist.

There is an urgent need for in-depth understanding of the role of irrigation water in contamination of fresh produce with ARB, in order to apply appropriate mitigation strategies comprising preventive measures and technological sanitation of irrigation water before usage in the field. Overall, there is a lack of controlled studies proving the link between ARB detected on the produce and irrigation water by tracing them back to their source. The presented greenhouse study pursued two aims: (1) to investigate the impact of irrigation water quality on ARB detected on fresh produce (exemplified by chive) under agricultural practice conditions by describing diversity and antibiotic resistance of total ARB and target ARB *E. coli* and *Enterococcus* spp. from water and plants, and (2) to investigate whether selected isolates of ARB from plants originate from the applied irrigation water, by tracing them back through the irrigation chain to the water source. Notably, the cultivation conditions under which chive was grown are commonly used for other leafy

greens as well, e.g. rocket salad or lamb's lettuce. To investigate to what extent irrigation water quality influences the diversity of ARB detected on fresh produce, two types of water were used for overhead irrigation of the chive: (a) sterile-filtered water obtained from a three-stage filtration unit and (b) water sourced from an open-top reservoir collecting rain water, greenhouse rooftop run-off water and surface drainage water.

MATERIALS AND METHODS

Bacterial culture conditions

The following media were used for bacterial cultivation: R2A (Sigma-Aldrich, St. Louis, USA) for determination of total heterotrophic plate count (HPC); eosine methylene blue (EMB) agar (Becton Dickinson, Franklin Lakes, NJ, USA) for *Enterobacteriaceae*; CHROMagar *E. coli* (CHROMagar, Paris, France) and ready-to-use Brilliance ESBL plates for *E. coli*; and m-Enterococcus agar (mEA) (Sigma-Aldrich), Compact Dry ETC plates (HyServe, Uffing, Germany) for direct incubation of water filters, and ready-to-use Brilliance VRE plates (Oxoid Ltd., Hampshire, UK) for isolation of *Enterococcus* spp.

For isolation of ARB, all above-mentioned media apart from ESBL and VRE were supplemented with antibiotics. With the exception of high-concentrated ampicillin chosen to avoid overgrowth of non-target bacteria, antibiotic concentrations were based on epidemiological cutoffs (ECOFFs) defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), as recommended by the European Food Safety Authority (EFSA 2012), while minimizing detection of false positives when using broad-spectrum antibiotics. For isolation of total ARB, R2A was supplemented with broad-spectrum antibiotics: combined trimethoprim and sulfamethoxazole (SXT, 4 and 76 mg L^{-1}, respectively) and tetracycline (TE, 8 mg L^{-1}) representing ancient antibiotics (Czekalski *et al.* 2012), ciprofloxacin (CIP, 1 mg L^{-1}) or ceftazidime (CAZ, 8 mg L^{-1}). Additionally, pimaricin was added (25 mg L^{-1}, ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) to each of the R2A/antibiotics combinations to suppress fungal growth. For isolation of target ARB, EMB agar and CHROMagar were supplemented with either ampicillin (AM, 100 mg L^{-1}), kanamycin (K, 16 mg L^{-1}), ciprofloxacin (CIP, 1 mg L^{-1}) or ceftazidime (CAZ, 8 mg L^{-1}), while mEA and ETC plates were supplemented with either erythromycin (ERY, 4 mg L^{-1}) or ciprofloxacin (CIP, 1 mg L^{-1}).

Total HPC and total ARB were determined by direct plating. For detection of target ARB, samples were enriched (24 h, 37°C) prior to cultivation: Buffered peptone water (BPW, 10.0 g of peptone, 5.0 g of NaCl, 3.5 g of anhydrous Na$_2$HPO$_4$ and 1.5 g of KH$_2$PO$_4$ (Sigma-Aldrich) per 1 liter of water, pH 7.0) was used for enrichment of *Enterococcus* spp. and EE broth Mossel (Becton Dickinson) for enrichment of *Enterobacteriaceae* and *E. coli*. Using a 10 μl loop, the enriched samples were then streaked onto the respective antibiotic-containing agar plates. R2A plates were incubated at room temperature for 72 h; ETC plates, EMB agar and CHROMagar at 37°C for 24 h; ESBL, mEA and VRE at 37°C for 48 h. All media were incubated under aerobic conditions.

Experimental setup and plant growth conditions

The experiment was setup in a greenhouse covering an area of 1000 m^2 (10 × 100 m, 6 beds) and equipped with an overhead irrigation system. Water from an open-top reservoir as also applied standardly in practice was used for irrigation. To investigate the influence of irrigation water quality on ARB detected on plants,

a second irrigation system (control irrigation) was installed by disconnecting the existing tubing and installing new tubing and sprinklers for control irrigation. To provide clean water for control irrigation, a closed water tank was filled with fresh tap water daily and the tank water was pumped through a three-stage filtration unit (pore sizes 1.2 μm, 0.65 μm and 0.2 μm; Sartorius AG, Goettingen, Germany) to generate sterile water before supplying the sprinkler system. Chive plants (*Allium schoenoprasum* L.) were grown from untreated seeds by placing about 25 seeds into 4 × 4 cm pots containing substrate compliant with Bio Suisse standards (Bio Suisse 2015). After a germination period of approximately 10 days at 20°C (relative humidity: 75%), the temperature was lowered to 12°C for 7 days (relative humidity: 60%). Thereafter, temperature was further lowered (5–10°C) for another 14 days to increase plant durability. Finally, the seedlings were transplanted to the greenhouse containing agricultural field soil. To minimize plant–soil contact, the beds were overlaid with an organic foil before planting the seedlings. After planting, the chive plants were irrigated overhead at different frequencies depending on their growth phase: three to four times per day during the first three weeks (each irrigation about 2 L m^{-2}), once per day thereafter for two weeks (about 5 liters m^{-2}) and twice per day during the last week before harvest (each about 1 L m^{-2}). The total field output at harvest was 600 kg.

Field sampling and bacterial culture preparation

Irrigation water and plant material were sampled in summer 2016 (July–August). The greenhouse planted with chive was sampled every two weeks during a whole growth period, i.e. from freshly planted seedlings to harvest of marketable plants, resulting in a total of four samplings for analysis of total heterotrophic bacteria as well as total and target ARB. Of note, plant material was collected before running overhead irrigation to collect the water samples.

Irrigation water

At each sampling, irrigation water samples were collected in sterile water sampling bottles (VWR, Radnor, USA) along the complete irrigation chain. For the control-water chain, water was sampled from the municipal tap (tap), the water tank (tank), the three-stage filtration unit (inF) and the corresponding greenhouse sprinklers (spF). For the reservoir-water chain, water was sampled from the tube draining surface waters into the open-top reservoir (drain), the open-top reservoir itself (R) and the greenhouse inlet (inR) and sprinklers (spR) sourced from the open-top reservoir. Notably, reservoir water (R) was pumped through a particle filter (F-600 Gravel Filter; Netafim, Tel Aviv, Israel) before entering the greenhouse (inR). Water samples were transported at approximately 8°C and processed within 10 h. For determination of HPC, serial 10-fold dilutions were plated in duplicate on R2A. Further, depending on water sample clarity largely varying from sterile-filtered water to surface water, 5–500 mL were concentrated through nitrocellulose filters (0.22 μm pore size, EMD Millipore, Billerica, USA) for direct incubation on the surface of antibiotic-containing R2A or ETC plates. Additionally, 300 or 500 mL were filtered depending on water clarity for subsequent enrichment in 5 mL BPW and EE broth. For cultivation of target ARB, the enrichment broths were finally streaked onto the respective antibiotic-containing selective media.

Seeds and seedlings

Chive seeds as well as seedlings before entering the greenhouse were sampled and analyzed. Twenty grams of seeds or

seedling leaves were weighed into a stomacher bag containing 100 mL of either BPW or EE broth and homogenized in a Smasher (Biomérieux, Marcy l'Etoile, France) for 3 min. From the resulting BPW supernatant, appropriate volumes and dilutions as determined in pre-experiments were plated on R2A plates with and without antibiotics. Both BPW and EE homogenates were then incubated for enrichment and streaked onto antibiotic-containing selective media as described above.

Plant

For sampling of plant material, the field area was divided into six plots, three plots per treatment (control- or reservoir-irrigation). A sample of at least 120 g plant material was collected per plot by randomly sampling parts of about 50 plants, resulting in three plant samples per treatment. The samples were transported at approximately 8°C and processed within 10 h: of each sample, 20 g were weighed into 100 mL of either BPW or EE broth and processed as described for seeds and seedlings. For analysis of chive at the end of its shelf life, plant material from the last field sample was stored for 6 days at 4°C.

MALDI biotyping

Representative colonies from all media containing antibiotics were identified by MALDI biotyping. Attention was paid to picking the different morphotypes from R2A (up to 10 colonies per sample and antibiotic) and each morphotype from the selective media at least once (minimum 3 colonies per sample and antibiotic). MALDI biotyping was performed by direct smearing as described previously (Gekenidis *et al.* 2014) using a microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) and the associated MALDI biotyper RTC Software (Version 3.1).

