




## Microbial safety and antibiotic resistance of crops after irrigation with reclaimed water

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### ABSTRACT

Water scarcity is a growing global challenge and agricultural water reuse offers a viable solution. However, microbial safety concerns persist, particularly regarding pathogens and antibiotic resistance genes (ARGs). This study assessed microbial contamination of crops irrigated with three water qualities: tap water, reclaimed water from Treatment Train A (conventional activated sludge [CAS] + filtration + UV disinfection), and from Train B (CAS + ceramic ultrafiltration + ozonation + biological activated carbon [BAC] + UV), using overhead and drip irrigation. Culture-based methods, MALDI-TOF-MS, and PCR were used to analyze microbial parameters, including *E. coli*, coliforms, and ARGs. Results showed no significant differences in *E. coli* and coliform levels between irrigation types, indicating that water quality did not impact microbial crop safety. MALDI-TOF-MS revealed no major shifts in bacterial communities. While bacterial regrowth occurred in some irrigation water samples, pathogenic and fecal bacteria were not detected on crops. ARGs were present in irrigation water, particularly in Train A effluent, but their transfer to crops was limited. Overall, irrigation water quality and method had little influence on crop contamination, whereas crop type, growing practices, and environmental conditions played a more prominent role. Long-term impacts of ARG dissemination in agriculture warrant further study.

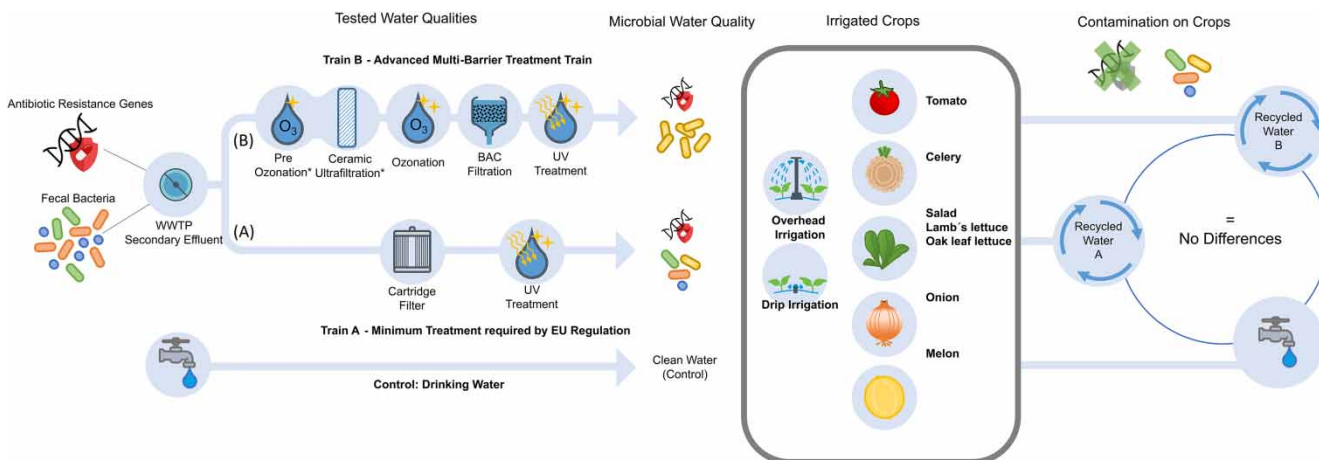
**Key words:** agricultural irrigation, antibiotic resistance genes, microbial contamination, reclaimed water, wastewater treatment, water scarcity

### HIGHLIGHTS

- Advanced treatment reduces bacterial loads and ARGs but does not fully eliminate them.
- Train B (ultrafiltration, ozonation, biological activated carbon, and ultraviolet) outperforms standard treatment; *sul1* and *tetA* persist.
- Irrigation water quality/method had minimal impact on crop microbial safety.
- Crop contamination mainly originates from soil and the environment.
- The long-term fate of ARGs in soil remains unknown and needs further study.

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## GRAPHICAL ABSTRACT



## 1. INTRODUCTION

## 1.1. Water scarcity and the role of water reuse

Climate change is exacerbating water scarcity, posing a major challenge to global food production. By mid-century, more than 83% of cropland is expected to be under increased water stress due to declining availability and increasing crop water demand (Liu *et al.* 2022a). Agricultural water reuse – the use of treated wastewater for irrigation – offers a sustainable solution to reduce freshwater dependence, particularly in arid and semi-arid regions. Several countries, such as Israel and Singapore, have successfully integrated advanced water reuse frameworks, while others struggle with regulatory and infrastructure challenges. Currently, only 2–15% of treated wastewater is used for irrigation worldwide (Jones *et al.* 2021).

## 1.2. Water reuse in the European union and Germany

In response to increasing drought risks, the European Union (EU) has recognized water reuse as a key measure for sustainable agriculture. Between 1981 and 2010, crop losses in Europe and the UK totaled €9 billion per year, with drought-related impacts tripling over the past five decades (Brás *et al.* 2021; Naumann *et al.* 2021). To mitigate these challenges, the EU Regulation 2020/741 sets minimum quality standards for reclaimed water used for irrigation (European Parliament & Council of the European Union 2020). Southern EU countries such as Spain, Italy, Greece, and France have been implementing water reuse regulations for years (Spain Royal Decree 1620/2007 2007; Alcalde-Sanz & Gawlik 2014; Water Reuse Europe), but Germany has yet to establish specific national guidelines.

Traditionally considered a water-rich country, Germany has faced unprecedented droughts in 2018, 2020, and 2022, marking the most intense dry spells in 250 years. These droughts led to widespread agricultural losses, declining groundwater levels, and severe forest dieback (Rakovec *et al.* 2022). Despite this, water reuse remains underutilized in Germany due to public perception and regulatory concerns (LAWA 2022).

## 1.3. Food safety concerns and knowledge gaps

Ensuring the microbial safety of reclaimed water is critical, particularly for crops that are consumed raw. Contaminated irrigation water has been linked to foodborne outbreaks (e.g., the 2011 outbreak of enterohemorrhagic *Escherichia coli* (EHEC) with 53 deaths in Germany (King *et al.* 2012), and the 2012 norovirus outbreak affecting 11,000 schoolchildren (Bernard *et al.* 2014)). While EU regulations set *E. coli* limits for irrigation water, they do not address emerging concerns like antibiotic resistant bacteria and antibiotic resistance genes (ARGs) (Purnell *et al.* 2016; Stange *et al.* 2016; Zhao *et al.* 2024). Studies have shown contradictory results – some reporting ARG accumulation in soils (Marano *et al.* 2019) and crops (Kumar *et al.* 2005), while others find no significant increase (Shamsizadeh *et al.* 2021; Liu *et al.* 2022b). This highlights the need for further research into the long-term risks of ARGs in food production.

#### 1.4. Research objective

The aim of this study was to evaluate the microbial quality of crops irrigated with reclaimed water from a multi-barrier pilot plant designed to meet EU Regulation 2020/741. Tomatoes, melons, celery, onions, lamb's lettuce, and oak leaf lettuce were grown under controlled conditions in both greenhouse and field environments. The crops were irrigated using three different water sources (drinking water and two reclaimed water qualities) and two irrigation methods (overhead and drip irrigation) to compare their respective impacts on microbial contamination.

A key focus of the study is to determine the presence of hygienically relevant microorganisms in crops irrigated with reclaimed water. Culture-based microbiological methods, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and quantitative polymerase chain reaction (qPCR) detection were used to assess potential contamination with pathogens and ARGs. Given the increasing concern about ARGs in wastewater-irrigated environments, this study aims to investigate whether ARGs accumulate in crops or irrigation water and whether their presence poses a potential risk to food safety. This research makes a significant contribution to the risk assessment of water reuse in agriculture within the framework of the EU Regulation 2020/741. It provides valuable insights into food safety risks, ARG dissemination, and sustainable water management. These insights help to inform regulatory developments and best practices for the safe implementation of water reuse in agriculture.

## 2. MATERIAL AND METHODS

### 2.1. Study site and water sources

To assess the quality of reclaimed water in terms of potential phytotoxic effects and hygienic/chemical impacts on edible crops, crops were cultivated in both a greenhouse and an open field. These crops were irrigated with two different reclaimed water qualities (Train A and Train B), along with tap water as a reference. The reclaimed water was derived from conventional wastewater treatment effluent and underwent different additional treatment steps.

- Train A consisted of secondary treatment followed by filtration and final ultraviolet (UV) disinfection, meeting the minimum requirements for non-potable water reuse under EU Regulation 2020/741.
- Train B consisted of secondary treatment followed by ceramic ultrafiltration (UF) membranes, ozonation, biological activated carbon (BAC) filtration, and final UV disinfection.

The details of treatment trains A and B are shown in [Figure 1](#) and more details are given in our previous study ([Ho et al. 2024](#)).

### 2.2. Sampling of irrigation water

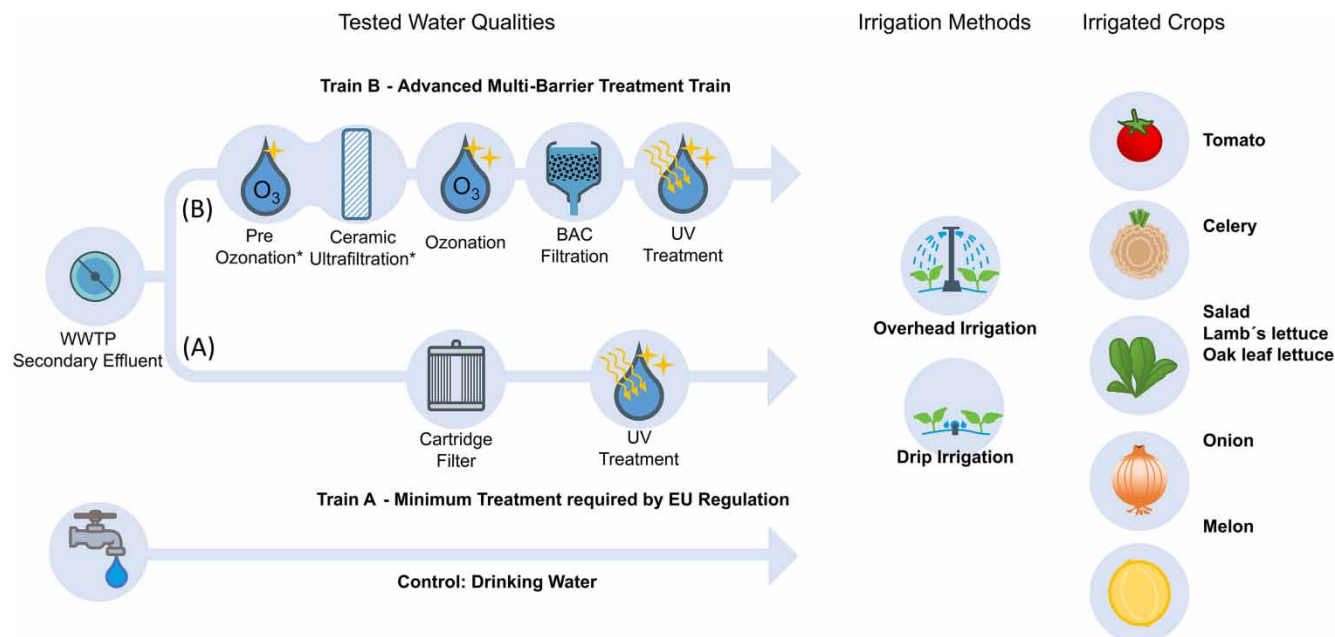
To assess microbial water quality, irrigation water samples were collected from both treatment trains (Train A and Train B) at multiple points, including the respective storage tanks where the treated water was stored prior to irrigation. Sampling followed the methodology described in our previous study [20], where 2 L samples were collected monthly in sterilized polypropylene bottles during multiple campaigns between September 2021 and February 2024.

