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Fresh produce as a reservoir of antimicrobial resistance genes: A case study of Switzerland

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HIGHLIGHTS

- Fresh produce is a reservoir of ARB (even multidrug-resistant) and ARGs.
- Cephalosporin-resistant Enterobacterales and carbapenem-resistant *P. aeruginosa* were found.
- 95 % of the fresh produce samples contained ARGs.
- *sul1* and *intI1* genes could be good indicators for AMR in fresh produce.
- Plasmidome of fresh produce contains resistances to antibiotics and antiseptics.

GRAPHICAL ABSTRACT

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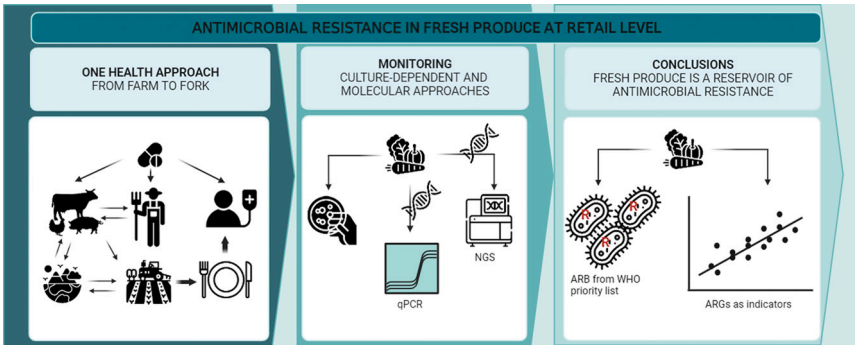

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ABSTRACT

Antimicrobial resistance (AMR) can be transferred to humans through food and fresh produce can be an ideal vector as it is often consumed raw or minimally processed. The production environment of fresh produce and the agricultural practices and regulations can vary substantially worldwide, and consequently, the contamination sources of AMR. In this study, 75 imported and 75 non-imported fresh produce samples purchased from Swiss retailers were tested for the presence of antimicrobial resistant bacteria (ARB) and antimicrobial resistance genes (ARGs). Moreover, the plasmidome of 4 selected samples was sequenced to have an insight on the diversity of mobile resistome. In total, 91 ARB were isolated from fresh produce, mainly cephalosporin-resistant Enterobacterales (n = 64) and carbapenem-resistant *P. aeruginosa* (n = 13). All *P. aeruginosa*, as well as 16 Enterobacterales' isolates were multidrug-resistant. No differences between imported and Swiss fresh produce were found regarding the number of ARB. In 95 % of samples at least one ARG was detected, being the most frequent *sul1*, bla_{TEM}, and *ermB*. Abundance of *sul1* and *intI1* correlated strongly with the total amount of ARGs, suggesting they could be good indicators for AMR in fresh produce. Furthermore, *sul1* correlated with the fecal marker *yccT*, indicating that fecal contamination could be one of the sources of AMR. The gene *sulI* was significantly higher in most imported samples, suggesting higher anthropogenic contamination in the food production chain of imported produce. The analyses of the plasmidome of coriander and carrot samples revealed the presence of several ARGs as well as genes conferring resistance to antiseptics and disinfectants in mobile genetic elements. Overall, this study demonstrated that fresh produce contributes to the dissemination of ARGs and ARB.

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1. Introduction

The rise in antimicrobial resistance (AMR) worldwide is one of the leading public health concerns of the 21st century. A recent report estimated that in 2019 a staggering 1.27 million deaths were directly attributable to bacterial AMR (Murray et al., 2022). In the past, studies focusing on AMR have been mostly in hospital and clinical settings, due to the direct effect of AMR to human health. This focus has been gradually shifting towards the One Health approach, which recognizes that human, animal, and environmental health are interconnected. Following this approach, it is indispensable consider foodstuff as vehicle in the spread of AMR from animals and the environment to humans (Robinson et al., 2016).

Fresh produce may pose a higher risk than other categories of foods in the transmission of foodborne pathogens and AMR as it is usually consumed raw or minimally processed. It may act as vehicle of dissemination of antimicrobial resistant bacteria (ARB) to the human gastrointestinal tract leading to direct infections, or they can contribute to horizontal gene transfer (HGT) of antimicrobial resistance genes (ARGs) to the gut microbiota (Kent et al., 2020). HGT may occur because ARGs are usually located in mobile genetic elements (MGEs) that promote intracellular DNA mobility (e.g., from the chromosome to a plasmid or between plasmids), as well as intercellular DNA mobility. Plasmids usually mediate the intercellular genetic exchange while insertion sequences (IS) and transposons (Tn) are able to move themselves (and their associated ARGs) within a single cell (Partridge et al., 2018). This means that, besides pathogens, all ARGs and MGEs have to be taken into account when studying the spread of AMR via the food chain. Moreover, because MGEs harbor genes conferring resistance to different antimicrobials, heavy metals and/or biocides (e.g. disinfectants), co-selection may occur, i.e. different compounds may indirectly select for resistance to unrelated antimicrobials or heavy metals and/or biocides (EFSA et al., 2021). In this regard, fresh produce could be a potential hotspot for AMR development and dissemination as it can be submitted to selection pressure exerted by a combination of antimicrobials and biocides throughout the food chain, starting in the farms and continuing in the industries (Oniciuc et al., 2019).

