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Research Article

Unde venis? Bacterial resistance from environmental reservoirs to lettuce: tracking microbiome and resistome over a growth period

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Abstract

Fresh produce is suggested to contribute highly to shaping the gut resistome. We investigated the impact of pig manure and irrigation water quality on microbiome and resistome of field-grown lettuce over an entire growth period. Lettuce was grown under four regimes, combining soil amendment with manure (with/without) with sprinkler irrigation using river water with an upstream wastewater input, disinfected by UV (with/without). Lettuce leaves, soil, and water samples were collected weekly and analysed by bacterial cultivation, 16S rRNA gene amplicon sequencing, and shotgun metagenomics from total community DNA. Cultivation yielded only few clinically relevant antibiotic-resistant bacteria (ARB), but numbers of ARB on lettuce increased over time, while no treatment-dependent changes were observed. Microbiome analysis confirmed a temporal trend. Antibiotic resistance genes (ARGs) unique to lettuce and water included multidrug and β-lactam ARGs, whereas lettuce and soil uniquely shared mainly glycopeptide and tetracycline ARGs. Surface water carried clinically relevant ARB (e.g. ESBL-producing *Escherichia coli* or *Serratia fonticola*) without affecting the overall lettuce resistome significantly. Resistance markers including biocide and metal resistance were increased in lettuce grown with manure, especially young lettuce (increased soil contact). Overall, while all investigated environments had their share as sources of the lettuce resistome, manure was the main source especially on young plants. We therefore suggest minimizing soil–vegetable contact to minimize resistance markers on fresh produce.

Keywords: antibiotic and biocide/metal resistance; fresh produce; irrigation water; manure; microbiome; mobile genetic elements

Introduction

Since the discovery of antibiotics, antibiotic resistance in pathogenic bacteria has increased dramatically due to over- and misuse in human and veterinary medicine (Davies and Davies 2010). As a result, antibiotic-resistant bacteria (ARB) are being detected more frequently, not only in clinics but also in the environment including soils and water bodies (Guenther et al. 2011, Graham et al. 2016, Gekenidis et al. 2018a, Cerqueira et al. 2019). In a nonclinical setting, transmission of ARB to humans occurs indirectly through handling of contaminated animal and natural products as well as directly via consumption of contaminated foods such as meat, fish, dairy products, or plant-based foods (Thanner et al. 2016). Fresh produce is of particular interest since its popularity as part of a healthy diet has risen in recent years (Food and Agriculture Organization of the United Nations 2020), and the frequent raw consumption of such products increases the probability of ARB transfer to the consumer (Rahman et al. 2022). This is all the more critical, as produce has been shown to harbor an impressive diversity of self-transmissible plasmids carrying multiple resistance genes (Blau et al. 2018).

The cultivation environment of fresh produce, including soil, irrigation water, and fertilizer, is a reservoir of ARB and antibiotic resistance genes (ARGs), which act as a potential source of contamination (Thanner et al. 2016). Soil is considered a natural reservoir of ARB and ARGs, where resistance determinants accumulate due to agricultural land-use and from where drainage into surface and groundwater can occur (Walsh and Duffy 2013). In turn, contaminated water reservoirs can serve as sources of ARB and ARGs when used for irrigation of fresh produce (Gekenidis et al. 2018b). Strikingly, wastewater treatment plants (WWTPs), which continuously release their effluent into nearby streams, are not able to completely eliminate antibiotics or resistance determinants and have been shown to enrich ARB and ARGs (Czekalski et al. 2012). As a result, WWTP discharge can lead to increased

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levels of ARB and ARGs in receiving rivers (Lekunberri et al. 2018, Lee et al. 2021), and combined sewer overflows can increase the contamination considerably (Lee et al. 2022). Further, organic fertilizers such as manure—especially when originating from animals having received antibiotic treatment in the past—can add to the soil resistome (Marti et al. 2013), thereby increasing the risk of fresh produce contamination with ARB and ARGs (Blau et al. 2019, Sanz et al. 2022). Finally, metal and biocide resistance should be considered jointly with antibiotic resistance, since co- and cross-resistance (i.e. distinct resistance determinants colocated physically in a bacterial genome or one resistance determinant conferring resistance to anti-infective compounds of several classes, respectively) is described frequently and is of clinical concern (Pal et al. 2015, Cândido et al. 2019).

It is well established that antibiotic resistance in the environment is an ancient phenomenon, while the more recently observed increased resistance levels are related to human activities (Rothrock Jr. et al. 2016, Iwu et al. 2020). There have been efforts to determine the natural occurrence of antibiotic resistance in pristine environments such as soil, seawater, or pristine plants, as opposed to antibiotic resistance of anthropogenic origin. A multitude of studies has demonstrated that soils contain high levels of native ARB carrying a broad arsenal of ARGs (Cytryn 2013). A study isolating bacteria from the deep terrestrial subsurface found resistance towards at least one of 13 antibiotics tested in 90% of isolated strains, with nalidixic acid, mupirocin, and ampicillin resistance being the most frequent (Brown and Balkwill 2009). A shotgun metagenomic study in 17 pristine and remote Antarctic soils identified 177 naturally occurring ARGs, the majority of which encoded single or multidrug efflux pumps (Van Goethem et al. 2018). Other common mechanisms related to aminoglycoside, chloramphenicol, and β-lactam antibiotic resistance. Interestingly, the researchers describe the lack of mobile genetic elements (MGEs) flanking ARGs. A metagenome-wide analysis comparing samples from pristine soils and seawater to human, chicken, and pig gut samples found a much higher concentration of intrinsic ARGs in pristine environments, while the abundance of total ARGs was significantly higher in gut samples including ARGs against last resort antibiotics (Zeng et al. 2019). The two dominant ARG types in both pristine environments were multidrug and aminocoumarin. In another study, psychrotrophic bacteria from Antarctic Marine waters were all resistant to ampicillin and more than half to chloramphenicol and streptomycin (De Souza et al. 2006). Finally, an investigation of 111 *Enterobacteriaceae* from pristine freshwater showed that resistance to multiple antibiotics was common (61%), most frequently toward β-lactams and chloramphenicol (Lima-Bittencourt et al. 2007). In contrast to soil or water, studies on the innate resistome of plants are rare. An investigation on the effect of struvite-application on the resistome of the *Brassica* microbiome revealed the presence of 25 ARGs from eight different classes unique to the phyllosphere [mainly macrolide–lincosamide–streptogramin B ARGs (MLSB) and multidrug], making them potentially phyllosphere-specific ARGs (Chen et al. 2017). A more extensive investigation of the same group comprising 12 plant species among which lettuce (*Lactuca sativa*), resulted in 172 ARGs unique to the plant phyllosphere while the plant host significantly affected ARG profiles (Chen et al. 2020). The ARGs shared between all samples conferred resistance to aminoglycoside, β-lactam, MLSB, multidrug, tetracycline, and vancomycin. Another work investigating *Sphagnum* moss as a plant growing in a pristine environment found a highly diverse resistome, targeting 29 antibiotics and covering all major resistance mechanisms with an extraordinarily high abundance of efflux pumps (≤96%) (Obermeier et al. 2020). As another environment with least anthropogenic influence, the primary vegetation of a retreating glacier was studied revealing a core set of phyllosphere ARGs across the successional sequence of plants, where multidrug and aminoglycoside ARGs were the most abundant (Li et al. 2023). Finally, a study of the native microbiome of two model indoor plants detected a variety of ARGs, with multidrug resistance (MDR) as the most prevalent resistance category followed by MLSB (Wicaksono et al. 2023).

The prevalence of a broad variety of ARB and ARGs in fresh produce including lettuce has been described in many studies (Blaak et al. 2014, Vital et al. 2017, Rahman et al. 2022, Yin et al. 2022, Kläui et al. 2024). In 2017, the World Health Organization defined a priority list of AR pathogens for which new antibiotics are urgently needed, divided into three priority classes: (1) critical priority, including carbapenem-resistant (CR) *Acinetobacter baumannii*, CR *Pseudomonas aeruginosa*, and CR and extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*; and (2) high priority, including vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus*, among others. Of these clinically significant ARB, many have also been isolated from food or environmental sources. For example, ESBL-producing *Enterobacteriaceae* and vancomycin-resistant *Enterococcus* spp. (VRE) have been isolated from a variety of fresh produce (Reuland et al. 2014, Kim et al. 2017). Resistant *Acinetobacter* spp. including *A. baumannii* have been isolated from hospital tap water, raw meat, and cattle manure (Shamsizadeh et al. 2017, Malta et al. 2020). *Pseudomonas aeruginosa* including CR isolates have been detected in fresh produce (Kläui et al. 2024). Multidrug-resistant as well as CR and ESBL-producing *Klebsiella pneumoniae*, a member of the *Enterobacteriaceae* family, have been isolated from raw milk, various meats, fruits, and raw vegetables (Hartantyo et al. 2020, Junaid et al. 2022). Nevertheless, more research is needed to better understand: (1) which environmental reservoirs contribute to the transfer of ARB and ARGs to foods and to what extent (especially to fresh produce, which is exposed to a wide variety of potential contamination sources; Drissner and Gekenidis 2023), (2) which bacterial genera and resistance genes are involved, and (3) whether resistance determinants persist until harvest in case of an early contamination event.

The aim of the present study was to identify the changes in microbiome composition of lettuce as well as potential source microbiomes in soil, manure, and irrigation water, and to track ARB and ARGs from the investigated environmental reservoirs to the beginning of the plant food chain in order to identify the main sources of fresh produce contamination. As a model system, we used lettuce (*L. sativa*, Salanova® Barlach), grown on a field of a conventional lettuce farm. The lettuce was grown under four conditions, representing all combinations of conventional, that is, nonmanured field soil (common practice) or manure-amended field soil with untreated or UV-treated river water irrigation. In a culture-based approach, we quantified total bacterial numbers and presumptive ARB present in the four environments lettuce leaves, soil, water, and manure, and monitored their development throughout the lettuce growth period. Our target bacteria included ESBL-, carbapenemase-producing, and fluoroquinolone-resistant *Escherichia coli* and *K. pneumoniae*; VRE; and *A. baumannii* and *P. aeruginosa*. We further characterized the microbiome of the same four environments using amplicon sequencing, and investigated their resistome including resistance to antibiotics, biocides, and metals. Such insights are essential to establish comprehensive resistance-monitoring programs along the plant food chain and to develop recommendations for improved agricultural practices.

Material and methods

Field site and experimental treatments

The field trial was conducted in summer 2017 (July and August) on a conventional salad farm in the canton of Berne (Switzerland), in a region known for its fertile peat soils. More specifically, the soil of the selected field site has been classified as calcaric cambisol. The selected field site is used for lettuce cultivation all year long and is shaded by a foil tunnel, which reduces the impact of environmental factors such as rain, wind, and UV radiation on the plants. The common practices of the farm included opening the foil tunnel on very hot days to avoid overheating, plowing the soil before each new culture, and sprinkler irrigating with water pumped from a nearby river (Saane) receiving up to 9.1% of effluents from a WWTP located about 2 km upstream (Federal Office of the Environment 2014). For the present trial, half of the plants were irrigated with UV-disinfected river water. To this purpose, a water disinfection device AQUASTERA/Aqua UVtron ASUV46 was used (aquatec solution GmbH, Schwarzenbach, Switzerland), providing a minimal UVC irradiance of 26.4 W/m^2. Lettuce seedlings (*L. sativa*, Salanova® Barlach) of 3 g were purchased from a local supplier. Stored pig manure was obtained from a private pig farm and used for manual soil amendment after lettuce planting while taking special care not to contaminate the seedlings with manure. The conventional, that is, nonmanured field soil (common practice) will be termed 'conventional soil' in the following. Overall, the lettuce was managed according to the farmer's recommended practice for planting, irrigation, and temperature regulation.

The selected field site consisted of two adjacent patches (total: 100 m × 4 m), and each was planted with four rows of lettuce heads (35 cm distance between heads). The patches were divided into four sectors from front to rear with buffer zones in between them, to compare the common practice to three alternative practices (four treatments A–D; Fig. S1): (A) river water and conventional soil (common practice), (B) river water irrigation and manure-amended soil, (C) UV-treated river water irrigation and manure-amended soil, and (D) UV-treated river water irrigation and conventional soil. Each of the four treatment sectors was further divided into 18 sampling plots to provide three harvest replicates for 6 weeks, each containing eight lettuce heads (576 lettuce heads in total). At the end of each treatment sector, four plots containing eight lettuce heads each served as buffer zones between treatments.