### 2.3. Crops, irrigation, and growth periods

A wide variety of leafy, fruit, and root vegetables were successfully cultivated during the 2022 and 2023 growing seasons. These included melon, lamb's lettuce, oak leaf lettuce, and bush tomatoes in the greenhouse, as well as celeriac and onions in the open field. The applied amounts of reclaimed water are summarized in [Table 1](#).

The irrigated crops were sampled at the end of each irrigation season and prepared for further analysis. Before implementing the cultivation experiments, the experimental setup was carefully designed, the irrigation infrastructure was installed, and crop-specific bed preparations were carried out. To investigate potential analytical differences between full plant exposure to irrigation water (via overhead irrigation) and water uptake solely through the roots (via drip irrigation), both irrigation methods were established in the greenhouse ([Figure 2](#)).

Each greenhouse experiment comprised 32 plots (4.8 m<sup>2</sup> per plot), with 8 plots allocated to each water quality, further subdivided into 4 plots per irrigation method. To minimize the risk of cross-contamination between different water treatments, the plot arrangement was not randomized; instead, a block design was used. Throughout the study, a single crop type was cultivated across all plots within each growth period before transitioning to the next crop. The irrigation system was managed using an automated irrigation controller (BEWAMAT-CA 12).



**Figure 1** | Treatment trains A and B with internal water treatment modules. Drinking water was used as a 'clean' control. All three water types were used to irrigate tomatoes, celery, lamb's lettuce, oak leaf lettuce, onions and melons using drip and overhead irrigation.

**Table 1** | Cultivated crops in the projects greenhouse and open field

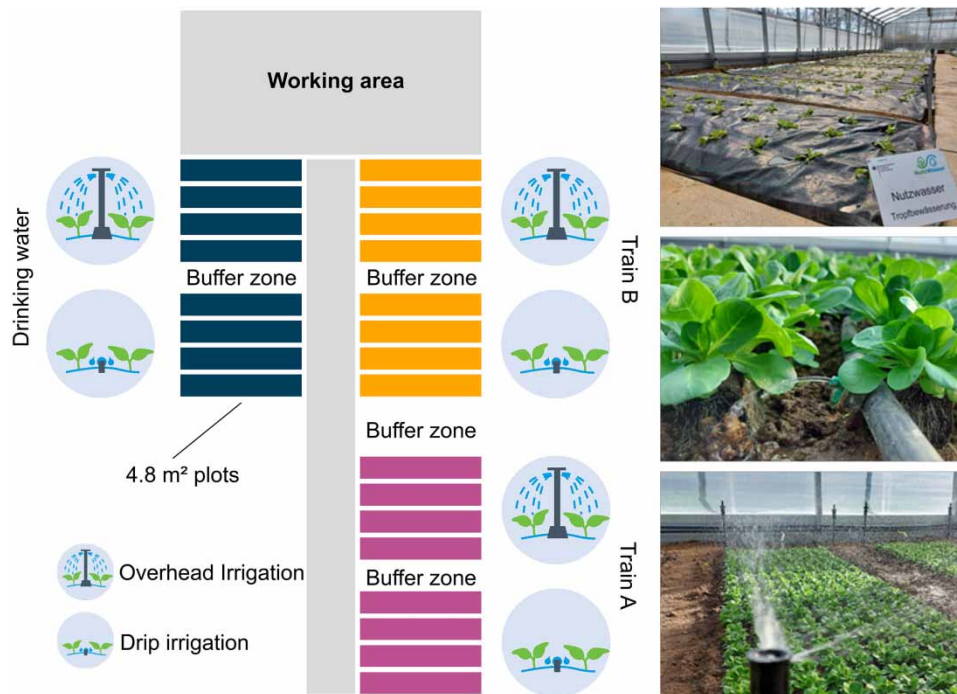
Crop	Growth period	Cultivation	Water per m <sup>2</sup>	Standing space	Exposition
Celery	Summer 2022	Open field	1,200 mm	0.16 m <sup>2</sup>	192 L
Melon	Summer 2023	Greenhouse	1,300 mm	0.8 m <sup>2</sup>	1,040 L
Lamb's lettuce	Winter 2022	Greenhouse	254 mm	0.01 m <sup>2</sup>	2.54 L
Tomato	Summer 2023	Greenhouse	555 mm	0.436 m <sup>2</sup>	242 L
Oak leaf lettuce	Spring 2023	Greenhouse	147 mm	0.126 m <sup>2</sup>	18.5 L
Onion	Summer 2023	Open field	820 mm	0.053 m <sup>2</sup>	43.5 L

Similar to the greenhouse experiments, field trials were conducted in an open field equipped with an automated irrigation infrastructure. Each water variant was applied to 7 plots, using only drip irrigation to prevent water drift caused by strong winds and to minimize the risk of cross-contamination. At the end of each irrigation season, the cultivated plants were sampled and analyzed for microbial parameters. During the cultivation period in the greenhouse, we observed incidental challenges, including mouse activity during tomato cultivation and aphid infestations on oak leaf lettuce. Although only healthy-looking crops were sampled, potential contamination from these organisms could not be entirely excluded.

#### 2.4. Lab preparation of crops

Microbial analysis was conducted on the surface of melons, oak leaf lettuce, lamb's lettuce, and bush tomatoes. To assess the highest possible concentration of contaminants – representing a worst-case scenario where crops are not washed before consumption – the wash water from these crops was analyzed. For celery and onions, this approach was not feasible due to the high amount of soil adhering to the crops. Instead, the inner part of the crops was analyzed. Five samples were analyzed for each crop and combination of water and irrigation type. Each sample included multiple plants or crop units. The preparation procedure for each crop type is described as follows.

**Melons:** Melons were collected at the sampling site and placed in sterile 2,250 mL low-density polyethylene (LDPE) bags, with preference given to melons with minimal soil contact. After cooling and transport to the laboratory, 170 mL of sterile



**Figure 2** | Left: Greenhouse block design for all three irrigation water qualities with buffer zones to avoid cross-contamination. Each plant species was grown simultaneously across all fields, ensuring that at any given time. Right: Photos from cultivation, drip irrigation and overhead irrigation (from top to bottom).

0.9% sodium chloride solution was added to each bag. The melons were thoroughly washed in the solution, which was then transferred to sterile 150 mL tubes for further microbial analysis.

*Oak leaf lettuce*: At the sampling site, 50–90 g of oak leaf lettuce was collected and placed in sterile 2,250 mL LDPE bags. Care was taken to minimize soil contamination. In the laboratory, 100 mL of sterile 0.9% sodium chloride solution was added to each bag. The leaves were washed in the solution, and the resulting wash liquid was transferred to sterile 50 mL centrifuge tubes for microbial analysis.

*Lamb's lettuce*: Lamb's lettuce leaves were collected at the sampling site and placed in sterile 2,250 mL LDPE bags. To minimize soil contamination, any soil adhering to the leaves was carefully removed. In the laboratory, six leaves per sample were weighed and placed in sterile 50 mL centrifuge tubes. Each tube was filled with 40 mL of sterile 0.9% sodium chloride solution, and the tubes were shaken to simulate a washing process. The resulting wash liquid was subsequently used for microbial analysis.

*Bush tomatoes*: Five tomatoes per sample were collected and placed in sterile 2,250 mL LDPE bags, selecting those with minimal soil contamination. In the laboratory, 100 mL of sterile 0.9% sodium chloride solution was added to each bag. The tomatoes were gently agitated in the solution, and the outside of the bag was rubbed against the tomatoes to simulate a washing process. The wash liquid was then transferred to sterile 50 mL centrifuge tubes for analysis.

*Celery*: One celery stalk per sample was transported to the laboratory in sterile 2,250 mL LDPE bags. Due to significant soil contamination, the celery was first washed with tap water in the laboratory. The washed celery was transferred to a sterile workspace and roughly peeled using a flamed knife. After peeling, the celery was washed again to remove any remaining soil. A central piece of the celery stalk was cut with a flamed knife to avoid contamination from the outer layers. The sample was then crushed in a sterile blender and placed in 200 mL sterile tubes for microbial analysis.

## 2.5. Microbial analysis

To ensure comparability of results, microbial concentrations were expressed per gram of crop, even though analysis was conducted on the wash water from crop surfaces. This approach reflects potential contamination levels, allowing direct comparison across crop types and referencing typical serving sizes.

*Enterococci* were detected using a membrane filtration method. For each sample, between 5 and 50 mL of wash liquid was filtered through a 0.45 µm membrane filter (EZ-Pak, Merck Millipore) using a vacuum pump. The filter was placed on the surface of Slanetz & Bartley selective agar plates and incubated at 36 °C for 48 h. After incubation, colonies indicative of enterococcal growth were identified by their characteristic appearance (red or maroon colonies).

*Coliform bacteria and E. coli* were quantified using the IDEXX Colilert<sup>®</sup>-18 System. For further analysis of coliform bacteria, wells showing positive detections in the Colilert system were processed. For each sample, twelve positive wells were opened, and their contents were streaked onto agar plates prepared according to German Standard Methods (Deutsches Einheitsverfahren; DEV nutrient agar, Thermo Fisher Scientific, BO1251T) using sterile inoculation loops. These plates were incubated at 36 °C for 24 h to allow single colonies growth. The resulting colonies were then analyzed using MALDI-TOF MS to identify the coliform bacteria. For positive *E. coli* detections, wells from the Colilert system were opened and their contents were transferred to Chromogenic Coliform Agar selective plates with sterile inoculation loops. These plates were used to confirm the presence of *E. coli* by examining the growth of characteristic colonies (metallic blue to violet).

*Salmonella* was detected via Salmonella Brilliance Agar (Oxoid<sup>™</sup> Brilliance<sup>™</sup>, ThermoFisher). Samples were vacuum filtered (0.45 µm pore size, EZ-Pak, Merck Millipore), placed on the agar, and incubated at 37 °C for 24 h. Colonies were identified by MALDI-TOF-MS.

*Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteria* were detected using a chromogenic selective medium (CHROMagar<sup>™</sup> ESBL, MAST Diagnostica). Samples were vacuum filtered (0.45 µm pore size, EZ-Pak, Merck Millipore), placed on the agar, and incubated at 41 °C for 24 h. Colonies were distinguished using the medium's color profiles and further analyzed with MALDI-TOF-MS.

## 2.6. Identification of coliforms and ESBL-producing gram-negative bacteria with MALDI-TOF-MS

Isolates were identified using MALDI-TOF MS (Bruker) according to the manufacturer's instructions and as previously published (Reitter *et al.* 2021). The identified isolates were only documented at the species level if the score value generated by the BioTyper Software Packet (Version: 4.1.80, Bruker Daltonik) was between 2.0 and 3.0. If the score value was between 1.7 and 1.99 or different species appeared among the first 10 hits, the identity of the isolates was noted at the genus level.

## 2.7. Molecular biological analysis

### 2.7.1. Sample preparation and extraction

For qPCR analysis, the flesh of celery, onions, tomatoes, and lettuce was used, as only small quantities are needed for qPCR. As with the culture-based tests, five samples for each irrigation and water type were analyzed (30 in total per crop).

*Oak leaf lettuce*: Leaves were frozen in sterile LDPE bags at -20 °C. After freezing, the leaves were crushed inside the bags. Approximately 0.4 g of shredded leaf material per irrigation type was weighed and used for extraction.

*Onions*: Onions were peeled and pureed with a flamed blender. Approximately 0.5 g of pureed onion was weighed and used for extraction.

*Celery*: From the blended material from the culture assays, 1 mL was placed in a reaction tube and centrifuged at 20,000 g for 20 min. The supernatant was discarded, and the pellet was used for extraction.

*Tomatoes*: Three to four tomatoes were placed in a sterile beaker and chopped using a flamed hand blender. The puree was transferred to 50 mL centrifuge tubes and centrifuged at 5,000 g for 30 min. The supernatant was discarded, and approximately 0.5 g of tomato puree was weighed into 2 mL test tubes for extraction.

Plant material was extracted using the FastDNA<sup>™</sup> SPIN Kit for soil (MP Biomedicals, Germany) with the FastPrep<sup>®</sup> instrument (MP Biomedicals, Germany) following the manufacturer's instructions (MP Biomedicals). The elution volume was 100 µL.

### 2.7.2. qPCR analysis

All qPCRs were performed using a Rotor-Gene cycler (Qiagen) with the SsoAdvanced Universal SYBR Green Supermix (BioRad). Primers and annealing temperatures are listed in Table 2. The general qPCR program consisted of: 2 min at 98 °C (enzyme activation), 45 cycles of 20 s at 98 °C (denaturation), 20 s at the primer-specific annealing temperature (TA), 20 s elongation time (tE) at 72 °C.

Quality controls were performed by melting curve analysis and capillary gel electrophoresis with the QIAxcel<sup>®</sup> Advanced system (Qiagen). The presence of polymerase chain reaction (PCR) inhibitors was evaluated by analyzing dilutions of DNA

**Table 2** | Primers used for qPCR detection of bacteria and antibiotic resistance genes

Parameter/Target/Primer	bp	Primer	PCR Annealing	Source
Total bacteria (16S)	160	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGGC	68 °C	Muyzer <i>et al.</i> (1995)
<i>ermB</i>	405	CATTTAACGACGAACTGGC GGAACATCTGTGGTATGGCG	63 °C	Gevers <i>et al.</i> (2003)
<i>sul1</i>	163	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	68 °C	Pei <i>et al.</i> (2006)
<i>bla</i> <sub>TEM</sub>	112	TTCCTGTTTTTGCTCACCCAG CTCAAGGATCTTACCGCTGTTG	66 °C	Bibbal <i>et al.</i> (2007)
<i>tetA</i>	150	CCTGCGCGATCTGGTTCCT GCCAGCGAGACGAGCAAGA	55 °C	Stanton & Humphrey (2003)

(deoxyribonucleic acid) samples. If the dilutions were 25% higher in concentration than less diluted samples, the diluted results were used, provided the melting points and product lengths were correct. All samples and standards were run in duplicate.

qPCR standards were prepared from serial dilutions of known quantities of linearized plasmid containing target genes. The limit of quantification (LOQ) was determined as the lowest concentration of DNA plasmid that the assay could quantify and maintain within the linear portion of the standard curve, with PCR efficiencies between 90 and 105%,  $R^2$  values above 0.99, and minimal dispersion around the curve. The overall LOQ for the qPCR assay was 10 copies/ $\mu$ L. Considering an elution volume of 100  $\mu$ L, a qPCR sample volume of 1  $\mu$ L, and the use of 0.5 g of crop material, the final LOQ for the complete qPCR method was calculated to be 2,000 gene copies per gram.

The ARG subtypes selected for this study – *ermB*, *sul1*, *bla*<sub>TEM</sub>, and *tetA* – are among the most frequently detected resistance genes across various environmental matrices (Zhuang *et al.* 2021). These genes were chosen to represent four major classes of antibiotics: macrolides, sulfonamides,  $\beta$ -lactams, and tetracyclines, respectively, enabling a broad assessment of resistance profiles.

## 2.8. 16S amplicon sequencing

Exemplary 16S rRNA high-fidelity (HiFi) gene sequencing was performed on samples from the influent and effluent of the wastewater treatment plant (WWTP) Schweinfurt, irrigation water from Train A and Train B, intermediate water after each process step from Train B, and crop samples. Sequencing was performed by CeGaT (Tübingen, Germany). Methodological details regarding demultiplexing, trimming, filtering, mapping, and taxonomic assignment are provided in the supplementary information (SI).

## 2.9. Statistics and visualization

Statistical analysis and visualization of the results were performed in Python (3.12.4) using the following packages: pandas (2.2.2), seaborn (0.13.2), numpy (1.26.4), pingouin (0.5.5), and matplotlib (3.9.1). To assess differences in microbial contamination across different fruit types, a one-way analysis of variance (ANOVA) was performed using rank-transformed microbial count data. Rank transformation was applied to account for potential non-normality and heteroscedasticity in the dataset. The analysis was conducted using the pingouin.anova function in Python, with the independent variables being the microbial parameter (e.g., *E. coli*, coliforms, enterococci, ESBL gram-negative bacteria, *Salmonella*) and the fruit type (e.g., lamb's lettuce, oak leaf lettuce, celery, tomato, and melon).

Following a significant ANOVA result ( $p < 0.05$ ), a post-hoc Tukey's Honest Significant Difference (HSD) test was applied using pingouin.pairwise\_tukey to determine which specific fruit-microorganism combinations differed significantly. Additionally, the Kruskal–Wallis test was chosen as a non-parametric alternative to check for significant differences between groups. If the Kruskal–Wallis test was significant ( $p < 0.05$ ), Dunn's test with Bonferroni correction was used as a follow-up pairwise post-hoc test to identify which groups differed.

The different weights of the crops also resulted in different detection limits for the microbiological and molecular biological tests. Values below the limit of detection were treated as samples with counts at the detection limit when calculating mean values. Comparative statistics were not performed if a high percentage of results were below the detection limit.

### 3. RESULTS AND DISCUSSION

#### 3.1. Coliforms, *E. coli*, enterococci and ESBL-producing gram-negative bacteria

##### 3.1.1. Quality of irrigation water

Both open-field and greenhouse crops were irrigated using three different water sources: drinking water and advanced treated wastewater from Train A and Train B. The quality of these water sources varied significantly (r-ANOVA and Tukey post-hoc in the SI). Train A had the highest detection rates and concentrations of coliforms, *E. coli*, enterococci, and ESBL-producing gram-negative bacteria (Figure 3). In contrast, Train B exhibited lower detection rates and bacterial counts, with only occasional detections of *E. coli*, enterococci, and ESBL-producing bacteria, all associated with temporary malfunctions in the UV treatment system.

Notably, ESBL-producing gram-negative bacteria in the WWTP influent comprised a mix of genera, including *E. coli* (39%), *Acinetobacter* (24%), *Pseudomonas* (22%), *Enterobacter* (5%), *Citrobacter* (5%), and *Klebsiella* (4%). In Train A, where 56% of the samples tested positive for ESBL-producing bacteria, *Pseudomonas* dominated (80%), followed by *Citrobacter* (15%) and *E. coli* (5%). In contrast, Train B exclusively contained ESBL-producing *Pseudomonas* (see the SI for details).

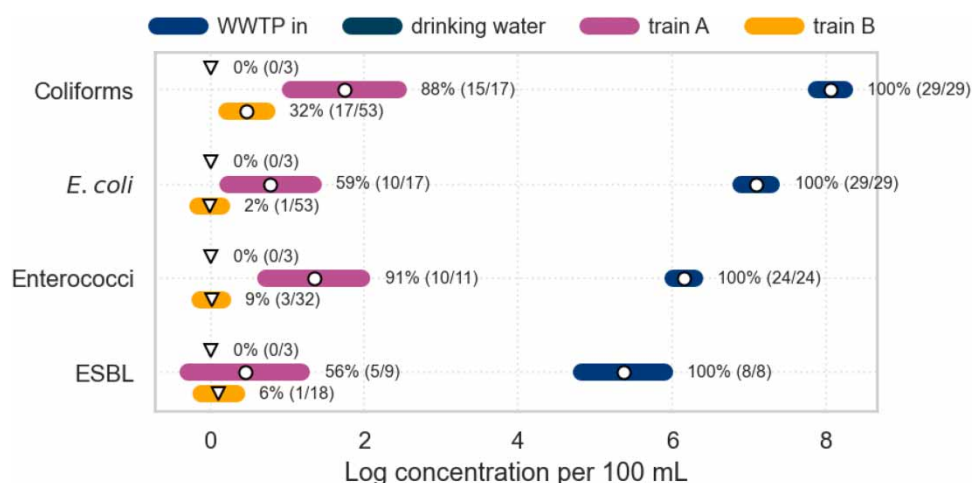
No detections of coliforms, *E. coli*, enterococci, or ESBL-producing bacteria were observed in the drinking water. Salmonella testing was conducted sporadically in both Train A and Train B, but no positive detections were recorded.

According to the minimum requirements for water reuse, *E. coli* concentrations must not exceed 10 colony-forming units (CFU) per 100 mL in at least 90% of samples. Train B met this requirement, with 52 out of 53 samples (98%) remaining below this threshold. However, Train A did not meet this requirement, as only 12 out of 17 samples (70%) were below 10 CFU per 100 mL. The only detection of ESBL-producing gram-negative bacteria in Train B occurred in the water storage system and was associated with elevated levels of coliforms and enterococci, though *E. coli* was not detected.