Since consumption of fruit and vegetables has increased during the last years following the recommendations of several health organizations such as the World Health Organization (WHO), their role as a vector of AMR to the consumer needs to be investigated (WHO, 2003). Hence, the European Food Safety Authority (EFSA) advises to continuously test bacteria for AMR during routine testing of fresh produce (EFSA et al., 2019). According to the WHO, fruits and vegetables are among the most readily available food groups with an annual per capita availability of 195 kg in the European Union and 177 kg in Switzerland (WHO, 2022), where this study was carried out. Swiss regulations limit the import of the majority of fruit and vegetable varieties cultivated in the country (Swiss Federal Council, 2011) changing customs quota for each product throughout the year, depending on sales possibilities and domestic supplies. Therefore, fresh produce from Swiss origin is mostly sold during the warmest months of the year while produce is commonly imported in the coldest months when domestic agriculture is not able to cover all the demands of Swiss consumers. Overall, fruit and vegetables are the most commonly imported foodstuffs in Switzerland, representing over 2500 million CHF in the last year (Swiss Federal Statistical Office, 2023). Despite the recognized role of international travel in the dissemination of AMR, clearly exemplified by the worldwide spread of the NDM-1 carried by a *K. pneumoniae* from a Swedish patient coming back from New Delhi (Yong et al., 2009), there is still a gap of knowledge of how international trade in food products contributes to the global dissemination of AMR (George, 2017). As the antibiotic consumption and agricultural practices, like using reclaimed water for irrigation or soil amendment, are subjected to different regulations among the countries, the difference in the prevalence of AMR according to the origin of fresh produce should be evaluated. A systematic review assessing the prevalence of ARB in retail food between 1996 and 2016 covering the Swiss agriculture sector and relevant imported food highlighted that 2.8 % and 7.6 % of plant-foods from global or Swiss origin, respectively, harbored ARB (Jans et al., 2018). Another experimental study analyzing ready-to-eat prepacked salads and fruits from Swiss markets showed that 5 % of these products carried Extended-Spectrum-Beta-Lactamases (ESBL)-producing Enterobacteriaceae. Hence, the authors highlighted the importance of surveillance of ARB in fresh produce, particularly in imported produce, which may be a vehicle for the global dissemination of multidrug resistant (MDR) pathogens (Nüesch-Inderbinen et al., 2015). Similar to surveillance programs, these studies focused mainly on identifying AMR in foodborne pathogens and indicator organisms, overlooking the resistome from environmental organisms and opportunistic pathogens. Regarding ARGs, very few studies tackled their abundances in food samples and there is currently no reference data about the genes to be used for food AMR monitoring (Abramova et al., 2023).

The main goal of this project was to assess if fresh produce can serve as a vehicle of dissemination of AMR from the food chain to humans. Both culture-dependent and molecular techniques were applied to study the prevalence of ARB and ARGs in several fruits, herbs and vegetables. As the contamination of fresh produce could be partly through their contact with soil, irrigation water, or biosolids used as fertilizers, ARGs that have been suggested as good markers of AMR in the environment, such as *sul1* and *intI1* (Berendonk et al., 2015), were hereby evaluated to test their potential role as indicators in fresh produce. Special emphasis was laid on the difference between food items from Swiss agriculture and imported from other countries to improve the understanding of how international trade in fresh produce contributes to the global dissemination of AMR. Moreover, the plasmidome of selected samples was analyzed by high-throughput sequencing to have an insight on the diversity of the mobile resistome in fresh produce.