Antibiotic susceptibility tests

Escherichia coli and *Enterococcus* spp. were screened for antibiotic resistance by disk diffusion assays against 32 and 11 clinically relevant antibiotics, respectively. Each bacterial strain was subcultured on Columbia agar with 5% sheep blood (BioMérieux, Marcy l'Etoile, France) at 37°C and 7.5% CO_2. Disk diffusion assays were performed according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST 2012): (1) bacterial suspensions with a turbidity corresponding to 0.5 McFarland were produced in saline (9 g L^{-1}) and streaked on Mueller Hinton E (MHE) agar (Becton Dickinson), (2) antibiotic disks were applied (i2a, Montpellier, France) and (3) the plates were incubated at 35°C for 18 h $\pm$ 2 h or 24 h (*E. coli* or *Enterococcus* spp., respectively). Special attention was paid to meeting the 3×15 min rule, i.e. none of the three steps should exceed 15 min. Finally, the inhibition zones were measured with a Sirscan instrument (i2a) (Hombach, Zbinden and Böttger 2013) and manually corrected on-screen whenever needed. For determination of antibiotic susceptibility, EUCAST's epidemiological cutoff (ECOFF) values were used based on EFSA recommendations for epidemiological antibiotic resistance screening (EFSA 2012). For antibiotics with no defined ECOFF value (cefpodoxime and fosfomycin for *E. coli*), EUCAST's clinical breakpoints were used. Where EUCAST guidelines did not define any value, breakpoints from the Clinical and Laboratory Standards Institute (CLSI) were applied (i.e. for colistin, minocycline, kanamycin, sulfonamide, tetracycline, temocillin and cefalotin for *E. coli*; gentamicin high concentration, erythromycin, tetracycline and chloramphenicol for *Enterococcus* spp.) (CLSI 2016). Species with intrinsic resistances as

defined by EUCAST expert rules were considered resistant (*Enterococcus faecalis* and *Enterococcus faecium* to erythromycin; *Enterococcus gallinarum* and *Enterococcus casseliflavus* to erythromycin and vancomycin) (Leclercq *et al.* 2013).

Phylogenetic groups (PG)

Escherichia coli phylogenetic groups (PG) were determined as described by Clermont and colleagues (2013) by quadruplex PCR amplification of three genes (*arpA*, *chuA* and *yjaA*) and a DNA fragment (TspE4.C2) using custom-synthesized primers (Microsynth, Balgach, Switzerland) and a DreamTaq hot start PCR master mix (Thermo Fisher Scientific, Waltham, USA). PCR conditions were as described by Clermont and colleagues (2013). Bands were visualized with GelRed (Biotium Inc., Fremont, USA) on a TBE gel (2% agarose, 35 min, 100 V), and strains with ambiguous band patterns were subjected to confirmatory C- or E-PCR.

Multilocus sequence typing (MLST)

Sequence types (ST) of MDR *E. coli* were determined by amplifying and sequencing seven housekeeping gene fragments (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) as described by Wirth and colleagues (2006). Briefly, custom-synthesized primers (Microsynth) were used for amplification by Phusion high-fidelity DNA polymerase (NEB, Ipswich, USA). PCR products were verified on an agarose gel and purified with a PCR clean-up kit (Macherey-Nagel, Düren, Germany). The purified amplicons were Sanger sequenced (Microsynth), and alleles and ST were determined using the MLST tool of BioNumerics 7.5 (Applied Maths NV, Keistraat, Belgium) and the *E. coli* MLST database (Warwick Medical School 2017).

Whole-genome sequencing and bioinformatics

Genomic DNA was extracted from two MDR *E. coli* strains of same sequence type, one isolated from drain water and one from irrigated chive plants, using the commercial GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's recommendations. Genomic DNA was sequenced on a Pacific Biosciences RSII instrument (20-kb insert library, P6/C4 chemistry, 360 min movie) at the Functional Genomics Center Zurich. The genomes were assembled de novo using the Hierarchical Genome Assembly Process (HGAP) (Chin *et al.* 2013) from SMRT Analysis run through the online platform SUSHI (Hatakeyama *et al.* 2016) with default settings. The resulting contigs (two per strain) were circularized and trimmed using AMOS and minimus2 to get the complete circular chromosome or plasmid (Schatz 2006; Sommer *et al.* 2007) and subsequently polished by remapping raw reads and creating consensus sequences using pbalign and Quiver (Huguet-Tapia *et al.* 2016). The generated fasta files, each containing two contigs, were submitted to Basic Local Alignment Search Tool (BLAST). The closest hits for each contig (one chromosome and one plasmid, respectively) were combined to generate a reference sequence (Ecoli_1943_pH2291) for subsequent usage in CSI Phylogeny 1.4, a freely available online tool for inferring phylogeny from single nucleotide polymorphisms (SNPs) developed by the Center for Genomic Epidemiology (CGE) of the Technical University of Denmark (DTU) (Kaas *et al.* 2014). Briefly, CSI Phylogeny is calling SNPs, then filtering them to remove low-quality SNPs, validating the sites, and finally inferring a phylogeny using the concatenated SNP alignment. Additionally to phylogenetic analysis

of the two strains, the CGE online tools MLST 1.8, ResFinder 3.0, PlasmidFinder 1.3 and pMLST 1.4 were used to confirm sequence type and identify antibiotic resistance determinants and plasmid replicons (Larsen *et al.* 2012; Zankari *et al.* 2012; Carattoli *et al.* 2014). All CGE tools were used with default settings (CSI Phylogeny: SNP pruning minimum distance 10 bp, minimum SNP quality 30; ResFinder: minimum 90% ID, minimum 60% length; PlasmidFinder: minimum 95% ID, minimum 60% length). Finally, the online service for Rapid Annotation using Subsystem Technology (RAST) was used to elucidate the potential of the plasmid to be transmitted to other bacteria (Aziz *et al.* 2008). The PacBio raw reads (ERR2535305, ERR2535306) and assembled nucleotide sequences (ERZ535013, ERZ535014) were submitted to the European Nucleotide Archive under BioProject PRJEB26426.

Biolog

Additionally to whole-genome sequencing, the two MDR *E. coli* strains were phenotypically characterized using Biolog Phenotype Microarrays in duplicate (Biolog Inc., Hayward, USA). Two microarrays for carbon sources (PM-1 and PM-2A), one for nitrogen sources supplemented with sodium succinate/ferric citrate as a carbon source (2 M/200 μM, PM-3B), and one for osmotic stress to simulate dry conditions of the phyllosphere as opposed to the water environment (PM-9) were selected. All microarray plates were prepared as described previously for *E. coli* (Mackie *et al.* 2014) and after incubation for 48 h at 37°C, the data were evaluated using the opm package in R (Vaas *et al.* 2013; R Core Team 2017).

Statistical analysis

For changes in bacterial numbers along irrigation chains or over time, ANOVA was performed on log-transformed data (Hirano *et al.* 1982) using GraphPad Prism 6 (GraphPad Software, La Jolla, USA) after replacing values below the limit of detection with the limit of detection of the measurement (Lorimer and Kiermeier 2007). Additionally, Tukey's multiple comparison test was applied. To compare the effect of the two irrigation regimes on bacterial numbers, Student's t test (unpaired, two-tailed, homoscedastic) was applied to log-transformed data. Significant differences are reported (*P* < 0.05).

RESULTS

Greenhouse climatic conditions

To detect whether growth conditions were comparable for all plants, climatic conditions in two distant parts of the greenhouse were recorded (one for control- and one for reservoir-irrigated plants) using data loggers measuring temperature and relative humidity (RH) at 15 min intervals. Overall, temperature and RH were virtually the same during the complete sampling period, as shown by overall maximal, minimal and average values (Fig. S1, Supporting Information). Development over time showed that daily average temperature and RH values were equal, although one sector displayed overall higher maximal temperatures and sometimes lower maximal RH values. Of note, samplings were conducted in the morning, long before the daily maximal temperature was reached (Fig. S1 A, Supporting Information).

Total HPC along the two irrigation water chains and on chive

Total HPC was determined for water from both irrigation systems each sampled at four sites, from water source to greenhouse sprinklers. Mean HPC values of the four sampling time points are displayed in Fig. 1 for control-water and reservoir-water system (Fig. 1A and B, respectively). Of note, the first site of the reservoir-water system (drain, Fig. 1B) could be sampled only once (S1), since the tube draining water into the open-top reservoir was submerged in water at the other three sampling occasions. Overall, total HPC varied greatly along both irrigation chains with average values between 2.4 and 6.0 log CFU mL^{-1} (Fig. 1). At the beginning of the control-water chain (Fig. 1A), the sampled tap water displayed low average HPC (3.2 log CFU mL^{-1}) with small deviations between sampling time points. Numbers in water from the tank were significantly increased (4.6 log CFU mL^{-1}, *P* < 0.01) as compared to tap water and very constant over the four sampling time points. Filtration resulted in a significant reduction of HPC to 2.7 log CFU mL^{-1} in filtered water compared to tank water (*P* < 0.0001), reaching levels below average HPC of tap water. Upon entering the corresponding greenhouse sprinkler system, a tendency towards increased HPC as compared to filtered water was observed (3.5 log CFU mL^{-1}, not significant (ns)). For the first sampling site of the reservoir-water chain (drain, Fig. 1B), HPC was around 5.1 log CFU mL^{-1}. Water from the open-top reservoir displayed an average HPC of 3.0 log CFU mL^{-1} with very small variation throughout all samplings (Fig. 1B). Counts were significantly increased in the water arriving at the greenhouse inlet as compared to open-top reservoir water (5.7 log CFU mL^{-1}, *P* = 0.001). Interestingly, HPC values from water recovered from the respective greenhouse sprinklers were reduced as compared to inlet water (4.2 log CFU mL^{-1}, *P* < 0.05), displaying, however, a large variability between samplings.