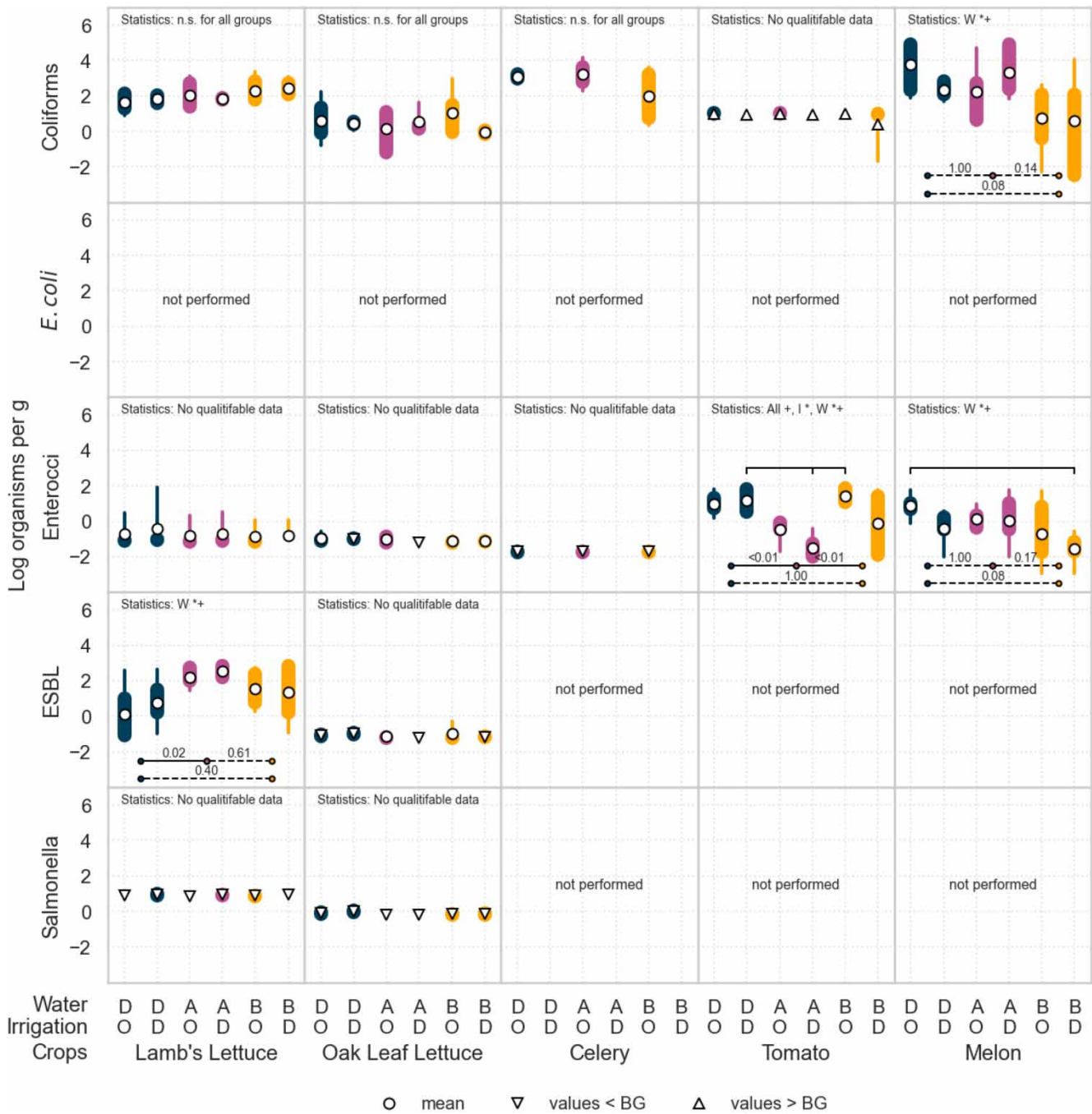
##### 3.1.2. Results for different crops

Lamb's lettuce, oak leaf lettuce, tomato, and melon were washed with buffer, and the wash water was analyzed. This method was intended to represent a worst-case scenario, considering that unwashed vegetables are often consumed raw, or in the case of melons, the inedible skin comes into contact with the edible portion. It was expected that Train A would result in the highest bacterial counts and that drip irrigation would generally result in lower contamination levels compared to overhead irrigation, as the latter allows for greater direct contact between the plant and the irrigation water.

Coliform bacteria were detected in the wash fluid of all crop types, with concentrations ranging from 0.01 to over 10,000 CFU/g (Figure 4). The highest counts were observed on melons, followed by celery, lamb's lettuce, oak leaf lettuce, and tomatoes. *E. coli* was only detected in the wash fluid of tomatoes (mean: 10 CFU/g) and two samples from melons



**Figure 3** | Mean (circles or triangles in case of low detection rate), 95% confidence interval (bars) and detection rate of coliform bacteria, *E. coli*, enterococci and ESBL producing gram-negative bacteria (ESBL) in the tree irrigation water types and WWTP influent (WWTP in).



**Figure 4** | Results from culture based analysis of washing liquid for different crops per gram irrigated with drinking water (D) or water from train A (A) or B (B) via drip (D) or overhead (O) irrigation. Symbols show mean values (circles or triangles in case of low detection rate), bars the 25–75% and 2.5–97.5%iles. Statistical tests are displayed above the plots, if significant ( $p < 0.05$ ) for the parameter irrigation (I), water (W), both parameters combined (W\*I) or all groups separately (all). Symbols indicate significance for ranked ANOVA (\*) and Kruskal–Walis-test (+). In case of significant differences ( $p < 0.05$ ) between water types, postHoc HSD test results are shown as lines below the plots including their  $p$  values. Dashed lines indicate  $p > 0.05$ . Significant differences between single groups (water/irrigation) are shown by lines above the plot (Dunn's test).

were irrigated via overhead irrigation with Train A water. Interestingly, *E. coli* was not found on tomatoes irrigated via drip irrigation using water from Train A or Train B, but it was detected on tomatoes irrigated with drinking water.

Enterococci were sporadically detected on lamb's lettuce (one positive sample per group) and were consistently present in nearly all wash fluid samples from tomatoes and melons, with mean concentrations ranging from 0.5 to 100 CFU/g. Tomatoes

irrigated with drinking water and Train B water had the highest enterococci counts (around 500 CFU/g), whereas tomatoes irrigated with Train A water had lower concentrations (around 1 CFU/g). On melons, enterococci levels were similar across the different irrigation waters but were slightly higher in overhead-irrigated samples compared to those irrigated via drip irrigation.

The wash water from lamb's lettuce and oak leaf lettuce was analyzed for ESBL-producing gram-negative bacteria. ESBLs were detected only on lamb's lettuce, with concentrations ranging from 1 to 200 CFU per gram. The highest levels were found in samples irrigated with Train A water, followed by Train B, with the lowest concentrations in drinking water-irrigated lettuce.

*Salmonella* was not detected in any lettuce samples.

The high prevalence of coliform bacteria was not unexpected, as these bacteria can be introduced through multiple pathways, including insects, soil, and the plant microbiome (Leclerc *et al.* 2001; Reitter *et al.* 2021). This fact is also confirmed by the microbiological findings in the plants that were irrigated with drinking water. The particularly high bacterial counts on melons may be due to their extensive contact with soil during growth and harvest. Tomatoes, which were frequently affected by aphid infestations – especially in overhead-irrigated plots – also showed relatively high bacterial loads. *E. coli*, which is linked to fecal contamination, was exclusively detected on tomatoes and melons. Notably, the presence of rodents in the tomato greenhouse suggests that the *E. coli* detected on tomatoes may have originated from mouse faeces. The sporadic *E. coli* detections on melons may be due to residual soil on their surface, which could also explain the elevated enterococci levels observed in both tomatoes and melons. While enterococci are often used as fecal indicators, they are also naturally present in the environment (Byappanahalli *et al.* 2012) and can be introduced by gastropods (Leister *et al.* 2023), even in the absence of direct soil contact.

ESBL-producing gram-negative bacteria were found on lamb's lettuce but not on oak leaf lettuce. The reason for this discrepancy is unclear, but ESBL-producing bacteria have been reported on ready-to-eat salads (Tresch *et al.* 2024). A potential explanation could be differences in harvest handling, seasonal variation, or environmental factors. Lamb's lettuce was grown in November, a time with lower temperatures and UV radiation, while oak leaf lettuce was grown in June, when temperatures and UV radiation are much higher, potentially affecting microbial survival on the surface. Additionally, oak leaf lettuce tends to grow larger and may have less soil contact compared to lamb's lettuce, which could reduce the likelihood of contamination from the soil. Certain bacteria, such as *Salmonella*, can persist on plant surfaces but also exhibit seasonal die-off rates. For example, *Salmonella* has been reported to survive better in the fall than in the spring (Oliveira *et al.* 2011).

The irrigation water sources showed differences in *e. coli*, coliform, and enterococci concentrations. If microbial contamination were primarily driven by irrigation water, one would expect consistently higher bacterial counts in crops irrigated with Train A water. However, the results do not follow a clear pattern: coliform, *E. coli*, and enterococci counts varied across water sources and irrigation methods, with no consistent trend. Surprisingly, *E. coli* and enterococci counts on tomatoes were lowest in the Train A irrigation group, while coliform counts on celery and ESBL-producing bacteria prevalence on lamb's lettuce were highest in Train A samples. Overall, only a few variables showed statistically significant differences. The water source was a significant factor (rank ANOVA and Kruskal–Wallis tests) for coliforms on melons, *E. coli* on tomatoes, enterococci on tomatoes and melons, and ESBL-producing bacteria on lamb's lettuce. However, post hoc tests were only significant for enterococci in tomatoes across all water sources and for ESBL-producing bacteria on lamb's lettuce when comparing drinking water and Train A ( $p < 0.05$ ). This suggests that in most cases, neither type of irrigation water nor method of irrigation had a significant effect bacterial concentrations on crops. In fact, when significance was observed, crops irrigated with the more contaminated water source (Train A) sometimes had lower bacterial counts (e.g., tomatoes).

The extant literature offers a somewhat equivocal picture with respect to the question of whether microbial contamination of crops irrigated with treated wastewater is an issue. Some studies have reported increased bacterial loads due to variations in water quality and irrigation method, while others have found no significant differences between irrigation with treated wastewater effluent and drinking water. For example, a study in Spain found high coliform and *Salmonella* counts on crops irrigated with treated wastewater effluent (Mañas *et al.* 2009). Conversely, another study on microbial indicators in broccoli and tomatoes reported that contamination levels were independent of the irrigation water type (treated wastewater effluent vs. groundwater) (Beneduce *et al.* 2017). Similarly, a Spanish study detected *E. coli* on plants irrigated with secondary effluent but not when additional treatments such as UF and granular activated carbon were applied (Intriago *et al.* 2018). Other research has shown that when pathogens are present in irrigation water, they are often detectable on crops as well (Truchado *et al.* 2021; Fernandes *et al.* 2023). A large multinational study conducted in Belgium, Brazil, Egypt, Norway, and Spain used regression models to analyze *E. coli*, *Salmonella*, *Campylobacter*, and STEC contamination. The authors reported that water type had a significant effect on crop contamination, but they also highlighted the influence of additional

environmental factors (Ceuppens *et al.* 2015). Another study identified risk factors for *Salmonella* and *Listeria* monocytogenes contamination in produce fields and showed that manure application, irrigation practices, wildlife presence, and recent soil cultivation increased the risk of contamination, while buffer zones had a protective effect (Strawn *et al.* 2013).