Sampling campaigns

Prior to the field trial, lettuce seedlings, conventional field soil, manure, and river irrigation water were sampled (week 0 or initial condition Z) as described in the respective section below. Starting 1 week after planting (week 1), lettuce, soil, and irrigation water were sampled weekly during the 6-week lettuce growth period, to study microbiome and resistome progression over time. Three biological replicates per treatment and environment (lettuce, soil, water, and manure) were collected each week. Plots were randomly selected for each time point using R (version 3.4.0). Gloves were worn at all times and changed between treatments. Samples were collected in order of cleanness, starting with the UV-treated river water irrigation/conventional soil treatment (D > A > C > B). Lettuces were always harvested before soil to avoid contamination of lettuce with soil. All samples were cooled during transport. Lettuce and soil samples were processed within 8 h, water samples within 36 h.

Lettuce harvesting

A stainless steel knife was used for harvesting and cleaned with 80% ethanol between lettuce heads. Per biological replicate, eight lettuce heads were cut ~1 cm above the soil, excluding the lower leaves. Special care was taken to ensure that the lettuce was not contaminated with soil during sampling. The lettuce leaves were directly transferred into sterile plastic bags for subsequent analysis. The plastic bags were sealed, including some air to minimize squeezing of lettuce leaves during transport.

Soil core collection

After lettuce heads were harvested, soil samples were collected from the top 10 cm of soil within 10 cm distance of the harvested lettuce heads using a soil corer (2.5 cm diameter). Between treatments, the soil corer was cleaned and disinfected with 80% ethanol. Per biological replicate, eight soil cores were pooled in a sterile plastic bag and mixed thoroughly.

Irrigation water sampling

Before sampling the untreated river water, 5-l sampling bottles were disinfected with 80% ethanol and rinsed twice with the water to be sampled. Additionally, two sterile 1-l bottles were filled with UV-disinfected river water by drawing off water at the outflow of the UV disinfection device.

Sample processing

Lettuce leaf washes

The plastic bags containing the lettuce leaves were shaken gently to ensure proper mixing. Eighty grams per replicate were weighed into sterile plastic containers and gently transferred into a plastic bottle containing 720 ml of phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 in 1 l distilled water; pH 7.3). The bottles were then sonicated for 7 min, rotating them after 3.5 min to ensure homogenous sonication. The resulting leaf wash was sieved into sterile glass bottles to avoid further soaking and disruption of the lettuce. Notably, this procedure was chosen to recover surface-attached bacteria while omitting internalized bacteria, since internalization from contaminated field soil has been described to be small or absent (Detert and Schmidt 2023), ARG numbers detected in root and leaf endophytes have been found to be markedly lower than those on the leaf surface (Zhang et al. 2019), and internalized pathogens seem to persist only in the short term (Erickson 2012). A 30-ml aliquot of each leaf wash was stored on ice for later cultivation. For DNA extraction, 200 ml of leaf wash were filtered through polycarbonate (PC) filters (0.2 µm pores, 47 mm diameter; Whatman plc, Buckinghamshire, UK). Each filter was then carefully transferred into DNeasy PowerWater Kit bead tubes (Qiagen, Venlo, Netherlands), top side facing inwards. Finally, the DNA tubes were shock frozen in liquid nitrogen and stored at −80°C until further processing.

Soil and manure processing

The plastic bags containing the soil samples were shaken gently to ensure proper mixing. Soil was sieved (mesh size 2 mm) onto clean aluminum foil, and the sieve was cleaned between samples using 80% ethanol. Thereof, 20 g (wet weight) were dissolved in 180 ml of PBS, vigorously shaken for 1 min, and allowed to settle for 5 min. The supernatant was transferred into 100 ml glass bottles and left to settle for another 5 min. The supernatant thereof was stored at 4°C for cultivation. Finally, three times 250 mg of soil (wet weight) were aliquoted into DNeasy PowerSoil Kit bead tubes

(Qiagen), shock frozen, and stored at −80°C for later DNA extraction. Manure samples (only sampled in week 0 = initial condition Z) were treated in the same manner as soil samples.

Irrigation water filtering

For bacterial culturing, 200 ml of river or UV-treated river water were filtered in duplicates through cellulose acetate (CA) filters (0.2 µm pores, 47 mm diameter; Sartorius AG, Göttingen, Germany). For DNA extraction, 700 ml of water were filtered using two PC filters (300 ml and 400 ml, respectively), to avoid filter clogging. The filters were transferred into DNeasy PowerWater Kit bead tubes, shock frozen, and stored at −80°C.

Bacterial cultivation

For determination of total heterotrophic bacteria, R2A agar was used (Merck Millipore, MA, USA). ARB were cultured using commercially available media. Brilliance™ ESBL and CRE agar (ESBL and CRE; Thermo Fisher Scientific, Waltham, MA, USA) were used for isolation of ESBL-producing and CR *E. coli* and *Klebsiella* spp. Fluoroquinolone-resistant *E. coli* or *Klebsiella* spp. were cultured on Brilliance™ *E. coli*/coliform Selective Agar (CM; Thermo Fisher Scientific) or Simmons Citrate Agar with 1% (g/g) myo-inositol (SCA; Sigma-Aldrich, St. Louis, MI, USA) supplemented with ciprofloxacin (1 mg/l), respectively. Brilliance™ VRE agar (VRE; Thermo Fisher Scientific) was used for isolation of VRE. Finally, *Acinetobacter* spp. and *Pseudomonas* spp. were cultured on Leeds *Acinetobacter* Medium (LEE; Hardy Diagnostics, Santa Maria, CA, USA) and Cetrimide Agar with 1% (g/g) glycerol (CTM; Thermo Fisher Scientific), respectively.

From the prepared leaf and soil washes, 100 µl of each sample were spread-plated in duplicate on each selective agar type. For R2A, 10-fold dilutions in PBS were prepared and the most appropriate two dilutions were plated. For water, duplicate CA filters were transferred to each agar directly after water filtering. All plates were incubated at 37°C for 24 h, except for LEE (35°C, 24 h) and R2A (25°C, up to 5 days). Thereafter, plates were inspected for growth and when no growth was visible, CRE, VRE, and SCA plates were incubated for another 24 h according to instructions. Total colonies as well as target colonies displaying coloration typical for each selective medium were enumerated.

MALDI-TOF biotyping

Randomly selected bacterial colonies displaying typical morphology as well as secondary colonies were identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) biotyping with the direct smearing technique described previously (Gekenidis et al. 2014). Identifications with a score above 2.0 were considered reliable on species level while identifications with a score between 1.7 and 2.0 were considered reliable on genus level, as recommended.

DNA extraction, amplicon, and shotgun metagenomic sequencing

Total DNA was extracted from the water- and leaf-wash-filters using the DNeasy PowerWater Kit, while the DNeasy PowerSoil Kit was used on the prealiquoted soil and manure samples, using the kit best adapted to each sample type in order to maximize DNA yield and quality. Both kits included a harsh lysis step using PowerBead tubes and specially formulated cell lysis buffers. All DNA extracts were checked using a Quant-iT™ High-Sensitivity dsDNA Assay Kit on a Qubit 3.0 Fluorometer (concentration measurement; Thermo Fisher Scientific) as well as a NanoDrop One Spectrophotometer (purity measurement; Thermo Fisher Scientific).

To study the microbial communities, Illumina MiSeq® sequencing of the 16S rRNA gene V3–V4 hypervariable regions was performed, using universal primers 341F and 805R (Herlemann et al. 2011). DNA extracts from soil and lettuce were diluted to a final concentration of 5 ng/µl, while low-concentrated DNA extracts from water were concentrated on an Eppendorf Concentrator plus centrifuge (Eppendorf, Hamburg, Germany). Sequencing libraries were produced following Illumina's 16S Metagenomic Sequencing Library Preparation guide, using Phusion Hot Start II High-Fidelity Master Mix (Thermo Fisher Scientific) for amplification. The libraries were pooled for paired-end sequencing on a MiSeq v3 cartridge (2 × 300 bp; Illumina, San Diego, CA, USA).

For resistome analysis, samples from weeks 0, 1, and 5 were selected. Biological replicates were pooled and metagenomic libraries were prepared using a NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA), following the high-input protocol including size selection. First, enzymatic digestion was performed during 10 min. After library preparation, fragment size distribution was checked on a D1000 ScreenTape System (Agilent Technologies, Santa Clara, CA, USA). Samples were barcoded using NEBNext Multiplex Oligos for Illumina (Unique Dual Index Primer Pairs; New England Biolabs), adjusted to 4 nM, and pooled equimolarly. The pool was sequenced on a NovaSeq™ 6000 System (2 × 250 bp, SP-type flow cell; Illumina).

Bioinformatics

Amplicon sequencing: microbial community data analysis

The demultiplexed 16S rRNA gene amplicon sequences were processed using USEARCH (detailed protocol: AmpSeq_DataPrepReport, see online Supplementary material). Briefly, in a first step, the 3′ ends of the reads were cut to improve the subsequent merging. In a next step, the primer regions of the merged reads were removed. Before clustering, size selection and quality filtering were performed. The amplicon sequences were then clustered into operational taxonomic units (OTUs) with 97% identity or into zero-radius zOTUs. In addition, the zOTUs were clustered at an identity of 99%, 98% and 97%. The SILVA SSU v128 database was used as a reference for taxonomic predictions. After annotation, clusters classified as mitochondria or chloroplasts were excluded from downstream analysis, while Archaea were retained for downstream analysis (detailed read statistics: AmpSeq_ReadReport, see online Supplementary material).

Microbial community compositions were investigated using the web-application Calypso (v8.84) (Zakrzewski et al. 2017) and R (v4.0.2) implemented in RStudio (RStudio Team. 2020). Upon import into Calypso, data was filtered as recommended by allowing only samples with more than 1000 sequence reads and taxa with less than 0.001% relative abundance across all samples, including maximally the top 20 000 taxa (filtered by mean). No samples were lost by the applied filtering. Additionally, cumulative-sum scaling with log2-transformation (Paulson et al. 2013) was applied to normalize the data. All downstream Calypso tools were used with default settings, unless otherwise indicated. Principal coordinates analysis (PCoA) was conducted for visualization in RStudio.

Metagenomics: resistome analysis

Two major approaches were used to analyse metagenomics read data: a read-based analysis to detect resistance genes after minimal data processing and thereby least information loss, and an

assembly-based analysis to explore the genetic context of resistance genes including potential for genetic mobility.

Read-based analysis

First, resistance genes were annotated to minimally processed reads using two reference databases. To do so, Illumina adapters were removed from raw paired-end reads using Cutadapt (v1.15) (Martin 2011), allowing 10% errors. Sequences were then trimmed with sickle (v1.33; paired-end, –qual-type Sanger) using a minimum quality score per window of 20 and a length threshold of 20 (Joshi and Fass 2011). The obtained trimmed reads were processed with DeepARG-SS (identity: 70%, e-value: 1e−10, target coverage: 60% and probability: 0.7) (Arango-Argoty et al. 2018) and ARGs-OAP (v2.0) (Yin et al. 2018) for ARG annotation. The resulting ARG tables were analysed using Microsoft Excel and RStudio (summary tables, PCoA, Venn diagram). PCoA was performed on 16S-normalized data using the ARGs-OAP v2.0 pipeline in RStudio.

Assembly-based analysis

Second, raw reads were assembled into contigs, open-reading frames (ORFs) predicted, and resistance genes and genetic mobility elements annotated using several reference databases (detailed protocol: MetaSeq_DataPrepReport, see online Supplementary material). Briefly, the raw paired-end reads were first processed to remove adapters, low complexity and low quality reads, correct overlaps of at least 30 bp, and discard reads shorter than 100 bp. *Lactuca sativa* (chromosome and chloroplast) DNA was then removed from all samples. The obtained clean reads were assembled, and protein-coding genes were predicted while retaining only ORFs longer than 50 amino acids. The obtained ORFs were searched against the profile hidden Markov model databases Pfam, Resfams (Gibson et al. 2015), and TIGRFAMs (Haft et al. 2001), as well as the BacMet database (Pal et al. 2014) containing antibacterial biocide and metal resistance genes (BMRGs). Only hits with bit score >50 and target coverage >60% were kept. All results tables were filtered to retain only the best annotation for each ORF. TIGRFAMs hits were additionally filtered using a list of keywords (transposase, transposon, conjugative, integrase, integron, recombinase, conjugal, mobilization, recombination, and plasmid) to retain annotations related to MGEs only, as described by Forsberg et al. (2014). Finally, plasmid tags were annotated. For better inter sample comparison, all counts were normalized by number of reads per sample to obtain counts per one million reads (CPM).