For control- and reservoir-irrigated plants, total HPC was determined in triplicate per treatment and sampling time point (Fig. 1C). HPC for seedlings before entering the greenhouse was around 7.5 log CFU g^{-1} (S0, Fig. 1C). After planting, HPC values were comparable for chive plants from both treatments, from sampling 1 to sampling 3 (S1–S3, Fig. 1C). As the plants grew during this period, counts decreased significantly from approximately 7.1 log CFU g^{-1} (S1) to an average of 5.3 and 4.9 log CFU g^{-1} (S3) for control- and reservoir-irrigated plants, respectively (*P* < 0.001 and *P* < 0.01). Only in the last sampling (S4), in which plants were harvested to be packaged and shipped to retailers, did the HPC of control-irrigated plants drop significantly below that of reservoir-irrigated plants (3.2 and 4.5 log CFU g^{-1}, respectively, *P* < 0.05; S4, Fig. 1C). Notably, HPC values of harvested chive at the end of shelf life (6 days) were significantly increased as compared to the last field sample (*P* < 0.0001 and *P* < 0.05 for control- and reservoir-irrigated plants, respectively), lying again around 6.0 log CFU g^{-1} for both treatments (end, Fig. 1C).

Counts of ARB on chive plants

Bacteria resistant to antibiotic combination sulfamethoxazole-trimethoprim-tetracycline (STT) displayed the lowest counts (Fig. 2A). Starting at 3.5 log CFU g^{-1} on seedlings, counts were still around 3.0 log CFU g^{-1} in the second sampling (S2) and decreased to undetectable levels thereafter (*P* < 0.01 and *P* < 0.001 for control- and reservoir-irrigated chive, respectively). Control-chive had lower loads of STT-resistant bacteria as compared to reservoir-chive (ns). For CIP-resistant bacteria compared to
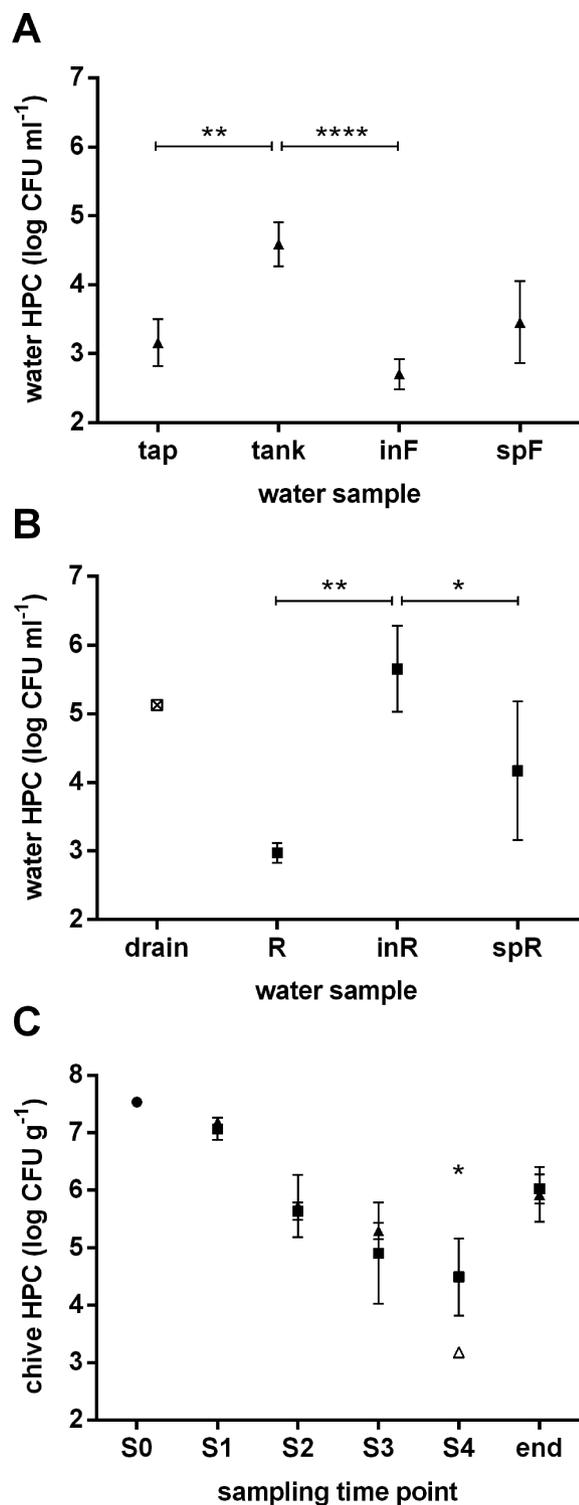
Figure 1. Total HPC for water samples from the control-water chain (triangles; **A**), the reservoir-water chain (squares; **B**), and from seedlings (dot) as well as greenhouse-grown control- and reservoir-irrigated chive plants (triangles and squares, respectively; **C**). Square with cross: value from a single sampling; open symbol: sample mean containing samples below the limit of detection. Tap, tap water; tank, tank water; inF, filter-sourced inlet water; spF, filter-sourced sprinkler water; drain, drain water; R, open-top reservoir water; inR, reservoir-sourced inlet water; spR, reservoir-sourced sprinkler water; S0, seedling sampling. S1–S4, sampling 1 to sampling 4; end, end of shelf life. Results are displayed as mean values (n = 4 and n = 3 for water and chive, respectively) and error bars show standard deviations. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

STT-resistant ones, overall higher counts were detected. Starting at 5.1 log CFU g^{-1} on seedlings, counts were around 5.4 log CFU g^{-1} in sampling 2 and decreased to 4.2 log CFU g^{-1} in sampling 3 for both control- and reservoir-irrigated plants (S2 and S3, Fig. 2B). In the last sampling (S4), CIP-resistant bacteria had further decreased by 1.7 log units on control-chive, while counts on reservoir-chive had slightly increased (0.2 log units). CIP-resistant bacteria were thus significantly less abundant on control- than on reservoir-chive (2.5 vs. 4.4 log CFU g^{-1}; $P < 0.05$). A similar trend was observed for CAZ-resistant bacteria, where numbers were around 5.3 log CFU g^{-1} on seedlings and were in the same range for plants from both treatments in samplings 2 and 3 (around 5.3 log CFU g^{-1} and 4.6 log CFU g^{-1}, respectively; Fig. 2C). As observed for ciprofloxacin, CAZ-resistant bacteria from sampling 4 were on average significantly lower for control- than for reservoir-chive (3.0 vs. 4.3 log CFU g^{-1}; $P < 0.05$). Overall, both CIP- and CAZ-resistant bacteria decreased significantly over the sampling period for control- but not for reservoir-chive (Fig. 2B and C). Finally, on chive at the end of shelf life (end, Fig. 2B and C), CIP- as well as CAZ-resistant bacteria were still significantly reduced on control- as compared to reservoir-chive (1.0 log unit lower; $P < 0.05$).

Assignment of ARB to species in the two systems

For qualitative comparison of ARB along the two irrigation chains as well as on the respective chive plants, total ARB and target ARB (*Enterobacteriaceae*, *E. coli* and *Enterococcus* spp.) were identified (Tables 1 and 2 for control- and reservoir-irrigation, respectively).

Overall, 40 and 45 different species of ARB were identified by MALDI biotyping in the control- and reservoir-system, respectively. From the 40 control-system species, half (20 species) were detected in water exclusively, 10 species on chive plants exclusively, and 10 species both in water and on chive plants (Table 1). From the 45 reservoir-system species, again half (22 species) originated exclusively from water, 7 species from chive plants exclusively and 16 species were detected in both water and on chive plants (Table 2). Among the species recovered from both water and chive—representing interesting candidates for microbial source tracking—were various *Enterococcus* spp. (one or four species from control- or reservoir-system, respectively) and *E. coli* (reservoir-system only). However, while antibiotic-resistant *Enterococcus* spp. were already isolated from chive seeds and seedlings, *E. coli* were not detected before the first irrigation event (data not shown). Regarding species unique to one of the two systems, 13 or 17 of the detected species from water and 5 or 4 of the detected species from chive plants were unique to the control- or the reservoir-system, respectively. Of the species detected both in water and on chive plants, 3 or 5 species were unique to the control- or the reservoir-system, respectively, with *E. faecium* and *E. gallinarum* among the five reservoir-species. In terms of antibiotic resistance, 5 or 8 species from the control- or reservoir-system, respectively, were isolated on at least three different antibiotics along the chain (two counts for STT; Tables 1 and 2). These included species from our target ARB, namely *E. faecalis* for the control-system and *E. casseliflavus*, *E. gallinarum* and *E. coli* for the reservoir-system. Interestingly, for the ones from the reservoir-system the same antibiotic/species combination was detected both in water and on chive (grey in Table 2), while *E. faecalis* from the control-system was isolated solely from chive. Notably, no ESBL *E. coli* or VRE *Enterococcus* spp. were detected at any time.