Many studies that report significant effects of irrigation water on crop contamination focus on irrigation with conventionally treated wastewater effluents. In contrast, the most contaminated irrigation water in our study (Train A) had already undergone additional treatment, including filtration and UV disinfection. This may explain why we did not observe any significant differences in bacterial counts across irrigation water sources. As seen in other studies, factors such as soil contact, animal activity, and harvesting practices likely played a more dominant role in shaping microbial contamination levels. To further investigate these influences, coliform bacteria were identified using MALDI-TOF-MS.

### 3.2. Microbial community of coliform bacteria

#### 3.2.1. Coliform bacteria in irrigation water

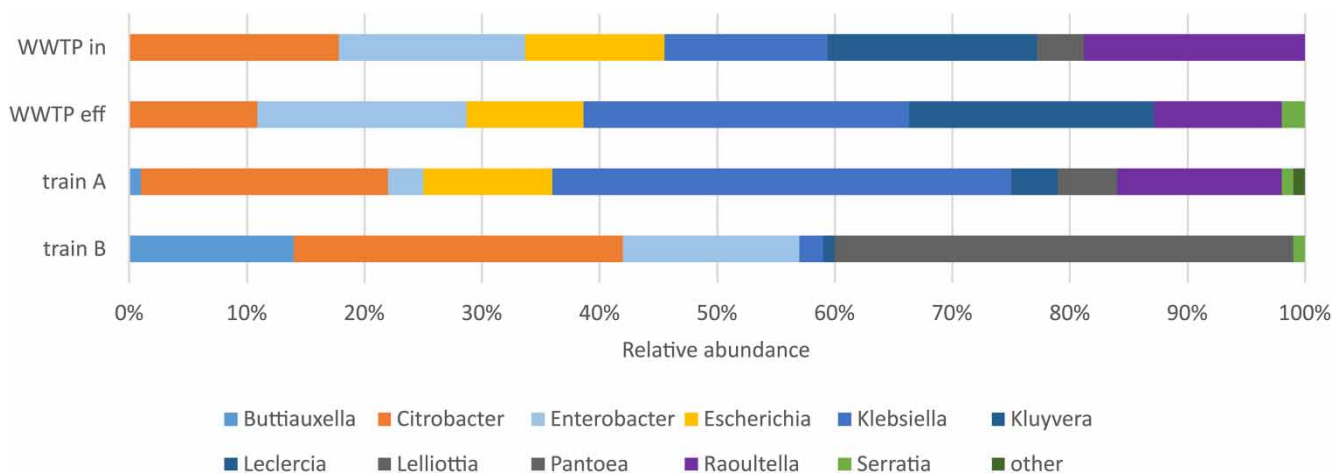
A total of 330 coliform isolates from the effluent of WWTP and the effluent of Train A and Train B were identified using MALDI-TOF-MS. In wastewater, the most frequently detected coliform genera were *Raoultella* (19%), *Citrobacter* (18%), *Kluyvera* (18%), *Enterobacter* (16%), *Klebsiella* (14%) and *Escherichia* (12%). In secondary treated wastewater effluent, we identified a higher amount of *Klebsiella* (28%) and *Kluyvera* (21%), similar counts of *Enterobacter* (18%), and reduced counts of *Citrobacter* (14%) and *Raoultella* (11%) (Figure 5). All these bacteria are commonly found in wastewater (Suzuki *et al.* 2018).

In reclaimed water from Train A (which includes filtration and UV treatment), we found higher counts of *Klebsiella* (39%) and *Citrobacter* (21%) as well as similar counts of *Raoultella* (14%) and *Escherichia* (11%). In contrast, reclaimed water from Train B contained almost no *Klebsiella* or *Raoultella* (0% in total) but had high counts of *Lelliottia* (39%) and *Citrobacter* (28%) (Figure 5).

It is important to note that coliforms in train B were only detected in 32% of all samples, and in nearly all cases, they were found in storage tank samples rather than directly after the final UV treatment step. These findings align with the general coliform concentrations observed in Train A and Train B: while Train A did not reduce coliform counts below the detection limit, UF in Train B effectively removed coliforms to undetectable levels. Consequently, coliforms detected in Train A water were likely of direct wastewater origin, whereas those in Train B were more likely the result of regrowth within the system. Specifically, *Lelliottia amnigena* was found to proliferate within Train B, as previously reported (Ho *et al.* 2024).

#### 3.2.2. Coliform bacteria on crops

Given the coliform composition in irrigation water, one might expect similar bacterial patterns on crops irrigated with this water. However, the results indicate that coliform communities on lamb's lettuce, oak leaf lettuce, melons, and celery were dominated by different genera with lower diversity (Table 3). Findings of non-coliforms, like high counts of *Priestia megaterium* on lamb's lettuce, a ubiquitous species, were not included in the analysis.



**Figure 5** | Composition of coliform bacteria identified by MALDI-TOF-MS in influent and effluent of the WWTP and irrigation water produced by Trains A and B.

**Table 3** | Relative abundance of bacteria identified by MALDI-TOF-MS from coliform detection in irrigation water and on different crops for irrigation with drinking water (T) and water from train A (A) and B (B) with drip (D) and overhead irrigation (O)

Fruit	Water				Celery			Lamb's lettuce						Oak leaf lettuce						Melon					
	Wi	We	A	B	T	A	B	T	T	A	A	B	B	T	T	A	A	B	B	T	T	A	A	B	B
Irrigation					O	O	O	D	O	D	O	D	O	D	O	D	O	D	O	D	O	D	O	D	O
<i>Buttiauxella</i>	0	0	1	13	3	16	0	0	0	0	0	0	0	0	0	0	0	0	33	2	0	0	0	0	0
<i>Citrobacter</i>	18	11	19	25	5	0	2	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	5	6	0
<i>Enterobacter</i>	16	18	3	14	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	39	60	87	29	48	75
<i>Escherichia</i>	12	10	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Hafnia</i>	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0
<i>Klebsiella</i>	14	28	35	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	6	2	46	0	0
<i>Kluyvera</i>	18	21	4	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	2	3	0	0
<i>Leclercia</i>	0	0	0	1	0	2	2	0	0	0	0	0	0	0	0	17	0	5	33	0	0	0	0	0	0
<i>Lelliottia</i>	4	0	5	35	10	2	0	17	0	20	31	0	23	43	100	17	0	0	33	18	0	2	2	18	2
<i>Pantoea</i>	0	0	0	0	33	39	44	17	0	0	0	0	0	57	0	50	0	58	0	36	3	0	2	28	23
<i>Rahnella</i>	0	0	0	0	0	0	0	0	0	0	6	0	8	0	0	0	0	0	0	0	0	0	0	0	0
<i>Raoultella</i>	19	11	12	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0
<i>Serratia</i>	0	2	1	1	50	27	51	0	0	0	13	0	0	0	0	0	0	16	0	0	18	7	7	0	0
No id.	0	0	8	10	0	0	0	67	100	80	50	100	69	0	0	17	0	21	0	0	0	0	2	0	0

*Celery*: *Serratia* (43%) and *Pantoea* (39%) were the predominant genera. The top 3 species were *Pantoea agglomerans* (38%), *Serratia plymuthica* (27%), and *Serratia liquefaciens* (12%).

*Lamb's lettuce*: A significant portion of samples (78%) could not be identified. The other coliforms mainly belong to *Lelliottia* spp. (15%).

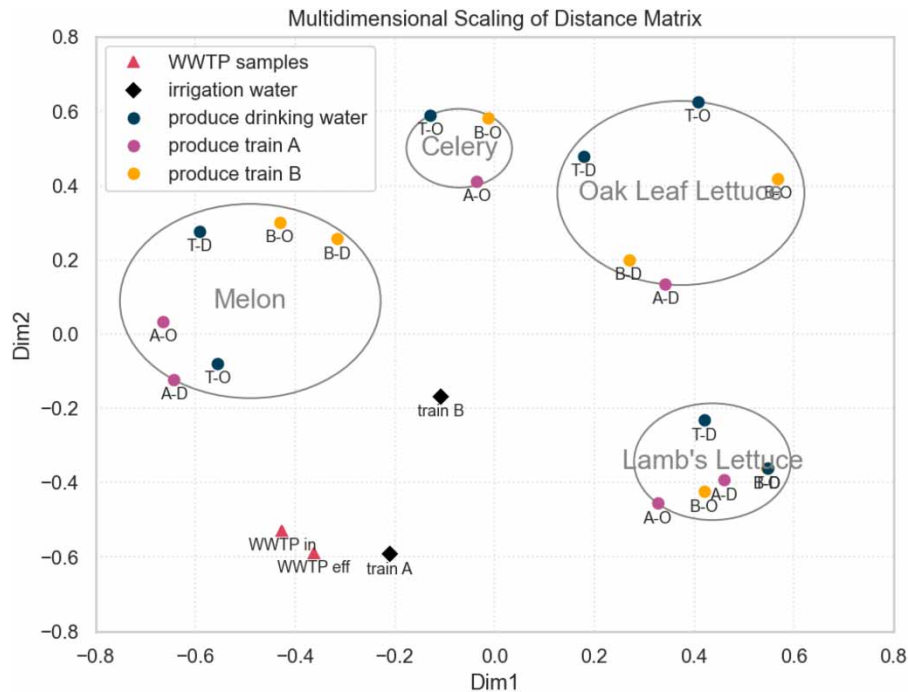
*Oak leaf lettuce*: *Lelliottia* (32%) and *Pantoea* (28%) were the most frequently detected genera. The top 3 species were *L. amnigena* (35%), *P. agglomerans* (33%), and *Leclercia adecarboxylata* (11%).

*Melons*: *Enterobacter* (56%) and *Pantoea* (15%) were the most prevalent genera. The top 3 species were *Enterobacter cloacae* (31%), *Enterobacter asburiae* (14%), and *P. agglomerans* (9%).

Notably, the most frequently identified bacterial species on crops were absent in both secondary treated wastewater effluent and reclaimed water from trains A and B, with *L. amnigena* being the only exception. These species are commonly found in soil, on plant surfaces, and in the broader environment (Compant *et al.* 2019; Wang *et al.* 2024), suggesting that their presence on crops is likely due to natural sources, such as soil contact, airborne deposition, or plant-associated microbiota.