A Circos plot for visualization of ARG distribution among environments was produced using a freely available online tool (Krzywinski et al. 2009). MDR contigs were defined as contigs carrying three or more ARGs assigned from the Resfams database. Non-metric multidimensional scaling (NMDS) of all samples was conducted in RStudio using Bray–Curtis distance measure and dimensions k = 3.

All R scripts used for data analysis are available from GitHub (https://github.com/maria-gekenidis/lettuce-resistome). The main R-packages used include ape, DESeq2, labdsv, microbiome, phyloseq, phyloseq.extended, plotly, and vegan.

Statistical analysis

For culture data analysis, bacterial counts were log10-transformed and statistically compared to detect an effect of time or treatment using two-way analysis of variance (two-way ANOVA) and adjusting *P*-values using Tukey's or Šídák's multiple comparison tests (GraphPad PRISM 8, GraphPad Software Inc., San Diego, CA). Where no visible growth was observed, half the limit of detection (LOD/2) was used. Statistical significance was assigned at $P < .05$.

In microbial community data analysis, differentially abundant (DA) taxa on lettuce were identified using Calypso at different taxonomic levels by pairwise comparisons {Student's *t*-test; *P*-values adjusted for multiple testing using Benjamini–Hochberg [False Discovery Rate (FDR)]-correction}. Additionally, the top 10 DA taxa at each level were identified using the R-package DESeq2 (FDR-adjusted $P < .05$). Alpha-diversity of lettuce communities was estimated in Calypso using different measures including Chao1, Shannon Diversity index, and Inverse Simpson's index (measuring species richness, richness and evenness, and dominance, respectively), and significant differences between time points or between treatments were detected by one-way ANOVA. Beta-diversity of lettuce communities was measured by Bray–Curtis, Jaccard, Unifrac, and weighted Unifrac distances (each taking into account presence/absence, taxa abundance, and/or phylogeny) and compared between time points and between treatments in RStudio by permutational analysis of variance (PERMANOVA) with 9999 permutations, for diversity measures with homogeneous multivariate dispersions among groups. All *P*-values were adjusted using FDR-correction.

To quantify the extent to which the observed resistome profiles in lettuce correlated with the respective microbial community structure, Procrustes analysis was performed using ResistoXplorer (default parameters; Dhariwal et al. 2021) and RStudio. For the microbiomes, zOTUs clustered at 99% and annotated with the SILVA database were used as input, after removing samples not represented in the resistome data, rarefying to the lowest depth, and merging biological replicates by summing in order to match the sample structure of the resistome dataset. For the resistome, abundance tables based on Resfams/BacMet annotations normalized by CPM were used.

Data accessibility

The Illumina MiSeq raw paired-end reads (ERR4552817–ERR4552843; ERR4552847–ERR4552882, ERR4552890–ERR4552950, ERR4554840–ERR4554892, and ERR4555363–ERR4555379) were submitted to the European Nucleotide Archive (ENA) under project number PRJEB36754.

The Illumina NovaSeq™ raw paired-end reads (ERR3929355–ERR3929377) as well as the trimmed reads (ERR3943975–ERR3943997) were deposited in ENA under project number PRJEB36754.

Results

Culturing and species identification

Heterotrophic bacteria and bacterial counts on antibiotic-containing media show temporal variation on lettuce and a strong impact of UV-treatment in water

First, the change in number of total heterotrophic bacteria on young compared to mature lettuce was assessed by counting colony forming units (CFU) on R2A plates for lettuce of all treatments harvested at weeks 1 and 5, but no systematic differences were observed. All counts were between 3×10^5 and 2×10^6 CFU/g. In soil, counts were significantly higher in week 5 than in week 1 for all treatments, increasing on average from $1 \times 10^6 \pm 3 \times 10^5$ to $4 \times 10^6 \pm 5 \times 10^5$ CFU/g wet weight (A: $P < .0001$, B: $P = .0019$, C: $P = .0015$, and D: $P = .0006$). Finally, as expected UV-treatment resulted in a reduction of total heterotroph counts (week 1: from

$4 \times 10^3 \pm 1 \times 10^3$ to $9 \times 10^2 \pm 7 \times 10^2$ CFU/100 ml, ns; week 5: from $7 \times 10^5 \pm 5 \times 10^5$ to $7 \times 10^3 \pm 4 \times 10^3$ CFU/100 ml, $P = .0029$).

In a next step, bacterial counts on chromogenic plates selective for ESBL-producing *Enterobacteriaceae*, CRE, and VRE were determined by plating and counting total CFU. Additionally, bacterial colonies displaying coloration typical of the target bacteria for each selective plate were enumerated as presumptive target bacteria, since typical coloration may be displayed by nontarget bacteria as well. Bacteria targeted by ESBL and CRE agar may include the *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., and *Citrobacter* spp. (KESC) group as well as *E. coli*. In the following, these counts are collectively referred to as ARB counts, although intrinsically resistant bacteria can grow on the mentioned selective plates when plating environmental samples.

Total counts from lettuce on ESBL agar significantly increased over time for all four treatments on average from $3 \times 10^4 \pm 1 \times 10^4$ to $3 \times 10^5 \pm 1 \times 10^5$ CFU/g (A[w1 versus w6]: $P = .0001$, B[w1 versus w6]: $P = .0385$, C[w1 versus w6]: $P = .0056$, and D[w1 versus w5]: $P = .0014$), while no clear trend was observed for either presumptive KESC or presumptive *E. coli* (data not shown). In soil, all counts were stable over time with only a few exceptions (data not shown). Finally, UV-treatment of river water always significantly reduced bacterial counts on ESBL agar (total: 4×10^1–5×10^2 CFU/100 ml; presumptive KESC: 2×10^{-1}–4×10^0 CFU/100 ml; presumptive *E. coli*: 2×10^{-1}–2×10^1 CFU/100 ml) below detection.

As for total counts on ESBL agar, total counts from lettuce on CRE agar consistently increased over time for all four treatments from $2 \times 10^4 \pm 4 \times 10^3$ to $3 \times 10^5 \pm 8 \times 10^4$ CFU/g on average (A[w1 versus w6]: $P < .0001$, B[w1 versus w6]: $P < .0001$, C[w1 versus w6]: $P = .0060$, and D[w1 versus w6]: $P < .0001$). A similar trend was observed for presumptive KESC counts though not significant, while presumptive *E. coli* were mainly detected in weeks 1 and 2, and were mostly below detection thereafter (data not shown). In soil, all counts fluctuated with no significant differences (data not shown). Finally, UV-treatment of river water reduced bacterial counts on CRE agar (total: 6×10^1–1×10^3 CFU/100 ml; presumptive KESC: 3×10^0–1×10^2 CFU/100 ml; and presumptive *E. coli*: 6×10^{-1}–7×10^0 CFU/100 ml) by up to nearly three log units.

As for ESBL and CRE plates, total counts from lettuce on VRE plates increased with time on average from $2 \times 10^3 \pm 2 \times 10^2$ to $1 \times 10^5 \pm 5 \times 10^4$ CFU/g (A[w1 versus w5]: $P = .0039$, B[w1 versus w5]: $P < .0001$, C[w1 versus w5]: $P = .0185$, and D[w1 versus w4]: $P <$ XXX). In soil, total VRE counts were mostly stable over time with a few exceptions (data not shown), while UV-treatment of river water significantly reduced total VRE counts (2×10^0–7×10^1 CFU/100 ml) to below detection (except week 1: reduction to $1 \times 10^1 \pm 4 \times 10^0$ CFU/100 ml). Notably, colonies displaying coloration typical of the target bacteria on VRE agar were never detected.

Overall, significant increases in ARB numbers were observed for lettuce over time within treatments, while significant differences between treatments of the same time point were rare and with no discernable patterns. In soil, ARB numbers were usually stable or fluctuated without correlation to time or treatment. However, UV-treatment of river water mostly significantly reduced total as well presumptive bacterial counts on all three antibiotic-containing media.

MALDI biotyping reveals rare occurrence of clinically relevant ARB

To further investigate bacterial composition on the chromogenic selective agar plates, representative colonies displaying typical coloration as well as secondary colonies were identified at genus and species level using MALDI biotyping (Tables S2–S6).

ESBL agar plates were dominated by *Pseudomonas* spp. Additionally, *Enterobacter cloacae* was detected once on lettuce grown on conventional soil with UV-treated river water irrigation (week 2), *E. coli* once in river water (week 4), and *Serratia fonticola* three times in river water (weeks 1–3; Table S2). Notably, *E. coli* was also identified in river water (week 4) and in manure on ciprofloxacin-supplemented CM plates (data not shown). Of the species targeted by CRE agar, only *Klebsiella oxytoca* was detected on lettuce grown on manure-amended soil irrigated with river water (week 1; Table S3). On the other hand, no ciprofloxacin-resistant *Klebsiella* spp. were identified on SCA plates (data not shown).

On VRE agar plates, *Enterococcus* spp. could not be identified at any time, while *Sphingomonas* spp., *Sphingobacterium* spp., and *Pseudomonas* spp. were the most frequent genera (Table S4). Finally, on CTM and LEE agar all identified species belonged to the respectively targeted genera *Pseudomonas* spp. and *Acinetobacter* spp. (Tables S5 and S6). Notably, the clinically important species *P. aeruginosa* was detected only sporadically and *A. baumannii* not at all. We therefore assumed that lettuce is not an important source for these species and did not investigate their resistance potential further. Of importance, other species of the genera *Pseudomonas* and *Acinetobacter* are also known as reservoirs of antibiotic resistance, including for example *P. putida* and *P. fluorescens* (Sabour et al. 2023), or *A. bereziniae* and *A. johnsonii* (Sheck et al. 2023) detected by culturing in this study.

Microbiome analysis

Microbial communities show temporal shifts in lettuce and a strong impact of UV-treatment in water

To complement culture-based findings, bacterial communities of lettuce, soil, water, and manure were characterized by sequencing of 16S rRNA gene fragments amplified from community DNA. On the leaves of lettuce seedlings before planting, the prominent phyla were Parcubacteria followed by Proteobacteria, Planctomycetes, and Actinobacteria (Fig. 1A, week 0). One week after planting, the latter three phyla were still dominant, while Parcubacteria were almost undetectable (Fig. 1A, week 1). Additionally, relative abundance of Chloroflexi had increased compared to week 0. As the plants grew, Proteobacteria became increasingly dominant until they accounted for more than 90% of the community in most samples by week 5. Finally, 6 weeks after planting, the communities most resembled 1-week-old lettuce communities, with overall a striking decrease in the relative abundance of Proteobacteria but increased Acidobacteria, Actinobacteria, Chloroflexi, and Planctomycetes as compared to week 5 (Fig. 1A, week 6). In contrast to this clear shift in communities over the growth period, no systematic differences were detected between the different treatments within each time point. Only notable exception was treatment A of week 6, with more than double the relative abundance of Proteobacteria compared to the other three treatments (Fig. 1A).

In manure samples, Proteobacteria dominated with a relative abundance of more than 90% (Fig. 1B, panel M). On the other hand, soil communities were very stable over time regardless of the applied treatment, and displayed Planctomycetes as dominant phylum, followed by Chloroflexi, Acidobacteria, Proteobacteria, and Actinobacteria (Fig. 1B). Notably, soil communities overall most resembled lettuce communities of weeks 1 and 6 (Fig. 1A).