Table 1. Total and target ARB from control-water chain and irrigated chive plants.

w	c	taxonomic assig.	tap	tank	inF	spF	chiveF	ABs[2]
		Achromobacter spp.	K	CIP, K		CIP	CIP, CAZ	
		A. xylosoxidans	K	CIP, K				2
		Acinetobacter spp.		CAZ	CAZ	CAZ	CAZ	
×	×	A. calcoaceticus				CAZ	CAZ	1
		A. pittii		CAZ			CAZ	1
		Aeromonas spp.		AM				
		Arthrobacter spp.		CIP		CIP, CAZ	CIP, CAZ	
		A. aurescens				CAZ	CIP	2
	×	A. chlorophenolicus					CIP	1
×		A. creatinolyticus				CIP		1
		A. nicotinovorans					CIP, CAZ	2
		Bacillus spp.			STT	CIP, CAZ	CAZ	
×		B. cereus group			STT			2
×		B. clausii				CIP		1
		Brevundimonas spp.	CIP, CAZ	CIP, CAZ	CIP, CAZ	STT, CIP, CAZ	STT, CIP	
×		B. aurantiaca	CIP			CIP		1
×		B. intermedia	CIP		CIP			1
		B. vesicularis			CAZ		CIP	2
		Cellulosimicrobium spp.				CIP		
		C. cellulans				CIP		1
		Chryseobacterium spp.		STT			STT	
		Citrobacter spp.				AM		
		C. amalonaticus				AM		1
		Cronobacter spp.					AM	
		Cupriavidus spp.	CAZ	CAZ		CIP, CAZ		
×		C. gilardii	CAZ	CAZ				1
×		C. pauculus				CAZ		1
		Delftia spp.		K				
×		D. acidovorans		K				1
		Enterobacter spp.		CAZ, AM, K			CAZ, AM, K	
		E. cloacae complex[1]		CAZ, AM, K			CAZ, AM	3
		Enterococcus spp.				CIP	CIP, CAZ, ERY	
		E. casseliflavus				CIP	CIP	1
		E. faecalis					CIP, CAZ, ERY	3
		Escherichia spp.					K	
		E. coli					K	1
		Kosakonia spp.					AM	
		Microbacterium spp.	CIP, CAZ	CIP, CAZ	CIP, CAZ	Van	CIP, CAZ	

Table 1. Continued

w	c	taxonomic assig.	tap	tank	inF	spF	chiveF	ABs[2]
×	×	*M. maritypicum*	CIP				CIP, CAZ	2
	×	*M. oxydans*					CIP	1
×		*M. resistens*				Van		1
×		*M. testaceum*		CIP				1
		Morganella spp.					AM	
		M. morganii					AM	1
		Ochrobactrum spp.				CIP, CAZ, AM, K	CAZ, AM, K	
		O. intermedium				CIP, CAZ, AM, K	CAZ, AM, K	4
		Paenibacillus spp.				CIP		
		Pseudoclavibacter spp.					CAZ	
		Pseudomonas spp.	CAZ, AM	CAZ, AM	CIP, CAZ, AM, K	CIP, AM	CIP, CAZ, AM, K	
		P. aeruginosa					AM, K	2
×		*P. chlororaphis*	CAZ, AM					2
×		*P. koreensis*			AM	AM		1
		P. monteilii				CIP, AM		2
		P. putida	CAZ, AM	CAZ, AM	AM, K	CIP, AM		4
		Psychrobacillus spp.					CAZ	
		Ralstonia spp.		CAZ, K		K	CAZ	
×	×	*R. mannitolilytica*		CAZ, K		CAZ, K	CAZ	2
		Raoultella spp.		AM, K	AM, K	AM		
		R. ornithinolytica		K	K	AM		2
×		*R. planticola*			AM			1
		Rhizobium spp.	CAZ		CAZ			
		R. radiobacter			CAZ			1
		Rhodotorula spp.		CIP				
		Sphingobacterium spp.				K		
		Sphingobium spp.				CAZ		
		Sphingomonas spp.					CAZ	
	×	*S. parapaucimobilis*					CAZ	1
		Staphylococcus spp.					CAZ	
		Stenotrophomonas spp.	CAZ		CIP, K	CIP, CAZ, K	CIP, CAZ, AM, K	
		S. maltophilia			CIP, K	CIP, K	CIP, CAZ, K	3
		Streptococcus spp.					ERY	
	×	*S. salivarius*					ERY	1
		Streptomyces spp.					CAZ	
	×	*S. violaceoruber*					CAZ	1

[1]At the time of writing this paper, bacteria from the *Enterobacter cloacae* complex cannot be distinguished using MALDI biotyping.
[2]Number of antibiotic classes.
Antibiotic/species combinations detected in water as well as on chive plants are highlighted in grey. Species detected exclusively in the control-system are marked (×): w, water; c, chive. STT, sulfamethoxazole-trimethoprim-tetracycline; AM, ampicillin; K, kanamycin; CIP, ciprofloxacin; CAZ, ceftazidime; ERY, erythromycin; tap, tap water; tank, tank water; inF, filter-sourced inlet water; spF, filter-sourced sprinkler water; chiveF, control-irrigated chive; taxonomic assig., taxonomic assignment.

Table 2. Total and target ARB from reservoir-water chain and irrigated chive plants.

w	c	taxonomic assig.	drain	R	inR	spR	chiveR	ABs[2]
		Achromobacter spp.	CIP	CIP, K	CIP, K	CIP, K	CIP, K	
	×	A. piechaudii					CIP	1
		A. xylosoxidans			K			1
		Acidovorax spp.			CIP	CAZ		
×		A. temperans			CIP			1
		Acinetobacter spp.		CAZ	CAZ	CAZ	CAZ	
×		A. baumannii			CAZ			1
×		A. lwoffii		CAZ				1
		A. pittii		CAZ	CAZ		CAZ	1
	×	A. protophormiae					CAZ	1
		Agromyces spp.					CIP, CAZ	
		Aeromonas spp.				CIP		
		Arthrobacter spp.	CIP	CIP, CAZ	CIP, CAZ	CIP, CAZ	CIP, CAZ	
		A. aurescens	CIP	CIP		CIP, CAZ	CIP	2
	×	A. mysorens					CIP	1
		A. nicotinovorans					CIP, CAZ	2
×	×	A. polychromogenes		CIP		CIP	CIP	1
×		A. saccharophilum			CAZ			1
×	×	A. ureafaciens	CIP			CIP	CAZ	2
×		A. woluwensis	CIP					1
		Bacillus spp.		CAZ			CAZ	
		Brevundimonas spp.		CIP	CIP, CAZ	CIP	CIP, CAZ	
		B. vesicularis		CIP		CIP, CAZ	CIP, CAZ	2
		Cellulosimicrobium spp.		CIP		CIP		
		C. cellulans		CIP		CIP		1
		Chryseobacterium spp.	STT			STT	STT	
×		C. gleum				STT		2
	×	C. scophthalmum					STT	2
		Citrobacter spp.	K	K	CAZ, K	CIP, AM, K	AM	
		C. amalonaticus				AM		1
×	×	C. freundii	K		CAZ	CIP	AM	4
×		C. gillenii	K	K	K	K		1
		Clostridium spp.			CIP			
×		C. glycolicum			CIP			1
		Comamonas spp.	CAZ					
		Cronobacter spp.					AM	
		Enterobacter spp.	AM	AM	AM	CAZ, AM	CAZ, AM	
×		E. aerogenes				CAZ		1

Table 2. Continued

w	c	taxonomic assig.	drain	R	inR	spR	chiveR	ABs[2]
		E. cloacae complex[1]	AM	AM		AM	CAZ, AM	2
		***Enterococcus* spp.**	**CIP, CAZ**	**CIP, CAZ, ERY**	**CIP**	**CIP, CAZ, ERY**	**CIP, CAZ, ERY**	
		E. casseliflavus	CIP, CAZ	CIP	CIP	CIP	CIP, ERY	3
		E. faecalis		ERY	CIP		CIP	2
×	×	*E. faecium*			CIP	CAZ	CIP	2
×	×	*E. gallinarum*			CIP	CIP, CAZ, ERY	ERY	3
×		*E. mundtii*		CAZ				1
		***Escherichia* spp.**	**CIP, AM, K**	**K**	**CIP, AM, K**	**AM**	**CIP, AM, K**	
		E. coli	CIP, AM, K	K	CIP, AM, K	AM	CIP, AM, K	3
		***Flavobacterium* spp.**			**CAZ**	**CAZ**		
		***Herbaspirillum* spp.**		**CIP**	**CIP**			
×		*H. huttiense*		CIP	CIP			1
		***Klebsiella* spp.**			**K**			
		***Kosakonia* spp.**					**AM**	
		***Lactobacillus* spp.**				**CIP**		
		***Microbacterium* spp.**			**CIP, CAZ**	**CIP, CAZ**	**CIP, CAZ**	
×		*M. liquefaciens*				CAZ		1
×		*M. paraoxydans*			CIP, CAZ			2
		***Morganella* spp.**	**AM**	**AM**	**AM**	**AM**		
		M. morganii		AM	AM			1
		***Ochrobactrum* spp.**	**CAZ, K**	**CAZ, K**	**CAZ, K**	**CIP, CAZ, K**	**CIP, CAZ, K**	
		O. intermedium	CAZ, K	CAZ, K		K	CIP, CAZ, K	3
×		*O. tritici*			CAZ, K	K		2
		***Pseudoclavibacter* spp.**					**CAZ**	
		***Pseudomonas* spp.**	**AM**	**AM**	**CIP, CAZ, AM**	**CIP, AM**	**CIP, CAZ, AM, K**	
		P. aeruginosa		AM	CAZ, AM	AM	K	3
		P. monteilii					CIP, CAZ	2
		P. putida		AM	AM		AM	1
		***Ralstonia* spp.**			**CAZ**			
		***Raoultella* spp.**		**STT, AM**	**AM**	**STT, AM**		
		R. ornithinolytica		STT, AM	AM	STT, AM		3
		***Rhizobium* spp.**					**CAZ**	
		R. radiobacter					CAZ	1
		***Rhodococcus* spp.**				**CAZ**		
×		*R. imtechensis*				CAZ		1
×		*R. opacus*				CAZ		1
		***Staphylococcus* spp.**					**CAZ**	
		***Stenotrophomonas* spp.**	**CIP**	**CIP**	**CIP**	**CIP**	**STT, CIP, CAZ, AM, K**	
		S. maltophilia		CIP			STT, CIP, K	4
		***Vagococcus* spp.**	**CIP**					
×		*V. fluvialis*	CIP					1
		***Weissella* spp.**					**CIP**	