2. Material and methods

2.1. Sampling and experimental design

A total of 150 fresh produce samples were included in this study, 75 coming from Swiss agriculture and 75 imported to Switzerland from other countries. Each category of Swiss and imported consisted of 15 samples of tomatoes (representing fruit vegetables), 15 samples of carrots (as root vegetables), 15 samples of iceberg lettuce (as leafy vegetables), 15 samples of strawberries (as berries), and 15 samples of coriander (as herbs). These products were selected as they are usually consumed raw and not peeled, therefore representing a higher risk of AMR transfer to the consumers. They were purchased between November 2020 and October 2021 from two Swiss retail companies that account for 80 % of fresh produce sales, thus representing well the fresh produce market in the country. All the produce came from conventional agriculture (non-organic) to avoid the introduction of a new variable, and packaged products were preferred in order to avoid cross contaminations from customers and supermarket staff. After purchasing, the samples were stored for a maximum of 24 h at 4 °C before the analyses. Once in the lab, a rinse technique was used to recover bacteria from the fresh produce' surface. Hence, around 25 g of each sample was aseptically weighted in a sterile beaker, with the exception of tomatoes, where the samples weighed 70 g on average as they were heavier than the other products and we avoided to cut them into smaller pieces to prevent that the released acids could affect the viability of bacteria in the downstream analyses. Afterwards, 95 or 190 mL (depending on the volume of the sample) of 0.1 % peptone water (PW) were added until the sample was totally immersed. Then, the beakers were shaked 20 min at 150 rpm and sonicated for 5 min at 42 kHz using a Branson 5510 device (Branson Power Company, USA). The resulting eluate was used for microbial enrichment and to extract microbial DNA for further molecular analysis.

2.2. DNA extraction

Part of the PW eluate of each sample (between 35 and 100 mL, depending on the debris content of the eluate) was filtered through a 0.22 μm-pore size Isopore filters (Merck, Germany) to concentrate the bacterial load on the filter. Each filter was stored at −20 °C until DNA extraction. Total DNA was extracted from the filters using the DNeasy PowerSoil Kit (Qiagen, Germany) according to the manufacturer's instructions and stored at −20 °C for further downstream molecular analysis.

2.3. Isolation and phenotypic characterization of antibiotic resistant bacteria

A second portion of the bacterial eluate in PW was non-selectively enriched by incubation for 20 h at 30 °C and 150 rpm. In order to isolate ARB, 100 μL of each enrichment were plated onto the following selective agar plates: CHROMagar KPC, CHROMagar VRE, CHROMagar Acinetobacter MDR (CHROMagar, France), ESBL Brilliance Agar (Oxoid Ltd., United Kingdom), MacConkey No. 3 agar (Oxoid Ltd., United Kingdom) with 8 mg/L ciprofloxacin and Pseudomonas Cetrimide Agar (Merck, Germany) with 0.06 mg/L meropenem. These media were selected as they allow the growth of bacteria such as *Escherichia coli*/coliforms, *Pseudomonas* spp. and *Enterococcus* spp., usually related with the acquisition of resistance to antibiotics, as well as their role as indicators and reservoir bacteria (Schwaiger et al., 2011). Moreover, these media support the growth of the ARB mentioned in the Global Priority List of ARB made by the WHO (WHO, 2017). The WHO lists the carbapenem-resistant and ESBL-producing Enterobacteriaceae as critical. In this study we also included species belonging to the order of Enterobacterales, to gain a better picture of the reservoir of resistances that could be potentially transferred between phylogenetically related species (Redondo-Salvo et al., 2020). After an incubation of 48 h at 37 °C, a maximum of 5 colonies per selective plate were isolated, making sure the five colonies showed different morphologies in order to minimize duplicates of the same clone. The isolates were identified by MALDI biotyping as described previously (Gekenidis et al., 2014) using a microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with the MALDI biotyper RTC Software version 3.1. To further minimize identical clones, isolates with identical taxonomy, as identified by MALDI biotyping, were discarded if they were isolated from the same media. The antibiotic resistant phenotypic profile of the target isolates and the minimum inhibitory concentration (MIC) was determined by broth dilution using the MicroScan autoSCAN-4 Systems (Beckman Coulter, USA). Isolates belonging to Enterobacterales were tested against 31 antibiotics using the Neg MIC 44 Panels (Beckman Coulter, USA) and isolates classified as non-fermenters (*Pseudomonas aeruginosa*) were tested against 23 antibiotics using the Neg MIC NF 50 panels (Beckman Coulter, USA). If isolates from the same sample that were isolated on different selective media showed identical resistance profiles, these were considered duplicates and only one isolate was regarded. The MIC values were exported into R version 4.1.3 (R Core Team, 2023) and phenotypes were interpreted using the EUCAST guidelines in the AMR (for R) package (Berends et al., 2022). MDR isolates were determined according to the definition by the European Center for Disease Prevention and Control (ECDC) (Magiorakos et al., 2012). Briefly, the ECDC defines antimicrobial categories for different pathogenic bacteria, and a MDR organism is defined as an organism that is non-susceptible (shows either intermediate or resistant phenotype) to at least one antibiotic from 3 or more antibiotic categories.