The microbial communities of the water samples were more diverse, even within time points (Fig. 1C). The dominant phyla in both untreated and UV-treated river water included Actinobacteria, Bacteroidetes, Parcubacteria, and Proteobacteria. In

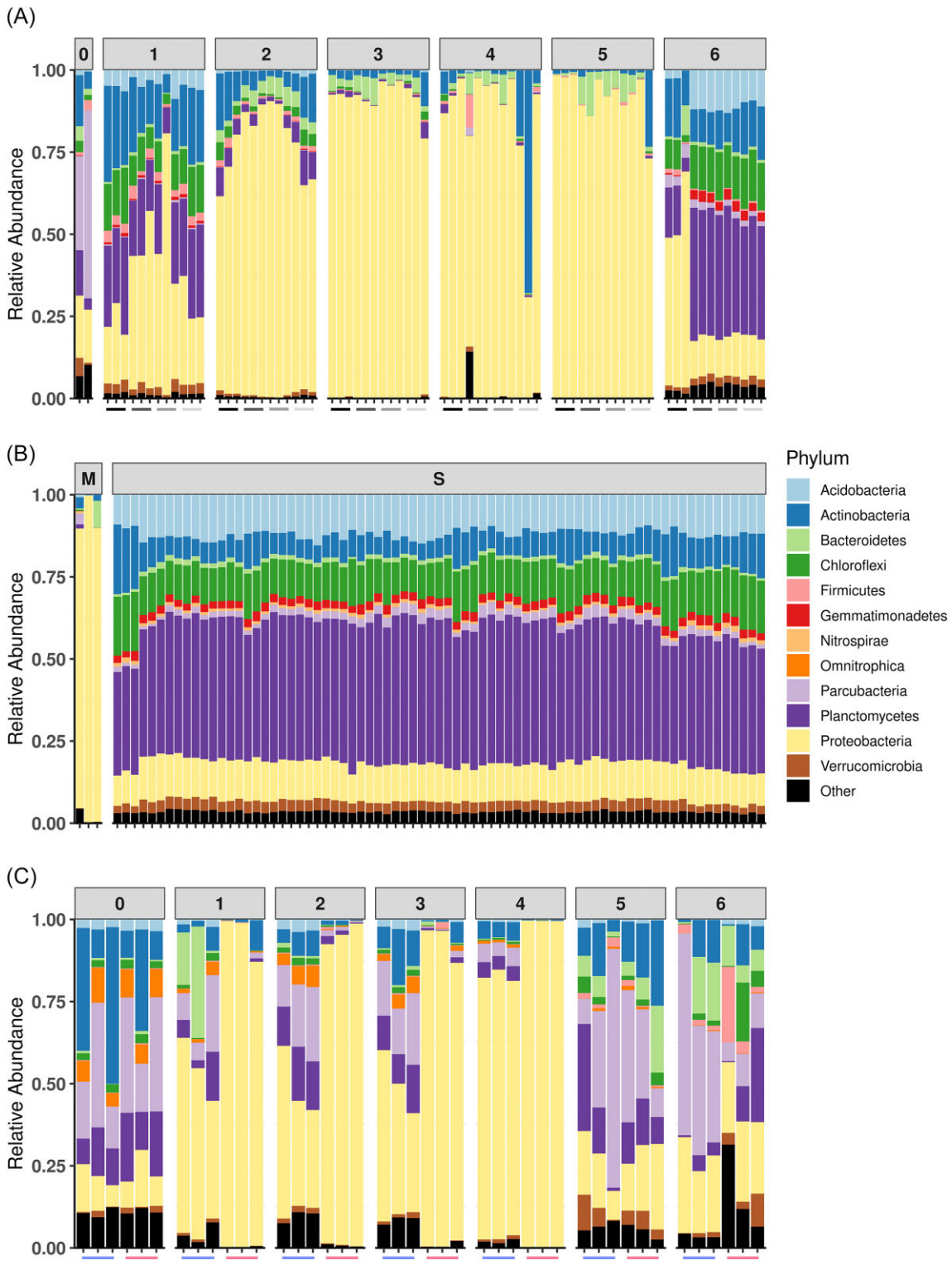

Figure 1. Relative abundance of the 10 most abundant microbial phyla in communities of (A) lettuce, (B) manure (M) and soil (S), and (C) water. For lettuce (A), the four treatments are shown with black to light gray bars below the bar plots (A: untreated river water/conventional soil; B: untreated river water/manure; C: UV-treated river water/manure; and D: UV-treated river water/conventional soil); for water (C), untreated or UV-treated river water is shown with blue or red bars, respectively. The numbers above the bar plots (A) and (C) indicate sampling time in weeks (0–6).

weeks 0, 5, and 6, no clear effect of UV-treatment was detected, while in weeks 1–4, UV-treated river water contained approximately twice as many Proteobacteria as untreated river water, accounting for nearly 100% in most cases (Fig. 1C). Notably, in the weeks with a clear effect of UV-treatment (weeks 1–4), Proteobacteria were the dominant phylum in the untreated river water already (that is, before UV-treatment), while in the weeks with no clear effect of UV-treatment, there was not one phylum clearly dominating the microbial community of untreated river water.

Clinically relevant bacterial families on lettuce show temporal variations in relative abundance

For a more detailed analysis of lettuce communities, relative abundance of the top 50 bacterial families was visualized in a heat map (Fig. 2). A temporal shift in the relative abundance of families targeted in the culture-based approach (*Enterobacteriaceae*, *Moraxellaceae*, and *Pseudomonadaceae* in cluster-3), characterized by an increase in relative abundance from medium to high from weeks 1 to 2, followed by a steadily high relative abundance through week 5, and a final steep decrease in relative abundance in week 6. Typical soil bacteria such as *Tepidisphaeraceae*, *Caldilineaceae*, and *Intrasporangiaceae* (cluster-1) or *Gemmatimonadaceae*, *Nitrosomonadaceae*, *Rhodospirillaceae*, *Cytophagaceae*, and *Verrucomicrobiaceae* (cluster-2) (Huang et al. 2019, Corrochano-Monsalve et al. 2020, Hannula et al. 2020) were abundant from planting until week 2, after which their relative abundance decreased until week 5, and was increased again in the last sampling (week 6). Important to note is that optically, the lettuces from week 5 (optimal harvest point) markedly differed from the oversized and partially already decaying lettuces from week 6. Further of note, treatment A of week 6 markedly differed from the other treatments of week 6 (Fig. 2), as already observed in the relative abundance bar plots (Fig. 1A). Finally, the relative abundance of target bacteria on lettuce was visualized in dot plots and at different taxonomic levels from genus to class (Fig. S3). At all levels, the observed differences between sampling time points were highly significant (one-way ANOVA, *P*-values in Fig. S3). For γ-Proteobacteria, including the target groups *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp., a common trend of increasing relative abundance from weeks 0 to 5 with a drastic decrease in week 6 was observed at all taxonomic levels. No clear temporal trend was observed for *Enterococcus* spp., except at class level where relative abundance of *Bacilli* increased during the first 2 weeks but dropped below initial levels in week 6. The class *Bacilli*, however, also includes many nontarget bacteria. When comparing treatments instead of time points, no significant differences were detected (one-way ANOVA, $P \geq .01$; data not shown).

Differential abundance analysis confirms increased abundance of clinically relevant taxa on mature lettuce

Next, the impact of lettuce age, irrigation water quality, and soil amendment on the abundance of taxa including clinically

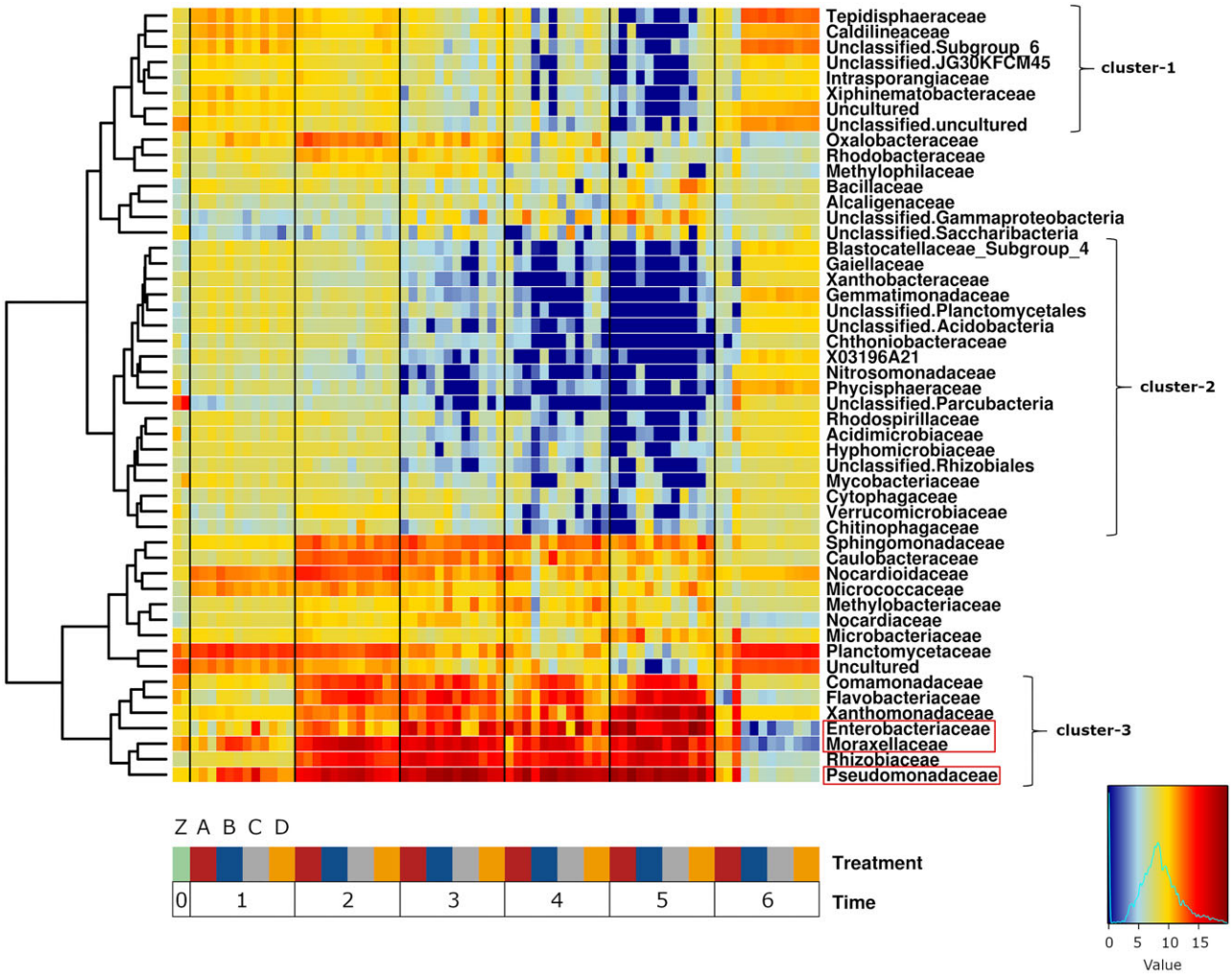

Figure 2. Relative abundance of the top 50 families detected by 16S rRNA gene amplicon sequencing on lettuce throughout a complete growth period (weeks 0–6). Blue to red coloration shows low to highly abundant families, respectively (arbitrary units). Families containing bacteria targeted in the culture-dependent approach are highlighted (red boxes). Samples are ordered first by sampling time in weeks (0–6), then by condition [initial condition Z: before planting; treatments on the field: A (untreated river water/conventional soil), B (untreated river water/manure), C (UV-treated river water/manure), and D (UV-treated river water/conventional soil)]. Three distinct clusters are marked with brackets.

relevant bacteria on lettuce was explored. DA taxa were identified among the 200 most abundant taxa at different taxonomic levels under three conditions: (1) young versus mature lettuce [week 1 (first samples after planting) versus week 5 (optimal harvest point)], (2) lettuce irrigated with untreated versus UV-treated river water, and (3) lettuce grown with or without manure was investigated. Several of the target taxa were significantly more abundant on mature compared to young lettuce of all treatments (Fig. 3A), with fold changes ranging from 1.4 to 4.6 (Table S7). Of these, *Enterobacteriaceae*, *Pseudomonadaceae*, and *Moraxellaceae* were among the top 10 families with increased abundance on mature compared to young lettuce (Fig. S4). On the other hand, manure application or irrigation water quality resulted in only few DA taxa on lettuce, never including any of our target taxa of clinical relevance (data not shown). Of note, species of the genera *Pseudomonas* and *Acinetobacter* other than our target species were not detected among these DA taxa.