[1]At the time of writing this paper, bacteria from the *Enterobacter cloacae* complex cannot be distinguished using MALDI biotyping.
[2]Number of antibiotic classes.
Antibiotic/species combinations detected in water as well as on chive plants are highlighted in grey. Species detected exclusively in the reservoir-system are marked (×): w, water; c, chive. STT, sulfamethoxazole-trimethoprim-tetracycline; AM, ampicillin; K, kanamycin; CIP, ciprofloxacin; CAZ, ceftazidime; ERY, erythromycin; drain, drain water; R, open-top reservoir water; inR, reservoir-sourced inlet water; spR, reservoir-sourced sprinkler water; chiveR, reservoir-irrigated chive; taxonomic assig., taxonomic assignment.
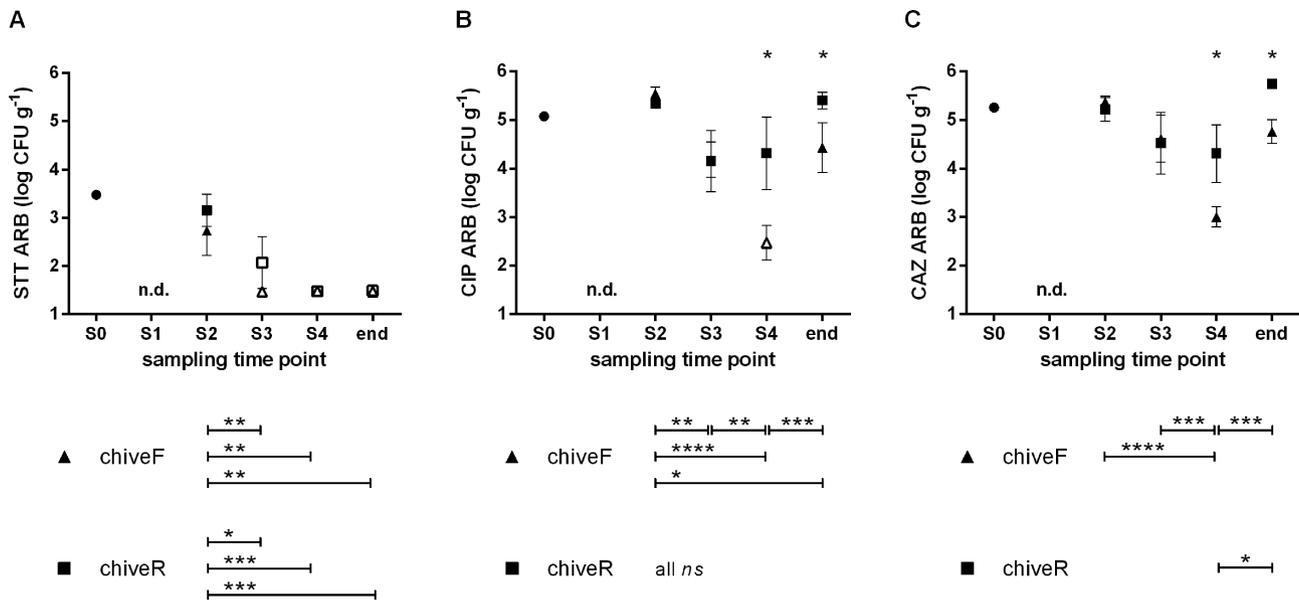
Figure 2. ARB determined for seedlings (dot) and for greenhouse-grown control- and reservoir-irrigated chive (triangles and squares, respectively). ARB isolated from R2A containing (A) sulfamethoxazole-trimethoprim-tetracycline (STT), (B) ciprofloxacin (CIP) or (C) ceftazidime (CAZ). S0, seedling sampling. S1–S4, sampling 1 to sampling 4; end, end of shelf life; chiveF, control-irrigated chive; chiveR, reservoir-irrigated chive; n.d., not determined; *ns*, not significant; ∗*P* < 0.05; ∗∗*P* < 0.01; ∗∗∗*P* < 0.001; ∗∗∗∗*P* < 0.0001. Results are displayed as mean values (n = 3) and error bars show standard deviations. Open symbols mark sample mean containing samples below the limit of detection.

Antibiotic resistance profiling of target ARB

For selected antibiotic-resistant *E. coli* and *Enterococcus* spp., resistance to clinically relevant antibiotics was determined in disk diffusion assays. Of note, for *Enterococcus* spp. the focus was on clinically relevant species (*E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. gallinarum*) (Leclercq *et al.* 2013).

In the control-system, *E. coli* was isolated once from chive (Table 1) and displayed resistance to kanamycin only (data not shown). In contrast, *E. coli* was isolated repeatedly from water of the reservoir-system along the whole irrigation chain as well as on chive (Table 2) and displayed resistances to up to six of the seven screened antibiotic classes (Table 3). Of the 45 investigated isolates, 36 (80%) were MDR, that is, resistant to 3 or more different antibiotic classes. Most resistances were attributed to the β-lactam antibiotics ampicillin and cefalotin (36 and 39 strains or 80 and 87%, respectively); the sulfonamide antibiotic sulfamethoxazole (37 strains, 82%) and trimethoprim (34 strains, 76%) or their combination (34 strains); and tetracycline (35 strains, 78%). Resistance to the quinolone antibiotic nalidixic acid was detected in two third of the isolates (30 strains, 67%) while resistance to fluoroquinolones was less frequent (norfloxacin: 19 strains, 42%; ciprofloxacin and levofloxacin: 16 strains each, 36%). No resistance was detected to piperacillin-tazobactam, ceftazidime, ceftriaxone, meropenem, ertapenem, imipenem, tobramycin, gentamicin, amikacin, colistin and nitrofurantoin.

In contrast to *E. coli* detected only after planting and irrigation, *Enterococcus* spp. were already detected on chive seeds and seedlings before entering the greenhouse (*E. faecium* and *E. mundtii* on seeds; *Enterococcus hirae* and *Enterococcus moraviensis* on seedlings; data not shown) and displayed resistance to one or two antibiotics (seeds and seedlings, Table 4). After planting, *E. casseliflavus* and *E. faecalis* were isolated from the control-system (Table 1): while *E. casseliflavus* were all resistant to norfloxacin, vancomycin and erythromycin, *E. faecalis* were only erythromycin-resistant (chiveF, Table 4). A higher species diversity was observed in the reservoir-system including additionally *E. faecium*, *E. gallinarum* and *Enterococcus mundtii* (Table 2). *Enterococcus casseliflavus* from drain water, reservoir-water and reservoir-irrigated chive were resistant to norfloxacin, vancomycin and erythromycin (Table 4). *Enterococcus faecalis* from reservoir water were resistant to erythromycin, streptomycin, tetracycline and chloramphenicol, whereas *E. faecalis* from reservoir-irrigated chive showed resistance to erythromycin only. *Enterococcus faecium* were resistant to up to three antibiotics including one ampicillin-resistant strain, and *E. gallinarum* were resistant to vancomycin, erythromycin, streptomycin and tetracycline (Table 4). Of the 39 investigated strains, 19 (49%) were MDR. Resistance to erythromycin was the most frequent (all 39 tested isolates), followed by norfloxacin (22 strains, 56%) and vancomycin (16 strains, 41%). Of note, all vancomycin-resistant strains from this study are known to carry intrinsic resistance and are therefore not considered VRE strains (Leclercq *et al.* 2013). Streptomycin and tetracycline resistances were observed in 4 strains (10%), resistances to chloramphenicol and ampicillin were rare (2 and 1 strains or 5 and 3%, respectively), and no resistance was observed to gentamicin, linezolid or tigecycline.