Among the four crop types, melons had the highest soil contact, which likely influenced their microbial composition. Indeed, the dominant bacterial genera in melons were predominantly associated with soil or rhizosphere environments. *Enterobacter*, in particular, was not found in high numbers on the other crops. In contrast, the dominant species on celery, *P. agglomerans* (38%), *S. plymuthica* (27%), and *S. liquefaciens* (12%), are typically linked to plants and roots, with some even exhibiting plant-growth-promoting or biocontrol properties (Poppe *et al.* 2003; Vleeschauwer & Höfte 2007). These findings further support the idea that soil contact, rather than the irrigation water, played a significant role in shaping the microbial communities on melons.

To further analyze these patterns, a Bray–Curtis dissimilarity matrix was computed and visualized to compare microbial communities across crops, irrigation water types, and irrigation methods (Figure 6). In the distance visualization, irrigation water samples clustered separately from crop samples, while plant-associated bacterial communities were primarily grouped by crop type rather than by irrigation water source (drinking water vs. Train A vs. Train B). Additionally, no distinct clustering was observed between crops irrigated via drip and overhead irrigation. This suggests that factors such as crop type,



**Figure 6** | Visualization of the Bray–Curtis dissimilarity matrix from coliform identification data from WWTP influent and effluent, irrigation water from trains A and B and from samples from melon, celery, oak leaf lettuce and lamb's lettuce. Groups of crops are circled.

environmental conditions, and soil microbiota play a greater role in shaping bacterial communities on plant surfaces than the irrigation water itself.

### 3.3. 16S sequencing

Exemplary 16S rRNA gene sequencing was performed on samples from raw and secondary treated wastewater effluent, reclaimed water from Train A and Train B, intermediate water after each process step from Train B, and crop samples. Unfortunately, sequencing failed for all crop samples, most likely due to the high proportion of eukaryotic DNA, which interfered with bacterial amplification. As a result, the sequencing findings are only briefly discussed in the main text, with detailed results provided in the SI.

In general, the bacterial community composition shifted throughout the treatment process. *Proteobacteria* dominated all samples, accounting for 50–98% of the bacterial communities.

**Raw wastewater:** The community consisted primarily of *Proteobacteria* (63%, almost exclusively *Epsilonproteobacteria*), *Bacteroidetes* (13%), and *Firmicutes* (21%).

**Secondary treated wastewater effluent:** A similar pattern was observed, with an increase in *Proteobacteria* (75%, predominantly *Betaproteobacteria*) and a decrease in *Bacteroidetes* (8%) and *Firmicutes* (2%).

**Train A reclaimed water:** The bacterial composition closely resembled that of the treated effluent.

**Train B reclaimed water:** The bacterial profile of Train B irrigation water differed substantially, with a mixture of *Alpha*-, *Beta*-, and *Gammaproteobacteria* (51% in total) and a notably high relative abundance of *Candidatus Peregrinibacteria*, more precisely *Candidatus Peribacter riflensis*. This recently identified, uncultured bacterium has limited information available regarding its physiology and ecological role.

Interestingly, the relative abundance of *Candidatus P. riflensis* increased progressively through the treatment stages of Train B – from 1% after ozonation to 18% after BAC filter 1, 44% after BAC filter 2, and finally reaching 45% in the irrigation water of Train B.

Microbial diversity, as measured by the Shannon index, declined significantly along the treatment process of Train B, dropping from 3.1 in raw wastewater to 0.86 after ozonation. This sharp decline was accompanied by a notable shift in bacterial dominance: after ozonation, 87% of the detected sequences belonged to *Pseudomonas abietaniphila*, suggesting a higher resistance of *Pseudomonas* species to oxidative treatments (Gholipour *et al.* 2024).

A Bray–Curtis dissimilarity matrix was used to visualize the microbial differences between the samples (S1). This analysis revealed a relatively short distance between WWTP effluent and irrigation water from Train A, indicating their similar bacterial communities and potentially carrying a higher likelihood of wastewater-associated bacteria and associated health risks. In contrast, irrigation water from Train B underwent substantial microbial shifts, dominated by *Candidatus P. riflensis* and exhibiting a sharp decline in microbial diversity. This shift is likely due to UF in Train B, which effectively removes fecal and potentially pathogenic bacteria.

### 3.4. Antibiotic resistance genes

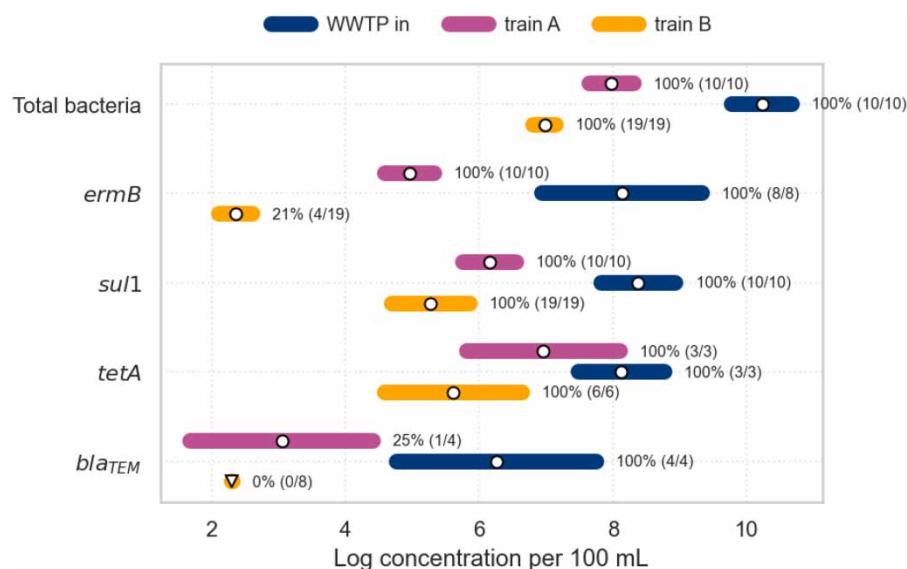
#### 3.4.1. Irrigation water

In addition to microbial indicators and pathogens, irrigation water, and fruit samples were analyzed via qPCR for selected ARGs. Generally, all differences between water types were statistically significant, except for the *tetA* gene (r-ANOVA and Tukey post-hoc in SI). Among the targeted genes, those encoding resistance to sulfonamide (*sul1*), erythromycin (*ermB*), and tetracycline (*tetA*) were detected at similar concentrations of approximately  $10^8$  genetic copies per 100 mL in wastewater (Figure 7). The beta-lactamase gene (*bla<sub>TEM</sub>*) was present at slightly lower concentrations, around  $10^6$  copies per 100 mL.

In reclaimed water from Train A, all selected ARGs except *bla<sub>TEM</sub>* were detected in 100% of samples, with concentrations ranging from  $10^5$  to  $10^7$  genetic copies per 100 mL. In contrast, Train B – due to its more advanced treatment process – showed lower detection rates and substantially reduced concentrations of *ermB*, while *sul1* and *tetA* were only slightly lower than in Train A. The *bla<sub>TEM</sub>* gene was not detected in the effluent of Train B.

During the treatment process of Train B, *ermB* and *bla<sub>TEM</sub>* genes, reached the detection limit after UF (achieving up to a 6-log reduction). While total bacterial load (16S rRNA gene copies) and the resistance genes *sul1* and *tetA* were significantly reduced by UF, their concentrations increased after ozonation and BAC filtration, with only a slight reduction following UV treatment. The overall bacterial regrowth observed in Train B – primarily *L. amnigena*, as previously reported (Ho *et al.* 2024) – likely contributed to this post-filtration increase.

The persistence of the tetracycline (*tetA*) and sulfonamide (*sul1*) resistance gene in Train B reclaimed water is likely due to resistant *Pseudomonas* species, which are known to survive oxidative treatments such as ozonation. This hypothesis is supported by the 16S sequencing results, where *Pseudomonas* populations declined after ozonation, alongside reductions in *tetA* and *sul1* gene copy numbers. Additionally, as demonstrated in previous studies, ARGs have been shown to persist even in wastewater that has undergone advanced treatment processes (Stange *et al.* 2019; Sauter *et al.* 2021). Given the presence

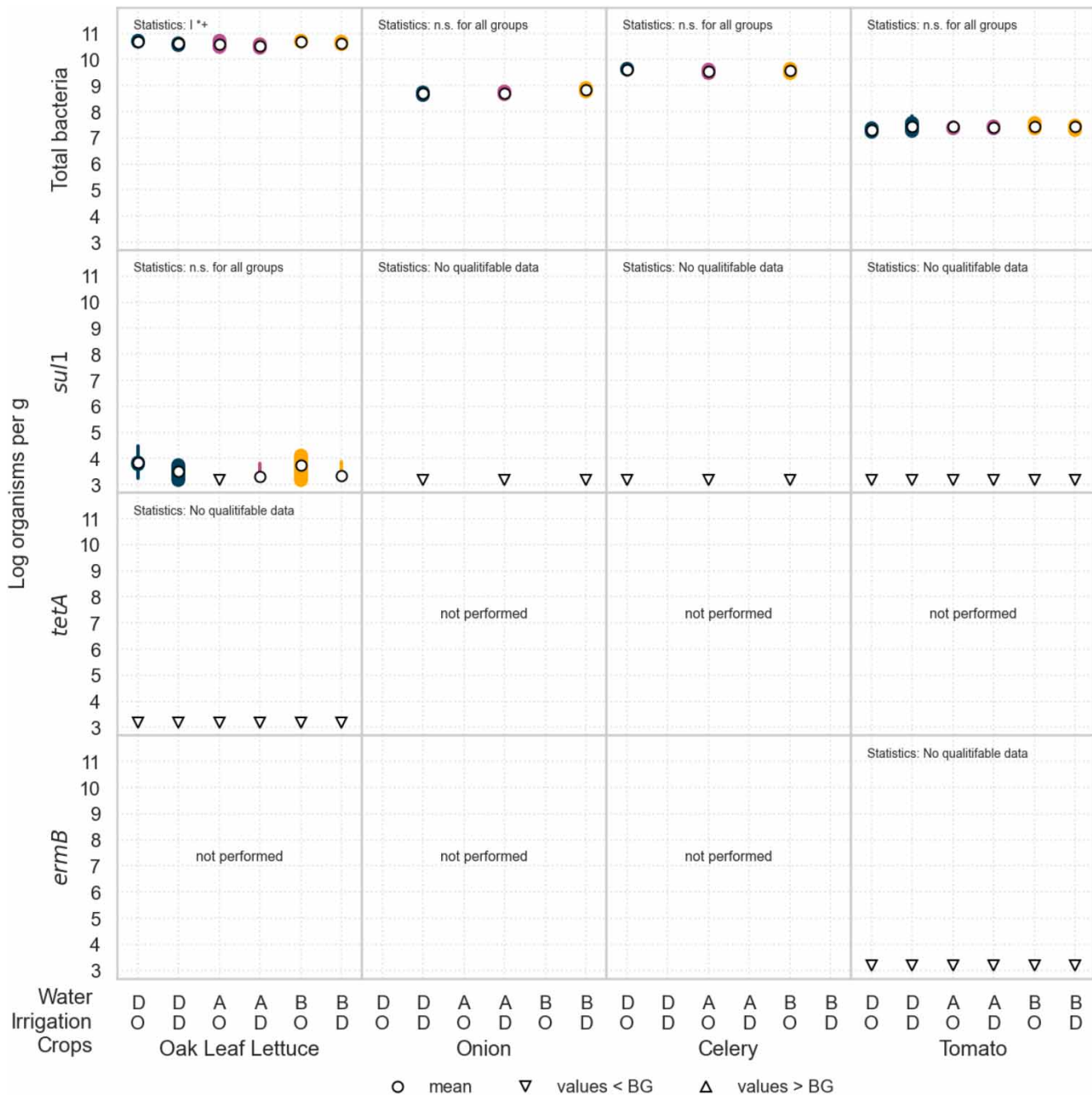


**Figure 7** | Mean (circles, or triangles in case of low detection rate), 95% confidence interval (bars) and detection rate of bacteria and the antibiotic resistance genes *ermB*, *sul1*, *tetA* and *bla<sub>TEM</sub>* in irrigation water from Train A and Train B, and WWTP influent.