Diversity in lettuce microbial communities decreasing over time, but no treatment effect

Microbial species richness, evenness, and dominance in lettuce communities were estimated with the Chao1, Shannon Diversity, and Inverse Simpson's index. A significant decrease was observed from weeks 0 to 5 (Fig. S5A). In the last sampling (week 6), all three indices increased again approximately to initial levels. This development was highly significant at all taxonomic levels (one-way ANOVA, $P < .001$) and coincides with the gradual increase and steep decrease of Proteobacteria proportions observed over time (Fig. 1A). Consistent with our observations when investigating individual taxa, there was usually no significant difference between treatments (one-way ANOVA, $P \geq .05$; Fig. S5B). In the marginally significant cases, initial condition Z showed increased diversity compared to treatments A–D.

Structural differences between microbial communities were measured considering taxa abundance and/or phylogeny

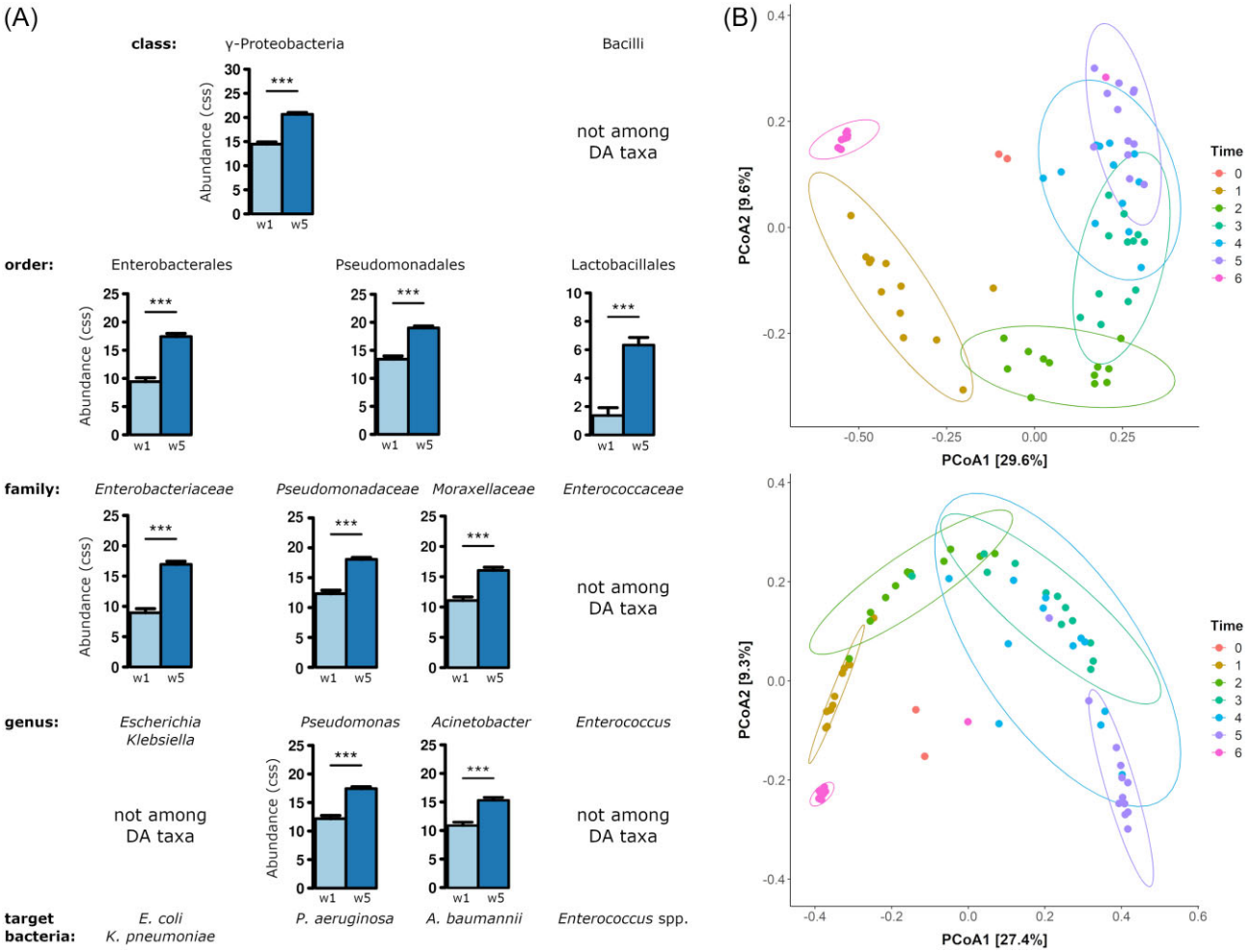

Figure 3. (A) DA target taxa on lettuce, identified among the 200 most abundant taxa. Lettuce treatments were pooled by time point to compare young lettuce (w1: week 1; $n = 12$) to mature lettuce (w5: week 5; $n = 12$). Significant differences were detected by pairwise comparisons using Student's *t*-test and *P*-value correction using FDR. ***: $P < .001$. In all cases, abundance was significantly increased on mature compared to young lettuce. (B) PCoA of lettuce microbial community structures over time, measured by Bray–Curtis (top) and Unifrac (bottom). Each point indicates a lettuce biological replicate sample (two samples for time 0; three samples per time point, and treatment for times 1–6). Time is indicated in weeks; confidence ellipsoids at 95%. The clustering revealed a clear temporal shift of lettuce communities. In contrast, no clustering was observed by lettuce treatment, wherefore no distinction of treatments within the time points was made.

(Jaccard, Bray–Curtis, Unifrac, and weighted Unifrac). Since all measures yielded similar results, only Bray–Curtis and Unifrac are presented. PCoA visually confirmed an effect of time on lettuce community structures: a time shift can be observed as well as clear separation of freshly planted from mature lettuce (week 1 versus week 5; Fig. 3B), with lettuce seedlings (week 0) in between the two. Lettuces of week 6 clustered tightly and were more similar to week 1 lettuces. When looking at all investigated systems jointly, it becomes evident that week 1/6 lettuce communities resemble soil communities in their structure (Fig. S6). For diversity-measures with homogeneous multivariate dispersions among groups, beta-diversity was significantly different among all time points with exception of pair week 3/4 (Table S8). In contrast, initial condition Z only marginally differed from the four treatments, becoming nonsignificant after *P*-value adjustment (Table S9).

Resistome analysis

Core ARGs detected in all environments mainly belong to class of multidrug ARGs

The antibiotic resistome of lettuce, soil, water, and manure was investigated. In total, 326 ARG subtypes were identified. An overview of the number of ARG subtypes [grouped by antibiotic (AB) class] detected in the four environments is shown in Fig. 4(A). The highest diversity of ARGs was detected in lettuce samples ($n = 256$), followed by water ($n = 141$), soil ($n = 102$), and manure ($n = 95$). In terms of shared ARGs, lettuce shared most ARGs with water and second most with soil ($n = 126$ and $n = 94$, respectively; Fig. 4B). All four environments had 30 ARGs in common (core ARGs; Table 1), most of which belonged to the class of multidrug ARGs encompassing mainly genes encoding efflux pumps. Further of interest, aminoglycoside (*aadA*, *aph(6)-I*), β-lactam (metallo-β-lactamase), glycopeptide (*vanR*, *vanX*), sulfonamide (*sul2*), and tetracycline (*tet*A) ARGs were among the core ARGs. The detailed lists of ARGs shared between lettuce and the other three environments are provided in Tables S10–S12. Among the ARGs uniquely shared between lettuce and water were mostly MDR and β-lactam ARGs (Table S10), whereas lettuce and soil uniquely shared mainly glycopeptide and tetracycline ARGs (Table S11), and only four ARGs were unique to lettuce and manure (Table S12). The distribution of ARGs by class among environments was very homogeneous, with efflux-related ARGs being the most prominent in all environments, followed by β-lactam related ARGs (Fig. S7).

Sample clustering by ARG profiles reveals impact of manure application on lettuce resistome

Samples were clustered using their ARG profiles with Bray–Curtis (accounting for ARG presence/absence as well as abundance) or Jaccard (accounting for ARG presence/absence only) as distance measures, to visualize sample similarities. Soil samples all clustered very tightly, irrespective of irrigation regime or amendment (Fig. 5). In young lettuce (week 1), a clear separation between treatments A/D and B/C was evident (without and with manure, respectively), which was more pronounced when only ARG presence/absence was considered (Fig. 5B). A similar tendency was observed in mature lettuce (week 5), though much less pronounced. UV-treatment of water lead to a clear separation that was more pronounced when considering ARG presence/absence only. Finally, manure clustered closest to young lettuce grown in manure-amended soil (Fig. 5B).

MDR proportions are increased manifold in mature lettuce and UV-treated river water

The effect of lettuce growth time, manure application, and UV-treatment on the proportions of MDR contigs (number of MDR contigs per total contigs) was investigated (Fig. 6A). MDR contig proportions in soil were very low irrespective of treatment (<0.15%), while manure had a proportion of roughly 2%. Of all samples, the highest proportions of MDR contigs were found in mature lettuce grown on manure-amended soil (L5B and L5C) and in UV-treated river water (W1B and W5B). Compared to untreated water, UV-treated water had at least four times increased MDR proportions, and mature lettuce samples had at least 2-fold increased MDR proportions compared to young lettuce samples (Fig. 6A), the difference being most pronounced when grown with manure.

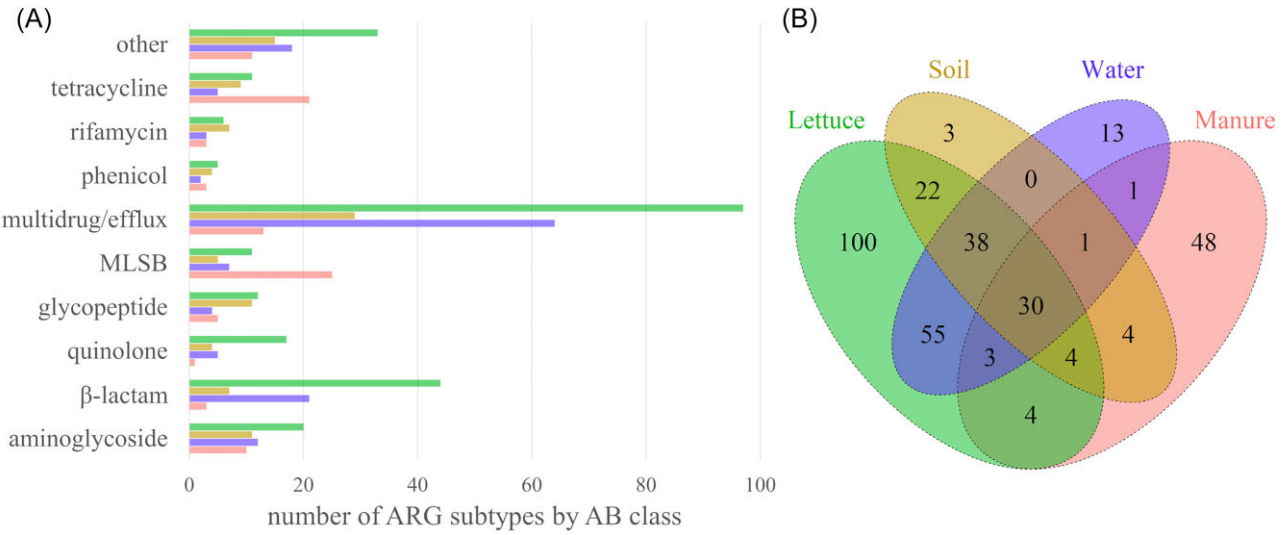

Figure 4. ARGs from lettuce, soil, water, and manure for sampling weeks 0, 1, and 5, as determined by deepARG and enumerated at subtype level (e.g. *aadA*, *vanR*, and *tetA*). (A) Number of ARG subtypes grouped by antibiotic (AB) class, detected in each environment. (B) Venn diagram showing distribution of ARG subtypes between lettuce, soil, water, and manure. Counts of unique as well as shared ARG subtypes are displayed in the respective intersections.

Table 1. Core ARGs shared between all four environments (lettuce, soil, water, and manure), as identified by deepARG (identity: 70%, e-value: 1e−10, target coverage: 60%, and probability: 0.7). Numbers are 16S-normalized read counts. L: lettuce; M: manure; S: soil; W: water; and avg: average of all positive samples. MLSB: macrolide–lincosamide–streptogramin B ARGs.