Tracing back MDR *E. coli*

Phylogenetic groups and sequence type

Escherichia coli was selected to investigate whether irrigation water constituted a source of MDR strains detected on the irrigated chive plants. To identify potential candidates for microbial source tracking, PG of 185 *E. coli* strains obtained from water or chive were determined. Strains belonging to groups A, B1, B2, C, D, E and F were detected (Clermont *et al.* 2013). The most frequent group was B1 (109 strains, 59%) followed by C (27 strains, 15%) and F (21 strains, 11%), while the remaining groups A, B2, D and E were represented by 3 to 5% of the isolates. PG of isolates from the antibiotic resistance profiling are shown in Table 3.

Table 3. Antibiotic resistance of ARB *E. coli* determined in disk diffusion assays.

Group	AM10	FEP	FOX	CPD	AMC	CXM	NA	NOR	SXT	MI	TGC	K	CIP	LEV	TMP	SMZ	FF	TE	TEMO	KF	CTX	ABs[1]	PG	
drain	×				×		×		×						×	×		×		×		5	B1	+
	×		×	×	×		×		×						×	×		×		×		5	B1	
	×						×		×						×	×		×		×		5	B1	
	×	×	×	×	×	×	×	×	×	×	×		×	×	×	×		×	×	×		5	B1	*
	×		×	×	×	×	×	×	×	×			×	×	×	×		×	×	×		5	B1	
	×		×	×	×	×	×	×	×	×			×	×	×	×		×		×		5	B1	
	×				×					×		×				×		×		×		5	C	
	×		×	×			×	×	×				×	×				×	×	×		4	D	
R																×						1	A	
																				×		1	C	
inR																×						1	A	
	×				×		×		×						×	×		×		×		5	B1	+
	×						×		×						×	×		×		×		5	B1	
	×	×	×	×	×	×	×	×	×	×	×		×	×	×	×		×	×	×		5	B1	*
	×		×	×	×	×	×	×	×	×			×	×	×	×	×	×	×	×	×	6	B1	
	×				×		×		×						×	×		×		×		5	B1	
	×						×	×	×						×	×		×		×		5	B1	
	×		×	×	×	×	×	×	×	×			×	×	×	×		×	×	×		5	B1	
	×		×	×	×	×	×	×	×	×			×	×	×	×	×	×	×	×		6	B1	
	×				×		×		×						×	×		×		×		5	B1	
	×		×	×			×	×	×				×		×	×		×		×		5	B1	
	×		×	×	×	×	×	×	×	×			×	×	×	×		×	×	×		5	B1	
	×		×	×	×	×	×	×	×	×			×	×	×	×	×	×	×	×	×	6	B1	
	×				×				×						×	×						2	D	
																				×		1	F	
spR									×									×	×			3	A	
	×				×				×	×					×	×		×				3	B1	
	×								×						×	×		×				3	B1	
	×	×	×	×	×	×	×	×	×	×	×		×	×	×	×		×	×	×		5	B1	*
	×		×	×	×	×	×	×	×	×			×	×	×	×		×	×	×		5	B1	
																				×		1	F	
																				×		1	F	
	×				×				×	×					×	×		×		×		4	F	
	×								×	×					×	×		×	×	×		4	F	
	×				×				×	×					×	×		×	×	×		4	F	
chiveR	×				×		×		×	×					×	×		×		×		5	B1	
	×				×		×		×	×					×	×	×	×		×		6	B1	
	×		×	×	×		×		×						×	×		×		×		5	B1	
	×				×		×		×						×	×		×		×		5	B1	+
	×		×	×	×	×	×	×	×	×			×	×	×	×		×	×	×		5	B1	
	×	×	×	×	×	×	×	×	×	×	×		×	×	×	×		×	×	×		5	B1	*
	×		×	×	×	×	×		×				×	×	×	×		×	×	×	×	5	B1	
	×		×	×	×	×	×	×	×	×			×	×	×	×		×	×	×	×	5	B1	
							×	×														1	B2	
																				×		1	F	

[1]Number of antibiotic classes.

Asterisk (*) and plus sign (+) mark strains selected for multilocus sequence typing.

AM10, ampicillin 10 μg; FEP, cefepime; FOX, cefoxitin; CPD, cefpodoxime; AMC, amoxicillin-clavulanic acid; CXM, cefuroxime; NA, nalidixic acid; NOR, norfloxacin; SXT, trimethoprim-sulfamethoxazole; MI, minocycline; TGC, tigecycline; K, kanamycin; CIP, ciprofloxacin; LEV, levofloxacin; TMP, trimethoprim; SMZ, sulfonamide; FF, fosfomycin; TE, tetracycline; TEMO, temocillin; KF, cefalotin; CTX, cefotaxime; R, reservoir; inR, reservoir-sourced greenhouse inlet; spR, reservoir-sourced greenhouse sprinkler; chiveR, reservoir-irrigated chive plants.

Table 4. Antibiotic resistance of ARB *Enterococcus* spp. determined in disk diffusion assays.

	AM2	VAN	NOR	ERY	STREP HC	TE	CM	ABs[1]	species
seeds				✖				1	*E. faecium*
seeds			✖	✖				2	*E. faecium*
seeds			✖	✖				2	*E. faecium*
sdl.				✖				1	*E. hirae*
sdl.				✖				1	*E. hirae*
drain		✖	✖	✖				3	*E. casseliflavus*
drain		✖	✖	✖				3	*E. casseliflavus*
R		✖	✖	✖				3	*E. casseliflavus*
R		✖	✖	✖				3	*E. casseliflavus*
R		✖	✖	✖				3	*E. casseliflavus*
R		✖	✖	✖				3	*E. casseliflavus*
R				✖	✖	✖	✖	4	*E. faecalis*
R				✖	✖	✖	✖	4	*E. faecalis*
spR	✖			✖	✖	✖		4	*E. gallinarum*
spR	✖			✖	✖	✖		4	*E. gallinarum*
spR		✖	✖	✖				3	*E. casseliflavus*
spR		✖	✖	✖				3	*E. casseliflavus*
chiveR				✖				1	*E. faecalis*
chiveR				✖				1	*E. faecalis*
chiveR				✖				1	*E. faecium*
chiveR				✖				1	*E. faecium*
chiveR			✖	✖				2	*E. faecium*
chiveR			✖	✖				2	*E. faecium*
chiveR			✖	✖				2	*E. faecium*
chiveR			✖	✖				2	*E. faecium*
chiveR			✖	✖				2	*E. faecium*
chiveR	✖		✖	✖				3	*E. faecium*
chiveF		✖	✖	✖				3	*E. casseliflavus*
chiveF		✖	✖	✖				3	*E. casseliflavus*
chiveF		✖	✖	✖				3	*E. casseliflavus*
chiveF		✖	✖	✖				3	*E. casseliflavus*
chiveF		✖	✖	✖				3	*E. casseliflavus*
chiveF		✖	✖	✖				3	*E. casseliflavus*
chiveF				✖				1	*E. faecalis*
chiveF				✖				1	*E. faecalis*
chiveF				✖				1	*E. faecalis*
chiveF				✖				1	*E. faecalis*
chiveF				✖				1	*E. faecalis*
chiveF				✖				1	*E. faecalis*

[1]Number of antibiotic classes.

AM2, ampicillin 2 μg; VAN, vancomycin; ERY, erythromycin; STREP HC, streptomycin high concentration; CM, chloramphenicol; sdl., seedlings; R, reservoir; spR, reservoir-sourced greenhouse sprinkler; chiveR, reservoir-irrigated chive plants; chiveF, filter-irrigated chive plants.

After determining PG, two sets of *E. coli* strains could be identified, each containing strains of same PG (group B1) and antibiotic resistance profiles (each set marked with asterisk or plus sign in Table 3, respectively). Within each set, strains originated from different water samples along the reservoir-irrigation chain as well as the respective chive plants. Multilocus sequence typing (MLST) revealed that all strains—from drain water over greenhouse inlet and sprinkler water to chive plant on the field—belonged to the same sequence type (ST1056).

Whole-genome sequencing

To prove strain identity indicated by MLST, the full genomes of the first and the last *E. coli*-ST1056 strain of the chain (drain water and chive plant) were sequenced. The number of obtained reads was 95′649 and 109′630 for the strain from water and chive, respectively, with a mean read length of 15′863 and 17′175 bp. Genome assembly using HGAP resulted in two contigs for each strain. A BLAST search revealed that for both strains the respective larger contig's (4.725 Mbp) closest match was the chromosome of *E. coli* strain 1943 (GenBank number CP023359.1), and the respective smaller contig's (116.5 kbp) closest match was *E.coli* plasmid pH2291-144 (GenBank number KJ484628.1). These two sequences combined (reference sequence Ecoli_1943_pH2291) were used for SNP-based phylogenetic analysis.

The sequence type of both strains was confirmed as ST1056 using the online tool MLST 1.8 (Larsen *et al.* 2012). The assembled sequences were then further analyzed using ResFinder 3.0 (known and unknown chromosomal point mutations and acquired ARG) as well as PlasmidFinder 1.3 and pMLST 1.4 (Zankari *et al.* 2012; Carattoli *et al.* 2014). Results generated by all tools were identical for the two strains. ResFinder results are shown in Fig. 3A. Acquired ARG were detected for aminoglycosides, β-lactams, sulfonamides, tetracyclines and trimethoprim on the contig matching the known plasmid pH2291-144 (Fig. 3A). Notably, all five genes were identical to the query sequence and covered its complete length (100% identity, query-to-HSP ratio of 1). A known chromosomal point mutation predicted to confer quinolone resistance was detected in both strains. Additionally, all detected unknown chromosomal point mutations were identical for both strains (Fig. 3A). Using PlasmidFinder, IncFIB and IncFII were detected in both strains (99.12% identity and 96.56%, query-to-HSP ratio 682/682 and 262/261, respectively; accession number AP001918 and AY458016; Fig. 3B). The replicon sequence type was identified as F24:A−:B6 using the pMLST tool (100% identity, query-to-HSP ratio of 1; Fig. 3B). Most relevantly, a complete and functional IncF plasmid conjugal transfer system consisting of 33 elements was identified using RAST on the plasmids of both strains (Fig. 3B).