of elevated copy numbers of multiple ARGs in various irrigation waters, particularly in Train A, it was imperative to undertake a comprehensive investigation of these ARGs within and on the irrigated plants.

### 3.4.2. Plants

Plant samples were analyzed for selected molecular markers, including total bacterial load (16S rRNA gene copies), and ARGs (Figure 8). Total bacterial counts were high across all crops, ranging from  $10^8$  (tomatoes) to  $10^{11}$  (oak leaf lettuce)



**Figure 8** | Results from qPCR analysis for different crops per gram irrigated with drinking water (D) or water from train A (A) or B (B) via drip (D) or overhead (O) irrigation. Symbols show mean values (circles or triangles in case of low detection rate), bars the 25–75% and 2.5–97.5% iiles. Statistical tests are displayed above the plots, if significant ( $p < 0.05$ ) for the parameter irrigation (I), water (W), both parameters combined (W\*I) or all groups separately (all). Symbols indicate significance for ranked ANOVA (\*) and Kruskal–Walis-test (+).

gene copies per gram. Bacterial concentrations were generally similar across different irrigation methods and water types, with only minor differences observed in oak leaf lettuce.

Surprisingly, ARGs were almost entirely undetectable in crop samples. Only *sul1* was detected in a few oak leaf lettuce samples, with slightly higher copy numbers observed in crops irrigated with drinking water and Train B reclaimed water. The generally low detection rate raised concerns about potential methodological issues. However, positive controls using extracted crop samples spiked with DNA from wastewater successfully detected all target genes, confirming that the qPCR results were reliable.

The absence of *sul1* and *tetA* genes in most crop samples suggests that these parameters did not persist on plant surfaces or within plant tissues at detectable levels, even when irrigation water contained high concentrations. The few positive detections of *sul1* in oak leaf lettuce may be explained by differences in leaf morphology affecting microbial retention – also reflected in the high total bacterial load (16S rRNA gene numbers) in this crop.

These findings suggest that ARGs from irrigation water do not readily accumulate on edible plants and may be subject to rapid degradation or removal. These findings align with studies indicating that even low-quality irrigation water containing high ARG loads does not necessarily lead to increased ARG accumulation in plants when compared to clean irrigation water sources (Marano *et al.* 2019; Shamsizadeh *et al.* 2021). Conversely, some studies have reported slightly elevated ARG levels in crop (Cerqueira *et al.* 2019), with soil type and cropping practices playing a role (Liu *et al.* 2022b). The detected ARGs in crop samples showed no significant differences between irrigation water qualities or irrigation types, suggesting that their presence is more likely influenced by cross-contamination and environmental sources rather than water quality.

### 3.5. Comparative assessment of treatment trains and process contributions

Our study clearly shows that Train B's advanced treatment processes achieved significantly greater log reductions of both microbial contaminants and ARGs compared to Train A. Importantly, Train B was specifically engineered to deliver exceptionally high water quality, including the removal of trace organic chemicals (Ahmadi *et al.* 2025). The different treatment steps of both trains, as well as their respective contributions to water quality, are summarized in Table 4.

Despite the advanced treatment applied in Train B, microbial loads on irrigated crops were statistically similar across all water sources. This suggests that environmental factors – such as soil contact, atmospheric deposition, and handling practices – play a dominant role in shaping microbial communities on produce. Additionally, the scalable nature of ozonation and UV disinfection enables dose adjustments to meet more stringent water quality targets, offering flexibility for future applications. In summary, while the enhanced performance of Train B significantly improves irrigation water quality, the final impact on crop microbiota is strongly influenced by post-treatment environmental interactions.

**Table 4** | Overview of treatment steps in train A and train B, their main targets, and contributions to water quality

Treatment step	Train A	Train B	Main targets	Contribution to water quality
UF (pore size: 0.03 µm)	✗	✓	Pathogens particles ARGs	Removes most microbial targets below detection limits
Filtration (pore size: 10 µm)	✓	✗	Particles	Removal of larger particles and particle-bound microorganisms
Ozonation	✗	✓	Pathogens ARGs Trace organic chemicals	Adjustable; removes pathogens (Ho <i>et al.</i> 2024) and micropollutants (Ahmadi <i>et al.</i> 2025)
BAC filtration	✗	✓	Trace organic chemicals	No pathogen removal; microbial regrowth possible (Ho <i>et al.</i> 2024); barrier for micropollutants (Ahmadi <i>et al.</i> 2025)
UV irradiation	✓	✓	Pathogens ARGs (partially)	Adjustable; The inactivation of ARGs requires higher doses than for pathogens (McKinney & Pruden 2012; Stange <i>et al.</i> 2019)

## 4. CONCLUSIONS

This study demonstrates that while advanced wastewater treatment significantly reduces bacterial loads and ARG concentrations, complete elimination is not always achieved. Train B, which included UF, ozonation, BAC filtration, and UV treatment, was particularly effective in reducing total bacterial loads and certain ARGs. However, the persistence of *sulI* and *tetA* in irrigation water suggests that even advanced treatment methods may not fully eliminate all potential risks.

Despite differences in irrigation water quality and application methods, our findings indicate no significant differences in bacterial communities on crops irrigated with drinking water compared to those irrigated with reclaimed water from Train A and Train B. This contrasts with other studies where irrigation water quality has been shown to influence crop-associated microbiota. The absence of coliforms and detectable ARGs on crop surfaces suggests that both reclaimed water sources in this study were of sufficient microbiological quality to mitigate major food safety concerns.

The long-term fate of ARGs in the soil after repeated irrigation remains largely unknown. Future research should focus on understanding their persistence, potential accumulation, and the risks associated with their integration into soil microbial communities.

Water reuse is a crucial strategy for sustainable agriculture, but its safety must be continually assessed. Strengthening regulations, optimizing treatment technologies, and implementing long-term monitoring programs will be essential to ensure that reclaimed water can be safely used without contributing to the spread of antimicrobial resistance.

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## DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

## CONFLICT OF INTEREST

The authors declare there is no conflict.

## REFERENCES

- Ahmadi, J., Hübner, U., Aumeier, B. M., Zumkeller, F. & Drewes, J. E. (2025) Risk-based chemical contaminant framework to comply with the EU nonpotable reuse regulation, *ACS ES&T Water*, 643.
- Alcalde-Sanz, L. & Gawlik, B. M. (2014) *Water Reuse in Europe: Relevant Guidelines, Needs for and Barriers to Innovation*. Luxembourg: Publications Office.
- Beneduce, L., Gatta, G., Bevilacqua, A., Libutti, A., Tarantino, E., Bellucci, M., Troiano, E. & Spano, G. (2017) Impact of the reusing of food manufacturing wastewater for irrigation in a closed system on the microbiological quality of the food crops, *International Journal of Food Microbiology*, 260, 51–58.
- Bernard, H., Faber, M., Wilking, H., Haller, S., Höhle, M., Schielke, A., Ducomble, T., Siffczyk, C., Merbecks, S. S., Fricke, G., Hamouda, O., Stark, K., & Werber, D. (2014) Large multistate outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012, *EuroSurveillance*, 19 (8), 20719. Berlin: Robert Koch-Institut.
- Bibbal, D., Dupouy, V., Ferré, J. P., Toutain, P. L., Fayet, O., Prère, M. F. & Bousquet-Mélou, A. (2007) Impact of three ampicillin dosage regimens on selection of ampicillin resistance in Enterobacteriaceae and excretion of blaTEM genes in swine feces, *Applied and Environmental Microbiology*, 73 (15), 4785–4790.
- Brás, T. A., Seixas, J., Carvalhais, N. & Jägermeyr, J. (2021) Severity of drought and heatwave crop losses tripled over the last five decades in Europe, *Environmental Research Letters*, 16 (6), 65012.
- Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R. & Harwood, V. J. (2012) Enterococci in the environment, *Microbiology and Molecular Biology Reviews: MMBR*, 76 (4), 685–706.