Class	Subtype	L_avg	M	S_avg	W_avg
Aminoglycoside	AADA	1.7E−03	1.4E−02	1.9E−03	1.2E−03
Aminoglycoside	APH(6)-I	1.4E−03	5.6E−03	3.4E−03	1.0E−03
Bacitracin	BACA	1.5E−01	3.6E−02	1.5E−01	2.1E−01
β-lactam	METALLO-β-LACTAMASE	4.8E−03	6.5E−04	7.5E−03	8.8E−03
Fluoroquinolone	QACH	7.2E−04	3.2E−03	3.1E−03	3.5E−03
Fosmidomycin	ROSA	2.6E−02	2.3E−03	3.8E−02	2.2E−02
Glycopeptide	VANR	3.2E−02	1.0E−02	1.7E−01	6.5E−03
Glycopeptide	VANX	1.9E−03	1.4E−03	7.8E−03	2.4E−04
MLSB	MACA	2.2E−02	1.2E−03	1.0E−02	4.1E−02
MLSB	VATF	4.4E−03	2.0E−03	1.1E−02	1.7E−02
Multidrug/efflux	ABES	1.3E−02	1.7E−03	4.3E−03	1.5E−02
Multidrug/efflux	ACRB	6.3E−02	1.2E−02	3.1E−02	3.5E−02
Multidrug/efflux	MEXA	3.2E−02	2.5E−03	2.7E−03	2.4E−02
Multidrug/efflux	MEXB	7.0E−02	6.0E−03	9.4E−03	8.9E−02
Multidrug/efflux	MEXF	6.3E−02	8.0E−04	8.8E−02	5.3E−02
Multidrug/efflux	MEXK	6.9E−02	2.6E−03	2.7E−02	5.9E−02
Multidrug/efflux	MTRA	3.4E−02	2.2E−03	8.0E−02	1.0E−02
Multidrug/efflux	OMPR	1.1E−01	5.1E−03	5.1E−02	1.6E−01
Multidrug/efflux	OPRM	5.2E−02	1.3E−03	3.7E−03	7.6E−02
Multidrug/efflux	RPOB2	1.0E−01	1.1E−01	4.3E−01	1.3E−01
Multidrug/efflux	SMEE	6.5E−03	2.4E−03	1.2E−02	6.2E−04
Peptide	UGD	1.7E−02	5.8E−02	1.3E−02	3.4E−02
Phenicol	CAT	1.7E−03	1.3E−03	2.2E−03	5.9E−04
Phenicol	CATB	5.8E−04	1.5E−03	1.7E−03	1.0E−03
Rifamycin	ARR	1.3E−02	1.7E−03	4.7E−02	1.9E−03
Rifamycin	RBPA	5.6E−03	2.8E−04	1.7E−02	4.2E−04
Rifamycin	RPHB	4.6E−03	1.8E−03	2.8E−02	9.7E−04
Sulfonamide	SUL2	4.3E−04	1.4E−02	2.7E−03	7.1E−04
Tetracycline	TETA	3.7E−03	3.2E−03	5.0E−03	6.6E−04
Unclassified	CAMP-REGULATOR	7.6E−02	2.7E−03	7.7E−03	2.4E−02

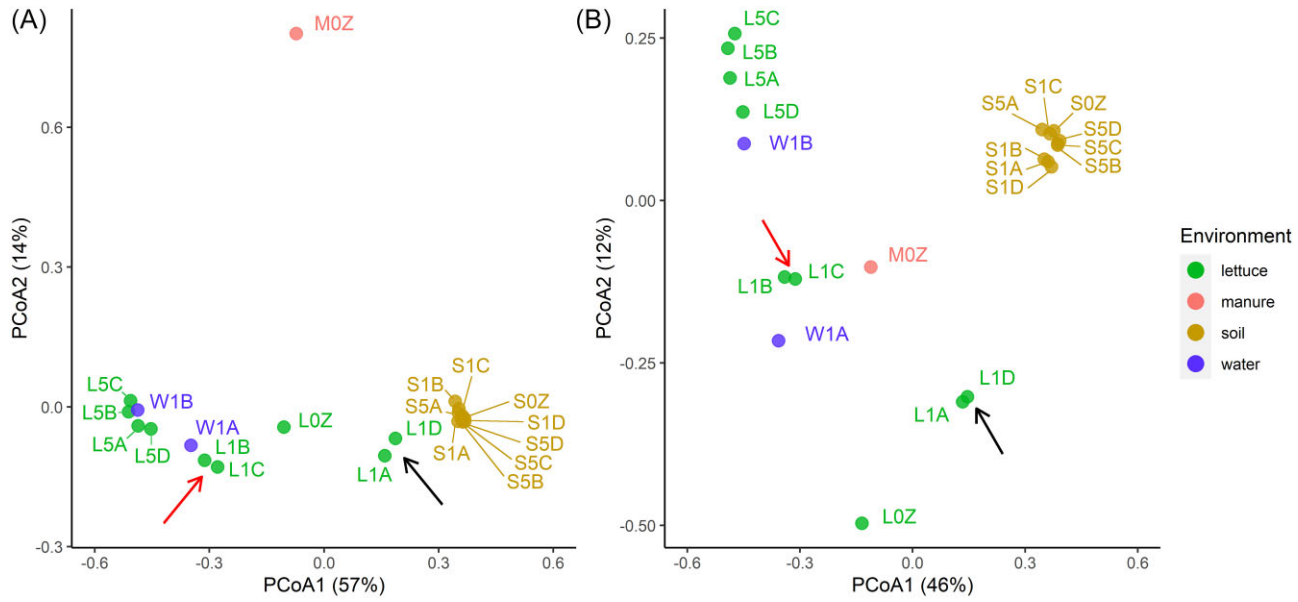

Figure 5. PCoA based on ARG annotation by ARGs-OAP v2.0 (16S normalized; subtype-level). (A) Bray–Curtis: sample distance measured taking ARG presence/absence and abundance into account; (B) Jaccard: sample distance measured taking ARG presence/absence into account only. Black/red arrows mark young lettuce grown without/with manure, respectively. Sample codes: initial letters indicate environment [L (lettuce), M (manure), S (soil), and W (water)]; numbers indicate sampling weeks; terminal letters indicate conditions [initial condition Z: before planting; lettuce treatments: A (untreated river water/conventional soil), B (untreated river water/manure), C (UV-treated river water/manure), and D (UV-treated river water/conventional soil); water treatments: A (untreated); B (UV-treated)].

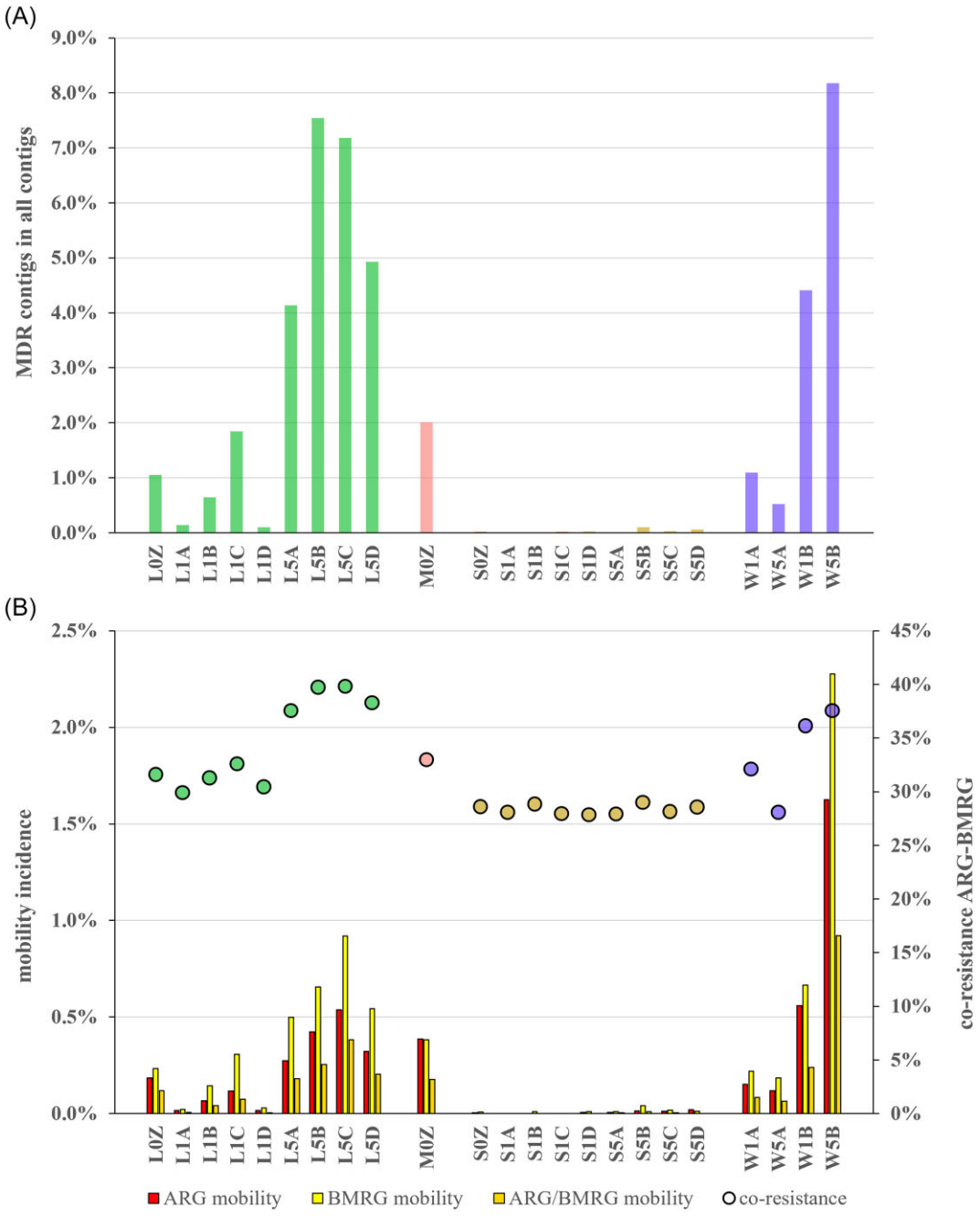

Figure 6. (A) Proportions of MDR contigs per total contigs. An MDR contig was defined as a contig carrying three or more ARGs annotated from the Resfams database. (B) Mobility incidence of ARGs and/or BMRGs (bars) as well as ARG–BMRG coresistance per sample (circles; green: lettuce; purple: manure; orange: soil; and blue: water). Sample codes: initial letters indicate environment [L (lettuce), M (manure), S (soil), and W (water)]; numbers indicate sampling weeks; terminal letters indicate conditions [initial condition Z: before planting; lettuce treatments: A (untreated river water/conventional soil), B (untreated river water/manure), C (UV-treated river water/manure), and D (UV-treated river water/conventional soil); water treatments: A (untreated); B (UV-treated)].

Increased abundance of antibacterial BMRGs in mature versus young lettuce

To investigate whether—additionally to antibiotic resistance—biocide and metal resistance contributed to differences between environments and treatments, BMRGs were annotated (see online Supplementary material for details). Overall, in all four environments the most abundant BMRG [counts per one million reads (CPM)] was *tupC*, conferring resistance to the metal tungsten (W), immediately followed by *znuC/yebM*, encoding zinc (Zn) resistance. Third most frequent was *wtpC* in lettuce, water, and manure and *modC* in soil, both conferring tungsten- and molybdenum (Mo) resistance. The top three BMRGs best discriminating young from mature lettuce when comparing average CPM values were *fbpC* [iron (Fe) and gallium (Ga) resistance], *wtpC*, and *adeL* [sodium dodecyl sulfate (SDS)/ethidium bromide/safranin O/acridine orange resistance], all detected at much higher abundance in mature than in young lettuce. In young lettuce, treatments A/D (no manure) could best be discerned from treatments B/C (manure) by *adeL*, *fbpC*, and *zraR/hydH* (Zn resistance), the two latter of which were among the top ten BMRGs detected in manure.

Lettuce BMRG-profiles reveal high coresistance to antibiotics, and confirm effect of growth time and manure application

The frequency of antibiotic–biocide/metal coresistance per sample was estimated, that is, the proportion of contigs carrying both ARGs and BMRGs in all contigs of a given sample (Fig. 6B). On average, mature lettuce had a higher proportion of contigs conferring coresistance to antibiotics–biocide/metals compared to young lettuce (38.9 ± 1.0% versus 31.1 ± 1.0%). Soil had on average the lowest proportion of coresistance (28%). In UV-treated river water, average coresistance was higher than in untreated river water (36.9 ± 0.7% versus 30.1 ± 2.0%).