Finally, using Ecoli_1943_pH2291 as a reference and the two assembled genomes, a phylogenetic tree and a SNP pairwise comparison table was generated. The SNP distance between drain water and chive isolate was eight, whereas the SNP distance to the reference was 7173 and 7171, respectively. Notably, the reference used was the closest one available based on BLAST results.

Phenotypic comparison

Escherichia coli-ST1056 from drain water and chive were tested on Biolog phenotype microarrays for utilization of carbon sources, nitrogen sources and osmotic stress. In total, 190 carbon sources, 95 nitrogen sources and 96 osmotic stress conditions were tested. Evaluation of end-points after incubation for 48 h revealed that the two strains were phenotypically identical. The only exception was D-allose, a sugar which could be utilized by *E. coli*-ST1056 from drain water, but not by the strain from chive (data not shown). Genome analysis revealed a frameshift mutation between the two strains in the gene encoding the periplasmic sugar binding protein of a ribose ABC-type transport system. Since D-allose can bind to ribose-binding proteins (Kim, Song and Park 1997), such a frameshift might cause the observed difference in D-allose utilization.

DISCUSSION

Irrigation water has been described as contamination source in agricultural systems (Olaimat and Holley 2012; Blaustein *et al.* 2015), and the microbiological quality of water used for fresh produce irrigation can vary considerably from tap water to various types of surface water such as rivers or lakes (Steele and Odumeru 2004). In addition to collecting various waters, rivers and lakes are prone to contamination through human activities or domestic and wild animals, and these pollutants can then be transferred to irrigated plants (Fonseca *et al.* 2011). Nevertheless, studies conducted under practice conditions are very scarce and mostly focus on human pathogens while testing artificially contaminated water (Erickson *et al.* 2010; Fonseca *et al.* 2011; Allende and Monaghan 2015). A very recent study on antibiotic-resistant *E. coli* suggested cross-contamination between water and vegetables but did not conclusively prove the link (Araújo *et al.* 2017). The present greenhouse study therefore described the population of ARB naturally occurring in irrigation water and chive under practice conditions and determined adequate ARB to demonstrate the potential of irrigation water as contamination source of fresh produce with ARB. Notably, while chive will not be consumed in such large quantities as other fresh produce such as lettuce it is a common ingredient in ready-made salads which can lead to a widespread distribution of the ARB.

From generic to ARB

Total HPC on chive continuously decreased over the growth period, as described previously in greenhouse-grown herbs (Gekenidis *et al.* 2017). A significant difference in total HPC, however, between control- and reservoir-chive was observed only at harvest (S4, Fig. 1C). Reasons for not detecting a significant difference earlier might be (a) greater soil proximity and thereby soil contamination of younger plants irrespective of irrigation regime, and (b) lower irrigation frequency of younger plants as compared to intensive irrigation one week before harvest—to reduce damage caused by *Thrips tabaci* (Poulsen 1989; Schuch, Redak and Bethke 1998)—both factors facilitating detection of an irrigation effect on older, that is, harvestable plants. It is worth noting that no significant difference was detected in total organic carbon, dissolved organic carbon and total nitrogen in sprinkler water of the two systems (data not shown). At the end of shelf life, total HPC on control-chive had risen to levels comparable to those of reservoir-chive. In contrast, numbers of bacteria resistant to ciprofloxacin and ceftazidime were significantly lower on control- compared to reservoir-chive at both harvest and end of shelf life (Fig. 2B and C). Thus, no conclusions could be drawn from total HPC on numbers of ARB, since ARB did not increase proportionally to total HPC on control-chive during storage.

A

acquired ARG	resistance gene	identity (%)	query/HSP	contig	resistance phenotype	accession no.
aminoglycosides	aadA1	100	792/792	plasmid	streptomycin, spectinomycin	JX185132
β-lactams	blaTEM-1B	100	861/861	plasmid	penicillins, 1st / 2nd gen. cephalosporins	JF910132
sulfonamides	sul1	100	927/927	plasmid	sulfonamides	CP002151
tetracyclines	tet(A)	100	1200/1200	plasmid	tetracycline	AJ517790
trimethoprim	dfrA1	100	474/474	plasmid	trimethoprim	JQ690541

chrom. point mutations	mutation	nucleotide change	amino acid change	contig	resistance phenotype	PMID
gyrase A	p.S83L	TCG → TTG	S → L	chrom.	quinolones, fluoroquinolones	15848289
DNA topoisomerase IV subunit A	p.E62K	GAA → AAA	E → K	chrom.	unknown	n.a.
sensor protein PmrA	p.G144S	GGC → AGC	G → S	chrom.	unknown	n.a.
sensor protein PmrB	p.D283G	GAC → GGC	D → G	chrom.	unknown	n.a.
	p.Y358N	TAC → AAC	Y → N	chrom.	unknown	n.a.
16S rRNA (rrsB)	r.250 A > T	A → T	n.a.	chrom.	unknown	n.a.
	r.253 A > T	A → T	n.a.	chrom.	unknown	n.a.
	r.273 T > A	T → A	n.a.	chrom.	unknown	n.a.
16S rRNA (rrsC)	r.226 A > G	A → G	n.a.	chrom.	unknown	n.a.
16S rRNA (rrsH)	r.250 A > T	A → T	n.a.	chrom.	unknown	n.a.
	r.253 A > T	A → T	n.a.	chrom.	unknown	n.a.
	r.273 T > A	T → A	n.a.	chrom.	unknown	n.a.
	r.1120 T > C	T → C	n.a.	chrom.	unknown	n.a.
ampC promoter	n.-1 C > T	C → T	n.a.	chrom.	unknown	n.a.
	n.-18 G > A	G → A	n.a.	chrom.	unknown	n.a.
23S rRNA	r.137 T > A	T → A	n.a.	chrom.	unknown	n.a.
	r.142 A > T	A → T	n.a.	chrom.	unknown	n.a.
	r.914 G > A	G → A	n.a.	chrom.	unknown	n.a.
	r.1174 T > G	T → G	n.a.	chrom.	unknown	n.a.
	r.1723 G > A	G → A	n.a.	chrom.	unknown	n.a.
	r.2203 T > G	T → G	n.a.	chrom.	unknown	n.a.
	r.2794 C > T	C → T	n.a.	chrom.	unknown	n.a.
	r.2796 T > C	T → C	n.a.	chrom.	unknown	n.a.
	r.2799 A > G	A → G	n.a.	chrom.	unknown	n.a.
	r.2802 G > A	G → A	n.a.	chrom.	unknown	n.a.

B

plasmid characteristics	plasmid size (kbp)	Inc (identity, %; query/HSP; accession no.)	sequence type (identity, %; query/HSP)
pC142, pC158	116.5	IncFIB (99.12; 682/682; AP001918)	FIB_6 (100; 373/373)
		IncFII (96.56; 262/261; AY458016)	FII_24 (100; 158/158)

IncF conjugal transfer system
conjugative transfer pilus assembly proteins (TraB, TraC, TraE, TraF, TraH, TraK, TraL, TraQ, TraU, TraV, TraW)
conjugative transfer proteins (TraD, TraG, TraN, TraP, TraR, TrbB, TrbC, TrbD, TrbE, TrbF, TrbG, TrbI, TrbJ)
conjugative transfer surface exclusion proteins (TraS and TraT)
conjugative transfer regulators (TraJ and TraY)
conjugative transfer DNA-nicking and unwinding protein TraI
conjugative transfer mating signal transduction protein TraM
conjugative transfer fertility inhibition protein FinO
conjugative transfer pilin acetylase TraX
conjugative transfer pilin protein TraA

Figure 3. (**A**) Acquired antibiotic-resistance genes and chromosomal point mutation identified in drain water and chive E. coli ST1056 using CSI Phylogeny. Note that all results were identical for both strains. Identity: % identical bases between query sequence and E. coli ST1056 sequence; query/HSP: query sequence length compared to alignment length; contig: location of detected resistance gene or chromosomal point mutation; PMID: PubMed ID; n.a., not applicable; chrom., chromosome. (**B**) Plasmid characteristics determined by PlasmidFinder, pMLST and RAST. pC142 and pC158 are the plasmids from drain and chive isolate, respectively.