- Cerqueira, F., Matamoros, V., Bayona, J. & Piña, B. (2019) Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial *Lycopersicon esculentum* fields under different agricultural practices, *Science of The Total Environment*, **652**, 660–670.
- Ceuppens, S., Johannessen, G. S., Allende, A., Tondo, E. C., El-Tahan, F., Sampers, I., Jacxsens, L. & Uyttendaele, M. (2015) Risk factors for *Salmonella*, Shiga toxin-producing *Escherichia coli* and *Campylobacter* occurrence in primary production of leafy greens and strawberries, *International Journal of Environmental Research and Public Health*, **12** (8), 9809–9831.
- Compant, S., Samad, A., Faist, H. & Sessitsch, A. (2019) A review on the plant microbiome: ecology, functions, and emerging trends in microbial application, *Journal of Advanced Research*, **19**, 29–37.
- European Parliament and Council of the European Union (2020) *Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020 on Minimum Requirements for Water Reuse*. Official Journal of the European Union. L 177, 5 June 2020, Brussels. Available at: <https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32020R0741>.
- Fernandes, L. S., Galvão, A., Santos, R. & Monteiro, S. (2023) Impact of water reuse on agricultural practices and human health, *Environmental Research*, **216** (Pt 3), 114762.
- Gevers, D., Danielsen, M., Huys, G. & Swings, J. (2003) Molecular characterization of tet(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage, *Applied and Environmental Microbiology*, **69** (2), 1270–1275.
- Gholipour, S., Nikaeen, M., Mehdipour, M., Mohammadi, F. & Rabbani, D. (2024) Occurrence of chlorine-resistant *Pseudomonas aeruginosa* in hospital water systems: threat of waterborne infections for patients, *Antimicrobial Resistance and Infection Control*, **13** (1), 111.
- Ho, J., Ahmadi, J., Schweikart, C., Hübner, U., Schwaller, C., Tiehm, A. & Drewes, J. E. (2024) Assuring reclaimed water quality using a multi-barrier treatment train according to the new EU non-potable water reuse regulation, *Water Research*, **267**, 122429.
- Intriago, J. C., López-Gálvez, F., Allende, A., Vivaldi, G. A., Camposeo, S., Nicolás Nicolás, E., Alarcón, J. J. & Pedrero Salcedo, F. (2018) Agricultural reuse of municipal wastewater through an integral water reclamation management, *Journal of Environmental Management*, **213**, 135–141.
- Jones, E. R., van Vliet, M. T. H., Qadir, M. & Bierkens, M. F. P. (2021) Country-level and gridded estimates of wastewater production, collection, treatment and reuse, *Earth System Science Data*, **13** (2), 237–254.
- King, L. A., Nogareda, F., Weill, F.-X., Mariani-Kurkdjian, P., Loukiadis, E., Gault, G., Jourdan-DaSilva, N., Bingen, E., Macé, M., Thevenot, D., Ong, N., Castor, C., Noël, H., van Cauteren, D., Charron, M., Vaillant, V., Aldabe, B., Goulet, V., Delmas, G., Couturier, E., Le Strat, Y., Combe, C., Delmas, Y., Terrier, F., Vendrely, B., Rolland, P. & Valk, H. d. (2012) Outbreak of Shiga toxin-producing *Escherichia coli* O104:H4 associated with organic fenugreek sprouts, France, June 2011, *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, **54** (11), 1588–1594.
- Kumar, K., Gupta, S. C., Baidoo, S. K., Chander, Y. & Rosen, C. J. (2005) Antibiotic uptake by plants from soil fertilized with animal manure, *Journal of Environmental Quality*, **34** (6), 2082–2085.
- LAWA (2022) *Endbericht der LAWA-Ad hoc AG/KG Water Reuse an die 163*. LAWAVollversammlung. Bund/Länder-Arbeitsgemeinschaft Wasser (LAWA), Dessau-Roßlau.
- Leclerc, H., Mossel, D. A., Edberg, S. C. & Struijk, C. B. (2001) Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety, *Annual Review of Microbiology*, **55**, 201–234.
- Leister, C., Reiner, J. E., Griefmeier, V., Gescher, J. & Hügler, M. (2023) Gastropods as a source for fecal indicator bacteria in drinking water, *Water Research*, **244**, 120494.
- Liu, X., Liu, W., Tang, Q., Liu, B., Wada, Y. & Yang, H. (2022a) Global agricultural water scarcity assessment incorporating blue and green water availability under future climate change, *Earth's Future*, **10** (4), 224.
- Liu, Y., Neal, A. L., Zhang, X., Fan, H., Liu, H. & Li, Z. (2022b) Cropping system exerts stronger influence on antibiotic resistance gene assemblages in greenhouse soils than reclaimed wastewater irrigation, *Journal of Hazardous Materials*, **425**, 128046.
- Mañas, P., Castro, E., Heras, L. & de, J. (2009) Irrigation with treated wastewater: effects on soil, lettuce (*Lactuca sativa* L.) crop and dynamics of microorganisms, *Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering*, **44** (12), 1261–1273.
- Marano, R. B. M., Zolti, A., Jurkevitch, E. & Cytryn, E. (2019) Antibiotic resistance and class 1 integron gene dynamics along effluent, reclaimed wastewater irrigated soil, crop continua: elucidating potential risks and ecological constraints, *Water Research*, **164**, 114906.
- McKinney, C. W. & Pruden, A. (2012) Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater, *Environmental Science & Technology*, **46** (24), 13393–13400.
- MP Biomedicals. (2021) FastDNA™ SPIN Kit for Soil: detailed protocol insert. Santa Ana, CA: MP Biomedicals, LLC. Available at: [https://www.mpbio.com/media/document/file/protocol/dest/f/a/s/t/d/FastDNA\\_SPIN\\_Kit\\_for\\_Soil\\_Detailed\\_Protocol\\_Insert\\_2021\\_WEB.pdf](https://www.mpbio.com/media/document/file/protocol/dest/f/a/s/t/d/FastDNA_SPIN_Kit_for_Soil_Detailed_Protocol_Insert_2021_WEB.pdf) (Accessed 25 February 2025).
- Muyzer, G., Teske, A., Wirsén, C. O. & Jannasch, H. W. (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments, *Archives of Microbiology*, **164** (3), 165–172.
- Naumann, G., Cammalleri, C., Mentaschi, L. & Feyen, L. (2021) Increased economic drought impacts in Europe with anthropogenic warming, *Nature Climate Change*, **11** (6), 485–491.
- Oliveira, M., Usall, J., Vifiñas, I., Solsona, C. & Abadías, M. (2011) Transfer of *Listeria innocua* from contaminated compost and irrigation water to lettuce leaves, *Food Microbiology*, **28** (3), 590–596.

- Pei, R., Kim, S.-C., Carlson, K. H. & Pruden, A. (2006) Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG), *Water Research*, **40** (12), 2427–2435.
- Poppe, L., Vanhoutte, S. & Höfte, M. (2003) Modes of action of *Pantoea agglomerans* CPA-2, an antagonist of postharvest pathogens on fruits, *European Journal of Plant Pathology*, **109** (9), 963–973.
- Purnell, S., Ebdon, J., Buck, A., Tupper, M. & Taylor, H. (2016) Removal of phages and viral pathogens in a full-scale MBR: implications for wastewater reuse and potable water, *Water Research*, **100**, 20–27.
- Rakovec, O., Samaniego, L., Hari, V., Markonis, Y., Moravec, V., Thober, S., Hanel, M. & Kumar, R. (2022) The 2018–2020 multi-year drought sets a New benchmark in Europe, *Earth's Future*, **10** (3), D05109.
- Reitter, C., Neuhaus, K. & Hügler, M. (2021) Draft genome sequences of *Enterobacter* spp., *Lelliottia* spp., and *Serratia* spp., Coliform bacteria from drinking water reservoirs and lakes, *Microbiology Resource Announcements*, **10** (32), e0062221.
- Sauter, D., Stange, C., Schumacher, V., Tiehm, A., Gnirss, R. & Wintgens, T. (2021) Impact of ozonation and biological post-treatment of municipal wastewater on microbiological quality parameters, *Environmental Science: Water Research & Technology*, **7** (9), 1643–1656.
- Shamsizadeh, Z., Ehrampoush, M. H., Nikaeen, M., Farzaneh, M., Mokhtari, M., Gwenzi, W. & Khanahmad, H. (2021) Antibiotic resistance and class 1 integron genes distribution in irrigation water-soil-crop continuum as a function of irrigation water sources, *Environmental Pollution (Barking, Essex : 1987)*, **289**, 117930.
- Spain. Royal Decree 1620/2007 (2007) Spanish Regulations for Water Reuse. English translation of Real Decreto 1620/2007, State Official Journal (Boletín Oficial del Estado, BOE No. 294, 8 December 2007). Madrid: Boletín Oficial del Estado. Available at: <https://www.asersagua.es/Asersa/Documentos/Spanish%20Regulations%20for%20Water%20Reuse%20EN.pdf> (Accessed 25 February 2025).
- Stange, C., Sidhu, J. P. S., Tiehm, A. & Toze, S. (2016) Antibiotic resistance and virulence genes in coliform water isolates, *International Journal of Hygiene and Environmental Health*, **219** (8), 823–831.
- Stange, C., Sidhu, J. P. S., Toze, S. & Tiehm, A. (2019) Comparative removal of antibiotic resistance genes during chlorination, ozonation, and UV treatment, *International Journal of Hygiene and Environmental Health*, **222** (3), 541–548.
- Stanton, T. B. & Humphrey, S. B. (2003) Isolation of tetracycline-resistant *Megasphaera elsdenii* strains with novel mosaic gene combinations of tet(O) and tet(W) from swine, *Applied and Environmental Microbiology*, **69** (7), 3874–3882.
- Strawn, L. K., Gröhn, Y. T., Warchocki, S., Worobo, R. W., Bihn, E. A. & Wiedmann, M. (2013) Risk factors associated with *Salmonella* and *Listeria monocytogenes* contamination of produce fields, *Applied and Environmental Microbiology*, **79** (24), 7618–7627.
- Suzuki, Y., Niina, K., Matsuwaki, T., Nukazawa, K. & Iguchi, A. (2018) Bacterial flora analysis of coliforms in sewage, river water, and ground water using MALDI-TOF mass spectrometry, *Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering*, **53** (2), 160–173.
- Tresch, S., Biggel, M., Schnyder, M., Nüesch-Inderbilen, M. & Stephan, R. (2024) Extended-spectrum  $\beta$ -lactamase (ESBL)- and carbapenemase-producing enterobacterales isolated from fresh herbs and salads at retail level in Switzerland, *Journal of Food Protection*, **87** (11), 100368.
- Truchado, P., Garre, A., Gil, M. I., Simón-Andreu, P. J., Sánchez, G. & Allende, A. (2021) Monitoring of human enteric virus and coliphages throughout water reuse system of wastewater treatment plants to irrigation endpoint of leafy greens, *Science of The Total Environment*, **782**, 146837.
- Vleeschauwer, D. d. & Höfte, M. (2007) *Using Serratia Plymuthica to Control Fungal Pathogens of Plants*. CABI Reviews, Wallingford, UK: CABI Publishing.
- Wang, M., Sun, H. & Xu, Z. (2024) Characterization of rhizosphere microbial diversity and selection of plant-growth-promoting bacteria at the flowering and fruiting stages of rapeseed, *Plants (Basel, Switzerland)*, **13** (2), 329.
- Water Reuse Europe. (2024) Policies and regulatory tools in place at national level across the European Union. Brussels: Water Reuse Europe. Available at: <https://www.water-reuse-europe.org/about-water-reuse/policy-and-regulations> (Accessed 25 February 2025).
- Zhao, Y., Fan, L., Gao, S.-H., Huang, F., Lei, Z., Wang, W., Gao, R., Dai, Z., Li, Q., Liang, B. & Wang, A. (2024) Strain-level multidrug-resistant pathogenic bacteria in urban wastewater treatment plants: transmission, source tracking and evolution, *Water Research*, **267**, 122538.
- Zhuang, M., Achmon, Y., Cao, Y., Liang, X., Chen, L., Wang, H., Siame, B. A. & Leung, K. Y. (2021) Distribution of antibiotic resistance genes in the environment, *Environmental Pollution*, **285**, 117402.

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