A clustering using NMDS was performed on all samples based on Resfams and/or BacMet annotations, to compare sample clustering when considering antibiotic or biocide/metal resistance either separately or jointly. The clustering was very similar whether considering either ARGs or BMRGs, which can be expected in case of a significant co- and cross-resistance to these compounds (clustering not shown). Overall, lettuce samples clustered as already observed in the PCoA analysis, that is, according to sampling time while within time clusters treatments A/D or B/C were more similar to each other.

Genetic mobility analysis shows high prevalence of Proteobacteria-associated resistance plasmids and dominance of multidrug/efflux ARGs in all samples

Since the transferability of resistance between bacteria is a major concern, the genetic context of ARGs and BMRGs was investigated by looking for proximal mobility elements. A mobility incidence (M%) per sample was calculated for ARGs, BMRGs, and their combination, defined as the percentage of resistance gene-encoding contigs flanked with at least one mobility indicator in all resistance contigs (Ju et al. 2019). Generally, BMRG mobility incidence was higher than ARG mobility incidence (Fig. 6B), especially in lettuce. On the other hand, contigs carrying both ARGs and BMRGs were always the less mobile of the three. In lettuce, mature plants displayed higher average mobility incidence than young plants, and within time points, treatments A and D (no manure) had the lowest mobility incidence. Soil samples had very low mobility incidences overall (Fig. 6B), just as was observed for proportions of MDR contigs (Fig. 6A). Finally, UV-treatment of water clearly increased mobility incidence of all—ARGs, BMRGs, and their combination—as compared to untreated river water.

The MGEs most typically linked to BMRGs were the tyrosine recombinases *xerC* and *xerD*, the recombination mediator *recR*, and the conjugative transfer ATPase *cagE*. These four jointly made up more than half (57.2%) of all detected MGEs. Interestingly, the above-mentioned four MGEs were likewise the most frequently linked to ARGs, again making up more than half (51.3%) of all detected MGEs.

Plasmid tag annotation using PlasFlow revealed proportions of ARG plasmid contigs in all ARG contigs in the different samples (Fig. 7A). On average, mature lettuce had higher ARG plasmid proportions than young lettuce (roughly 10%–12% versus 6%–8%). The ARG plasmid proportion of manure was comparable to mature lettuce, while ARG plasmid proportions of soil (whether containing manure or not) were in the range of young lettuce (roughly 6%–8%). ARG plasmid content of water varied from below 8% to 14%, while no clear difference between untreated and UV-treated and river water was detectable. Looking at ARG plasmid phylogeny, the phylum of Proteobacteria prevailed in all samples (Fig. 7B). Mature lettuce had higher proportions of Proteobacteria ARG plasmids compared to young lettuce (at least 80% versus around 60%). On the other hand, ARG plasmids from Firmicutes were more prominent in young than on mature lettuce. In manure, about 35% of ARG plasmids were from Proteobacteria, followed by about 14% of ARG plasmids from Firmicutes, while about half the ARG plasmids remained unclassified. In soil, ARG plasmids from Proteobacteria consistently prevailed (~60%), followed by much lower proportions of Firmicute and Actinobacteria plasmids. In water as well, ARG plasmids from Proteobacteria were most often identified. While additionally, ARG plasmids from Firmicutes were clearly detected in untreated river water, almost none were present in UV-treated river water (about 4% versus 0.2%). Further, ARG plasmids from Bacteroidetes, Cyanobacteria, and Spirochaetes were identified in different samples but at very low proportions (<1%) and only sporadically.

Finally, the ARGs localized on plasmid contigs were counted and grouped by AB class (Fig. 7C). Consistently, multidrug/efflux ARGs dominated the picture, followed by glycopeptide and β-lactam ARGs. Minor differences between sample groups include (1) higher proportions of multidrug/efflux and chloramphenicol ARGs in mature versus young lettuce, (2) lower proportions of β-lactam ARGs in mature versus young lettuce, (3) practical absence of chloramphenicol ARGs in soil and manure, and (4) lower proportions of multidrug/efflux but slightly higher proportions of MLSB ARGs in untreated versus UV-treated river water.

Procrustes analysis reveals high correlation between lettuce microbial community structure and resistome

At all phylogenetic levels, the correlation between resistome and microbial community structure was highly significant (shown for phylum and genus in Fig. S8; P at least <2e−4), with the highest significance reached at genus level (Fig. S8B; P < 1e−5). Further, a clear separation of the three time points (weeks 0, 1, and 5) was obtained at all phylogenetic levels. Finally, a separation of treatments A/D (no manure application) from B/C (manure application) was evident in young lettuce (week 1), in accordance to previous analyses (Fig. 5).

Discussion

The boundaries of culture-based techniques

The suitability of diagnostic plates—developed for clinical samples—for use with environmental samples is often discussed, and our study confirmed this problem. In the present study, bacteria culturable on antibiotic-containing selective agar plates from lettuce consistently showed a dependence on plant growth time. However, whether the lettuces were grown with or without pig manure, or irrigated with untreated or UV-treated river water showed no significant effect. Identification of presumptive ARB colonies from all environments further revealed very low abundance of clinically relevant bacteria, and the plates were overgrown with typical environmental bacteria such as *Pseudomonas* spp. or *Sphingomonas* spp. (White et al. 1996, Crone et al. 2020). As very recent work by Schreiber et al. (2021) demonstrated, adaptation of incubation conditions such as increasing temperature can help to suppress unwanted background microbiota, and thereby increase sensitivity. It still remains to be shown, however, whether such environmentally optimized culturing can yield enough insight to narrow the gap between culture-based and molecular techniques significantly.

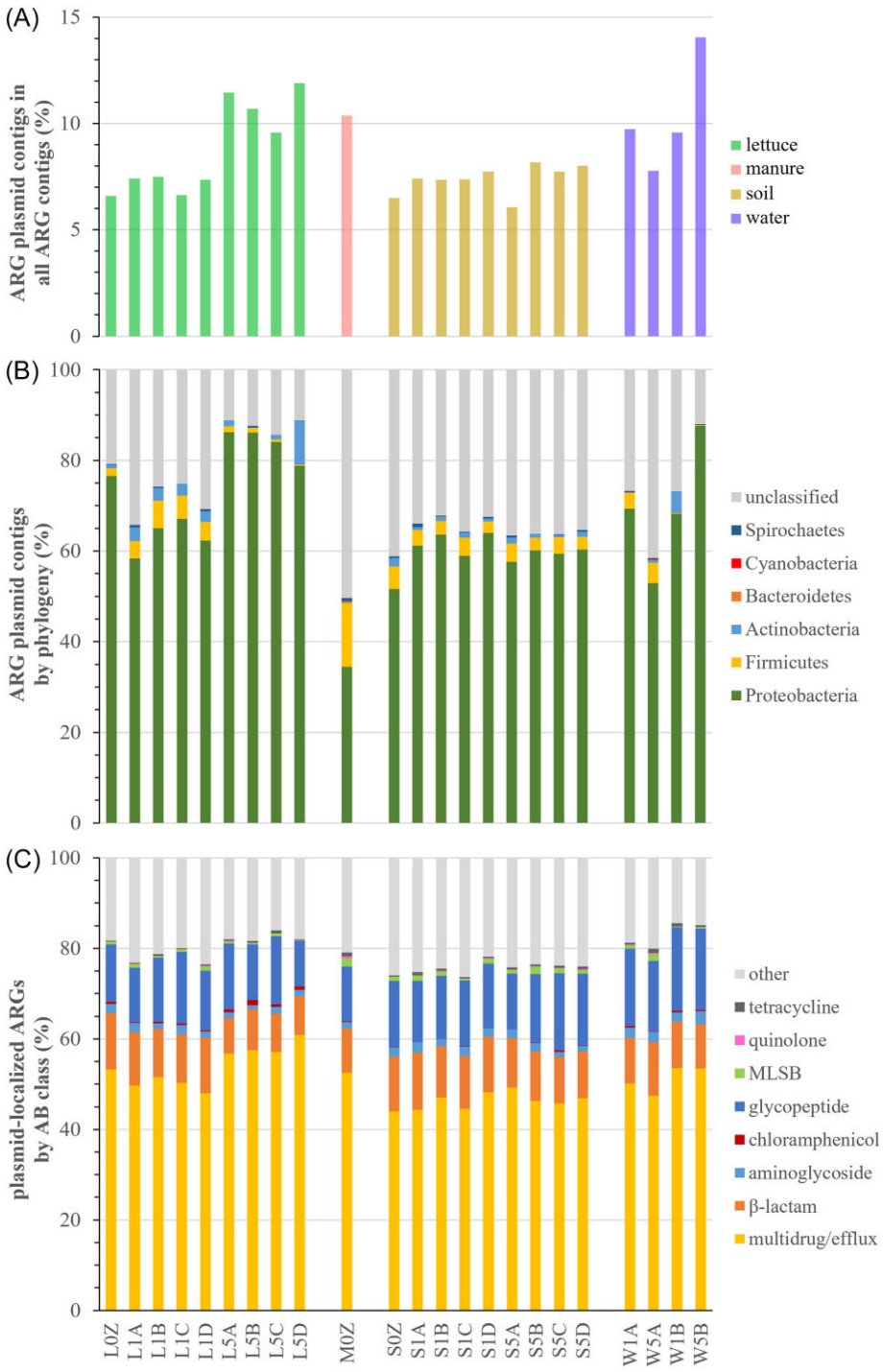

Figure 7. (A) Proportion of ARG plasmid contigs in total ARG contigs; (B) phylogeny of detected ARG plasmid contigs; and (C) ARGs (Resfams) localized on plasmid contigs, grouped by antibiotic classes. MLSB: macrolide–lincosamide–streptogramin B ARGs. Sample codes: initial letters indicate environment [L (lettuce), M (manure), S (soil), and W (water)]; numbers indicate sampling weeks; terminal letters indicate conditions [initial condition Z: before planting; lettuce treatments: A (untreated river water/conventional soil), B (untreated river water/manure), C (UV-treated river water/manure), and D (UV-treated river water/conventional soil); water treatments: A (untreated); B (UV-treated)].

Environment-specific microbiome structures

The main determinant of microbial community composition was environment (lettuce, soil, water, or manure). Thus, pig manure was heavily dominated by Proteobacteria, in accordance with a very recent study by Wang et al. (2023), who found 85% relative abundance of Proteobacteria in fresh pig manure. In contrast, soil was dominated by Planctomycetes, a phylum typically encountered abundantly in soil (Buckley et al. 2006). The overall soil community composition was not significantly affected by either time or treatment, despite the overwhelming dominance of Proteobacteria in manure as well as UV-treated river water of weeks 1–4. Confirming these findings, soil microbial communities have been described as very resilient to change (Griffiths and Philippot 2013).

In contrast, river water was much more variable with major differences between samplings. While in weeks 0, 5, and 6 communities were more diverse, in weeks 1–4 Proteobacteria clearly dominated. Precipitation data made evident that the more diverse river water samples were collected on days preceded by zero precipitation on at least 4 days prior to sampling and the sampling day itself (data not shown). In contrast, weeks 1–4 samplings were all preceded by at least 1 day of light to heavy rainfall (from 2.4 to 21.6 mm/h). While Parcubacteria and Actinobacteria are prevalent in various water systems like lakes, groundwater, or freshwater ecosystems (Warnecke et al. 2005, Proctor et al. 2018, Tian et al. 2020), rainfall has been shown to increase the abundance Proteobacteria, in particular *E. coli*, especially in the proximity of upstream WWTPs (Shibata et al. 2014, Tornevi et al. 2014). Finally, in river water with predominant Proteobacteria (i.e. after rainfall), UV-disinfection led to a steep increase of their relative abundance, in accordance with Becerra-Castro et al. (2016) who found that Proteobacteria become predominant by regrowth after water disinfection.