Species diversity of ARB and resistance profiles

Comparing control- and reservoir-system in terms of species richness of ARB, the reservoir-system overall displayed only a slightly higher diversity (45 vs. 40 species), although the irrigation waters used were expected to differ greatly. The difference in species diversity of ARB was just as little pronounced on the irrigated plants: Merely three more species of ARB were detected on reservoir-chive (23 vs. 20 species). This is most probably due to numerous other contamination sources to which field-grown fresh produce is exposed beside irrigation water such as soil (Olaimat and Holley 2012) and which were common to both control- and reservoir-chive. Such might be for instance typical soil bacteria like Achromobacter spp., Arthrobacter spp., or Strepto-myces spp. (Ma et al. 2011). Since no analogous studies describing the diversity of ARB along a complete irrigation chain from water source to irrigated plants seem to exist, direct comparisons to existing data cannot be drawn. In terms of ARB described to occur in different kinds of water and the environment, many of the species detected in our study have been described before:

Achromobacter spp., *Acinetobacter* spp., *Citrobacter freundii*, *Enterobacter cloacae*, *E. coli*, *Flavobacterium* spp. and *Pseudomonas* spp. in water (McKeon, Calabrese and Bissonnette 1995; Messi, Guerrieri and Bondi 2005), or *Acinetobacter* spp., *Enterobacteriaceae*, *Enterococcus* spp., *E. coli* and *Streptomyces* spp. in different environments, including soil (Kümmerer 2004).

Potential candidates for bacterial tracing from plant to water source (10 and 16 in control- and reservoir-system, respectively) included both *E. coli* and *Enterococcus* spp. However, in the control-system only *E. casseliflavus* was isolated both from water and chive on ciprofloxacin (Table 1), whereas in the reservoir-system *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. coli* were isolated both from water and chive and on all antibiotics tested (exception: no *E. coli* detected on ceftazidime; Table 2).

Enterococcus spp.

In disk diffusion assays of *Enterococcus* spp., norfloxacin and erythromycin were the most frequent resistances (incl. known intrinsic resistance to erythromycin (Leclercq *et al.* 2013)). Among chive isolates these two resistances were the only ones detected, apart from intrinsic vancomycin-resistance and one ampicillin-resistance (Table 4)—in good agreement with previous studies on *Enterococcus* spp. from fresh produce, detecting frequent resistance to erythromycin and fluoroquinolones (Johnston and Jaykus 2004; Abriouel *et al.* 2008). Among water isolates, a few displayed additional resistances to high-level streptomycin, tetracycline and/or chloramphenicol, which have equally been described previously for waterborne *Enterococcus* spp. (Abriouel *et al.* 2008). In terms of antibiotic/species combinations, norfloxacin- and/or erythromycin-resistant *E. faecium* were detected on reservoir-chive and on seeds. An ampicillin-resistant *E. faecium* was isolated from reservoir-chive (chiveR; Table 4), however, this combination was not detected in reservoir-water (drain, R, and spR; Table 4) to suggest water as contamination source. *Enterococcus faecalis* on the other hand was isolated in the control-system but only from chive, and *E. faecalis* from reservoir-chive strongly differed in antibiotic resistance profiles compared to *E. faecalis* from reservoir-water. Only *E. casseliflavus* resistant to vancomycin, norfloxacin and erythromycin might have originated from irrigation water (drain and reservoir water, Table 4).

E. coli

In *E. coli* antibiotic resistance was observed towards up to six of seven antibiotic classes with a high proportion of MDR strains (80%, Table 3). Such high proportions of MDR in environmental *E. coli* have been described previously (Marinescu *et al.* 2015). The resistances frequently detected in our study towards β-lactams (especially ampicillin and cefalotin), sulfamethoxazole, trimethoprim and tetracycline have been described in previous studies for *E. coli* from different waters directly or indirectly related to wastewater treatment plant effluents (Hu *et al.* 2008; Rizzo *et al.* 2013; Marinescu *et al.* 2015) as well as from fresh produce (Holvoet *et al.* 2013; Lima *et al.* 2017). Finally, as opposed to other studies describing occurrence of ESBL-producing *E. coli* in different waters and fresh produce (Zurfluh *et al.* 2013; Nüesch-Inderbinen *et al.* 2015), no ESBL-producing strains were detected on the investigated farm.

The two whole-genome sequenced MDR *E. coli*-ST1056 harbored plasmid-borne ARG as well as a chromosomal point mutation known to confer quinolone resistance (Fig. 3A), covering most resistances observed in disk diffusion assays. Only exception was resistance to the third and fourth generation cephalosporins (cefpodoxime and cefepime, respectively) and amoxicillin-clavulanic acid, which cannot be explained by the presence of bla_{TEM-1B} alone. Notably, resistance to cefpodoxime and cefepime was marginal, i.e. one to three mm below the resistance cutoff. Additionally, plasmid-borne *aadA1* encoding resistance to the aminoglycosides spectinomycin and streptomycin was identified, which were not tested in disk diffusion assays.

Tracing back E. coli

Escherichia coli was chosen for source tracking for various reasons. First, a variety of antibiotic-resistant *Enterococcus* spp. were already detected on chive seeds and seedlings before coming into contact with irrigation water. Second, *E. coli* displayed much more diverse and clinically relevant phenotypic resistance profiles, with resistance to up to six antibiotic classes (Table 3). Finally, antibiotic-resistant *E. coli* were not present at any time in control-water whereas they were detected at all stages of the reservoir-irrigation system (Tables 1 and 2). Sequence typing of *E. coli* revealed that selected strains from water and chive belonged to ST1056, described previously as the main sequence type in poultry (Zurfluh *et al.* 2014b; Maamar *et al.* 2016). Its presence in wild birds can therefore be assumed to be likely, from where it could easily have entered our investigated water system, e.g. from the greenhouse rooftops draining rain water into the reservoir. Roof-harvested rain water has been described recently to be contaminated often with *E. coli* (Jongman and Korsten 2016b). Notably, evidence for water being the source of chive contamination had been found previously in a pilot study (summer 2015), where MDR *E. coli*-ST1432 had been detected on the same farm in drain water as well as on the irrigated chive plants (unpublished).

MLST has been recognized as a reliable method reflecting microevolution of the *E. coli* core genome and has therefore been used to determine phylogenetic relationships (Guenther, Ewers and Wieler 2011). However, albeit being widely used for microbial source tracking (Foley, Lynne and Nayak 2009), it might fail to distinguish very closely related but non-clonal strains. We therefore fully sequenced two MDR *E. coli*-ST1056 from beginning and end of the chain (marked with asterisk in Table 3). One of the two contigs assembled for each strain matched a known transmissible plasmid (Wang *et al.* 2014a), which was also isolated in Switzerland and described to harbor the same ARG detected in this study. SNP pairwise comparison yielded a SNP distance of only eight between the two isolates, while SNP distance to the reference was 7173 and 7171, respectively. As described by the developers of CSI Phylogeny using *Salmonella enterica* outbreak strains, it is difficult to define a general cutoff for pairwise SNP comparison to determine clonality (Leekitcharoenphon *et al.* 2014). However, by comparing outbreak strains and closely related background strains, they could show that SNP distances within outbreak strains were smaller than between outbreak and background strains. Furthermore, analysis of an *E. coli* test set provided by the CGE (five *E. coli* strains, including three clonal outbreak strains) (Cavaco and Leekitcharoenphon 2017) yielded a maximum SNP distance within clonal strains of 73, whereas the minimum SNP distance between outbreak and non-outbreak strains was 461. The SNP distance of 8 between MDR *E. coli*-ST1056 from drain water and chive, combined with the fact of identical sequence type, ARG profile including unknown chromosomal point mutations, and presence of a completely assembled plasmid leads to the conclusion that the two strains are clonal. As for the plasmid it must be pointed out that the IncFII plasmid family can replicate in many species of the *Enterobacteriaceae* family and is important

in the dissemination of plasmid mediated antimicrobial resistance, e.g. carbapenemase bla_{KPC} in *Klebsiella pneumoniae* (Chen *et al.* 2014).

In conclusion, our findings show that while edible plants are exposed to several potential contamination sources, irrigation water quality significantly influences the frequency of ARB on fresh produce under normal agricultural practice conditions. This finding is vital in the control of ARB and ARG transmission from the environment to humans via the food chain. We could prove the transmission of a MDR *E. coli*-ST1056 carrying a completely assembled transmissible IncFII-IncFIB resistance plasmid from drain water to the irrigated chive plants through the complete irrigation chain, including open-top reservoir, particle filter, greenhouse inlet and overhead sprinklers. This underlines the urgent need to establish guidelines for agricultural practice as well as monitoring recommendations regarding ARB in irrigation water. Transmission of antibiotic resistances in non-pathogenic bacteria via food to humans may be an unfavorable event as they can—upon establishment in the intestine—spread ARG or contribute to inactivation of antibiotics, whereas transmission of antibiotic-resistant human pathogens is a risk. Currently, regulations are established to ensure the minimization of the risk from pathogenic bacteria, however, the long-term risk to human health from non-pathogenic ARB is currently unknown. Further, for regions where good-quality water is scarce, affordable sanitation technologies must be developed to eliminate ARB and ARG from the water before applying it to the plants. Such technologies might include UV-based disinfection, but should ideally be combined with other methods to improve their efficiency (McKinney and Pruden 2012). Wastewater treatment plants may also profit from such technological advances to mitigate the amount of ARB and ARG released by them into surface waters, subsequently used for irrigation. Finally, responsible use of antibiotics must be propagated and controlled better, to at least slow down if not stop the constant increase in environmental ARB of utmost clinical relevance.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

ACKNOWLEDGEMENTS

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