In-depth analysis of microbial communities on lettuce consistently showed a significant effect of plant growth time, while treatment made no significant difference except in a few exceptions, where seedlings before planting differed from lettuces of the four treatments on the field. The transfer from the environmental sources investigated (soil, water, and manure) to the lettuce was therefore not the decisive factor in shaping the lettuce microbial community or resistome. Taken together, abundance of clinically relevant ARB families (*Enterobacteriaceae*, *Moraxellaceae*, and *Pseudomonadaceae*, including the genera *Pseudomonas* and *Acinetobacter*) highly increased from very young lettuces (week 1) to mature lettuces (week 5), but was strongly decreased in oversized and partially already decaying lettuces (week 6). Overall, the observed time trend seemed related to the decreasing soil contact as plants grow, and the strongly reincreased soil contact in week 6, when the lettuces were already beyond the optimal harvest point. Soil thereby seems to serve as initial inoculant of young plants, whose communities thereafter develop independently of the stable communities in the surrounding soil. In conclusion, the observed temporal dynamic is relevant with regards to clinically relevant ARB taxa.

To compare our findings, we could not find another work investigating the development of lettuce's microbial communities over the growth period of the vegetable, as one-time samplings of the vegetable grown under different conditions (e.g. different amendments) are usually performed. To our knowledge, the only study including a time series was conducted in 2013 by Holvoet et al. (2013) who sampled lettuce and its production system from different farms, four times within the production cycle (seedlings, and 2 weeks before, 1 week before, and at harvest). They focused, however, on AR *E. coli* and did not discuss any changes related to plant growth time. In contrast to the lack of time series, many studies have investigated the microbiome of fresh produce to relate it to its production environment and different modes of culture. They especially detected an effect of various types of amendment or a weak impact of irrigation on the microbiome as well as the resistome of the vegetables (Blau et al. 2019, Cerqueira et al. 2019, Fogler et al. 2019, Zhang et al. 2019, Summerlin et al. 2021, Sanz et al. 2022, Seyoum et al. 2022).

The impact of amendment and UV-disinfection on the lettuce resistome

Of all investigated environments, manure displayed the lowest diversity of ARGs. Interestingly, although the microbial community was heavily dominated by Proteobacteria, only a third of all identified plasmid contigs were assigned to this phylum, while about half remained unclassified. The high proportion of unclassified plasmid contigs remains elusive, since PlasFlow was developed to identify bacterial plasmid sequences in environmental samples, and is therefore not biased toward clinical specimens. While many studies have investigated the effect of soil amendment with manure on the resistome of fresh produce, manure on its own is rarely analysed or discussed, while the focus rather lies on the manure-amended soil as an entity. Wang et al. (2023) quantified ARG abundance in raw pig manure by targeted qPCR of nine ARGs, showing high relative abundance of *sul2* followed by *tetM*, as opposed to dominance of *sul1* in stored pig manure. In the present study, *tetM* and *sul2* were among the top 15 most abundant ARGs in manure, including mainly other tetracycline-, MLS-, and aminoglycoside ARGs such as *aadA*, *aadE*, *tetQ*, *tetW*, or *ermF*, in good overall accordance with other reports (Wang et al. 2017, Blau et al. 2019, Zhang et al. 2020).

Soil was overall very poor in AR determinants, and comparable to young lettuce only in terms of ARG plasmid proportions. The majority of the assigned plasmids belonged to Proteobacteria, followed by Firmicutes, and Actinobacteria, a ranking not analogous to the relative abundance of these taxa in the respective microbial communities. Of importance, these phyla have been described previously as typical plasmid hosts in soil (Smalla et al. 2015). Notably, soil samples did cluster by sampling time for unknown reasons when considering BMRGs additionally to ARGs, in contrast to their very tight clustering when considering ARGs only. Comparable to pristine soils, multidrug/efflux and aminoglycoside resistance were the most prominent in our soil samples as well, whereas we additionally detected frequent glycopeptide resistance. The overall low resistance numbers in soil are unexpected, since soil is generally acknowledged as a diverse reservoir of antibiotic resistance (Séveno et al. 2002), including even resistance toward synthetic antimicrobials (Marshall et al. 2009), although significant differences between different types of soil have been reported (Popowska et al. 2012). However, exactly that high complexity of soil might explain this result since soil samples assembled poorly, probably due to insufficient sequencing depth, as has been described previously (Wind et al. 2021). When designing a study including a variety of environments, one main dilemma therefore is whether to apply the same protocol to all for best comparability, or whether to optimize the protocols by environment for maximum information.

In water, the second highest number of ARG subtypes of all environments was identified. A recent study by Lee et al. (2023) suggested using 'co-occurrence' of *aadA*, *sul1*, and class A β-lactamase genes as an indicator of wastewater-related pollution in river water. In good accordance, these three ARG subtypes were detected in our study in river water. Notably, class A β-lactamase genes were detected only in the Resfams annotation, underlining the importance of combining several databases. The most frequently detected resistance mechanisms included multidrug and β-lactam, which are frequently detected in pristine waters as well,

while the herein investigated river water additionally contained aminoglycoside ARGs frequently. Proportion of MDR contigs, ARG–BMRG coresistance, and mobility incidence were always markedly higher in UV-treated compared to untreated river water. An increase in ARG total relative abundance after UV-disinfection has been described earlier (Hu et al. 2016). Mobility incidence (M%) on the other hand has been quantified by Ju et al. (2019) in WWTP resistomes. The authors found an M% of 8.6% and 20.0% for ARGs and MRGs, respectively. These are 64-fold and 99-fold higher, respectively, than what was found in the present work in untreated river water. Mobile ARGs and BMRGs seem therefore to be enriched by wastewater treatment processes (as observed in the present work for UV-disinfection of river water), but diluted again with less mobile resistance determinants when introduced into the environment like a nearby river. Finally, Firmicute plasmids were detected practically only in untreated river water, although this phylum showed very low relative abundance in these samples.

Lettuce had the highest ARG diversity of all investigated environments, independently of the treatment. In common with pristine plants, lettuce harbored multidrug/efflux, β-lactam, and aminoglycoside ARGs most frequently, while it additionally contained an important number of quinolone ARGs. Taken together, the lettuces ready for harvest and consumption compared to young lettuce had increased resistance markers on all examined levels, with manure slightly exacerbating the effect. Regarding our central question, unde venis, it seems from our findings that soil and manure (when present) were the main source environments for the microbiome and resistome of young plants, but that the leaves of the growing plants enriched taxa and resistance determinants, which were below detection in the source environments.

Clearly, in lettuce as in all other environments, community abundance of a taxon did not correlate with plasmid proportion of that taxon. We therefore conclude that Proteobacteria were the main contributors of plasmid-borne ARGs in all environments, irrespective of their community abundance. A study investigating minimally processed vegetables-producing facilities analysed ARGs in initial and final product, contact surfaces, and operator swabs by taxonomy and plasmid-association (Valentino et al. 2022). As in the present study, the proportion of plasmid-associated ARGs was relatively low, and many of these were associated with Proteobacteria such as *Acinetobacter*, *Pantoea*, *Pseudorhodoferax*, or *Rahnella*, while on surfaces and operator swabs often additionally with Firmicutes like *Staphylococcus* and *Bacillus*.

When comparing the environments for commonalities, we found in all of them (1) similar distribution of ARGs to the different types, in accordance to the findings of Wang et al. (2023) finding dominance of efflux-related ARGs, (2) similar distribution of plasmid-associated ARGs to the different types, (3) dominance of the same few BMRGs encoding resistance toward W, Zn, and Mo, and (4) higher BMRG mobility compared to ARG mobility, in accordance to the findings of Ju et al. (2019), who used quantitative metagenomic and metatranscriptomic approaches to study antibiotic, biocide, and metal resistance in different compartments of 12 WWTPs. Core ARGs detected in all four environments mainly belonged to MDR (efflux). Further ARGs of interest included aminoglycoside (*aadA*, *aph(6)-I*), β-lactam (metallo-β-lactamase), glycopeptide (*vanR*, *vanX*), sulfonamide (*sul2*), and tetracycline (*tetA*) ARGs.

Interestingly, lettuce shared most ARGs with water and then with soil. A study by Shen et al. (2019) investigated the effect of water (with or without pharmaceuticals) applied by overhead versus soil-surface irrigation on the resistome and microbiome of lettuce in controlled greenhouse conditions. While exposure to pharmaceuticals did not result in consistent patterns of change in soil and lettuce, overhead irrigation resulted in greater abundance as well as diversity of ARGs and MGEs in lettuce shoots. Of the 42 ARGs detected in their study, 17 (41%) were also detected in the present work, among which *bacA* (bacitracin), *oleC* (MLSB), and *mexF* (multidrug/efflux), which were of middle to high relative abundance in both studies. Another study investigating the use of municipal wastewater effluents also described an irrigation effect on the resistome of romaine lettuce (Summerlin et al. 2021). In contrast, Seyoum et al. (2022) compared irrigation of tomatoes with treated wastewaters to freshwater irrigation and could not detect propagation of the investigated ARGs to the tomatoes. Finally, Cerqueira et al. (2019) found a minimal effect of irrigation on ARG abundance by qPCR, while crop type was more decisive. Taken together, findings on the effect of irrigation on the resistome of fresh produce are contradictive, with some studies detecting an effect while others do not. It must be kept in mind, however, that the type of water investigated and thereby varying loads of contained resistance determinants, combined with the type of produce under investigation may contribute to this discrepancy.

As mentioned earlier, a higher sequencing depth for complex environments such as soil should be considered, to comprehend the plethora of soil-borne ARGs in more detail. Nevertheless, an indirect effect of soil via amendment could be shown in the present work: Clustering by two methods (PCoA and NMDS) using either ARGs alone or combined with BMRGs revealed an effect of manure on lettuce, especially on young plants with increased soil contact. When considering ARG presence/absence only, manure clustered closely with young lettuce grown in amended soil. These findings are in good accordance with the work of Sanz et al. (2022), who concluded that fertilizers rather than soil were the main source of clinically relevant ARGs detected in foods. Additionally in the present study, of the five BMRGs most discriminatory for amendment in young lettuce, three were among the top 10 BMRGs in manure. An effect of amendment on the resistome of fresh produce has also been described in other studies (Fogler et al. 2019, Zhang et al. 2019, Huang et al. 2021, Wind et al. 2021). In the study by Zhang et al. (2019), the ARG transmission pathways between soil and lettuce were explored 90 days after application of poultry and cattle manure. They found mainly multidrug/efflux-related ARGs followed by β-lactam and aminoglycoside ARGs as in the present study, and of the 32 ARGs, which were shared between soil with poultry manure and the lettuce leaf surface, 10 (31%) were detected as well in the present study on lettuce and in soil/manure, including a variety of tetracycline resistance genes, *bacA*, *mexE*, and *aadA* (aminoglycoside). They further noted a stronger impact of poultry than cattle manure, suggesting that the type of manure can be an important factor to consider with respect to ARG abundance in fresh produce grown with such fertilizers. Taken together, in the presented study manure was revealed as the main source shaping the resistome of lettuce, especially that of the young plants.

Finally, we detected a strong correlation between microbial community structures and resistome, especially in lettuce, as has been recently described in a very similar system (Sun et al. 2021). Therefore, measures impacting the lettuce microbiome, e.g. by decreasing the abundance of Proteobacteria, are very likely to result in shifts in the lettuce resistome. The sort and effectiveness of such measures, however, must be subject to further studies.

Conclusions

In the present work, the effect of manure and irrigation on the microbiome and resistome of field-grown lettuce was investigated. By culturing, only few clinically relevant ARB could be recovered, including *E. cloacae*, *E. coli*, and *S. fonticola* on ESBL agar as well as *K. oxytoca* on CRE agar. Of note, adding an enrichment step prior to plating can enhance bacterial detection, whereas direct plating allows quantitative detection. Microbiome analysis showed a clear shift in microbial communities as a function of lettuce growth time, but no effect of soil amendment or type of irrigation water. Resistome analysis confirmed the effect of growth time, but additionally revealed an impact of manure, especially in young lettuces with increased soil contact. An impact of type of irrigation water on the lettuce resistome could not be observed although lettuce shared most ARGs with water. However, UV-disinfection did increase the proportion of examined resistance markers such as MDR, ARG–BMRG coresistance, and mobility incidence. Moreover, although surface water irrigation did not affect the overall lettuce resistome significantly, it can carry clinically relevant ARB to the produce, and should therefore be subject to monitoring. Overall, lettuces ready for harvest and consumption had increased resistance markers compared to young plants on all examined levels, with manure slightly exacerbating the effect. With regard to our central question, unde venis, our study revealed manure as the main source of resistance determinants on lettuce. Careful handling of organic fertilizers such as manure as well as measures minimizing soil contact of the vegetables is therefore key to minimize antibiotic resistance markers on fresh produce.

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Author contributions

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Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

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