2.4. Quantification of antibiotic resistance genes

Twelve gene targets associated to MGEs and conferring resistance to vancomycin (*vanA* and *vanB*), macrolides (*ermB*), beta-lactams (bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{CMY}, bla_{OXA_48}), fluoroquinolones (*qnrS*), sulfonamides (*sul1*), streptomycin (*aadA*), tetracyclines (*tetW*) were quantified by quantitative PCR (qPCR) using a magnetic induction cycler qPCR instrument (Bio Molecular Systems, Australia). The class 1 integron-integrase gene (*intI1*) was quantified as a proxy for anthropogenic pollution and horizontal gene transfer (Gillings et al., 2015; Stalder et al., 2014). In addition, the 16S rRNA gene was quantified and used for the normalization of ARG copy numbers. All these genes were amplified in duplicates using several multiplex qPCR assays, as detailed in table S1. Although all the primers and probes were previously described, different combinations of them were tested in silico and in vivo to develop several multiplex qPCRs (Table S1). Multiplex qPCR were only counted as valid when the efficiency to quantify each gene was between 90 and 110 %. Each qPCR reaction was performed in a total volume of 20 μL containing 10 μL of 2 × Kapa Probe Fast Master Mix (Kapa Biosystems, Wilmington, USA), 0.1 μM of each primer (forward/reverse), 0.05 μM of each probe, 2 μL of DNA template and PCR grade water to reach 20 μL. The cycle conditions were 1 cycle at 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s.

Standard curves were generated using known quantities of cloned target genes. Briefly, synthetic gBlocks® containing fragments of the selected ARGs (Integrated DNA Technologies, USA) were ligated into pCR2.1-TOPO cloning vectors (Invitrogen, USA) and transformed into *E. coli* competent cells. Plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen, Germany), and the concentration was determined using Qubit 2.0 Fluorometer (Life Technologies, USA). Copy number was then calculated as described previously (Ritalahti et al., 2006). Tenfold serial dilutions of plasmid DNA were amplified over ten orders of magnitude and in triplicate to generate a standard curve for each qPCR assay. The limit of quantification (LOQ) was determined as the copy number from the lowest dilution giving three positive results in the linearity range (Table S1). Moreover, plasmid dilutions with known quantities of target genes where spiked with DNA extractions from fresh produce' eluates to test the presence of qPCR inhibitors.

2.5. Determination of fecal contamination

The abundance of *E. coli* was determined as this species is a common indicator of fecal contamination. Total *E. coli* were quantified by a singlex qPCR, targeting the *yccT* gene, which is exclusive for this species and was previously used as indicator for fecal load in environmental samples (Heß et al., 2018). This qPCR assay was performed following the same protocol detailed in the previous section for ARGs, but in this case, the concentration of the primers and the probe were 0.4 and 0.2 μM, respectively, and the annealing temperature was 56 °C. Primers and probe sequences are shown in the supplemental material S1.

2.6. Statistical analysis

All statistical analyses were performed using R Statistics version 4.3.0 (R Core Team, 2023). The relative abundance of ARGs in this study was calculated as ((ARG copies) / (16S rRNA gene copies)) to normalize for different amounts of extracted bacterial DNA per sample. Six samples were excluded, as the 16S rRNA gene could not be quantified. To detect differences in the mean abundances of the quantified genes of imported versus Swiss food items, the quantities were pooled by origin, resulting in two unpaired groups for each sample type (i.e. imported carrots vs. Swiss carrots). As the data did not meet the assumption of normality, these groups were tested against each other by performing non-parametric non-paired Wilcoxon rank sum tests. In addition to comparing each quantified gene individually, certain genes were pooled before performing the statistical test for better comparison: all 12 ARGs were summed up for comparison of the overall ARG abundances, *vanA* and *vanB* were cumulated for comparison of vancomycin conferring resistance genes, and lastly, resistance genes conferring resistance to beta-lactams were cumulated (bla_{SHV}, $bla_{OXA\text{-}48}$, bla_{TEM}, $bla_{CTX\text{-}M}$, and bla_{CMY}).

Correlations were measured by calculation of the Pearson correlation coefficient *R* and corresponding *p*-values. The data was log10 transformed to meet assumptions of normality as well as homogeneity of variance. The correlation coefficients were interpreted as described previously as negligible ($R < 0.1$), weak (R between 0.1 and 0.39), moderate (R between 0.40 and 0.69), strong (R between 0.70 and 0.89) and very strong ($R \geq 0.90$) (Overholser and Sowinski, 2008). For all statistical testing significance level was set at $p < 0.05$. Barplot, boxplots and scatterplots were created in using the ggplot2 package (Wickham and Chang, 2016) circular Chord diagram was created using the circlize package (Gu et al., 2014).

2.7. Plasmidome analysis

2.7.1. Plasmid isolation

The categories of fresh produce that showed higher loads of ARGs, such as carrots and coriander, were selected to further study their plasmidome (i.e. the overall plasmid population of each sample) and thus determine the diversity of ARGs harbored in these MGEs. Bacterial fraction of 2 samples of coriander and 2 of carrots were eluted as described above. The eluate was then filtered through a 0.22 μm durapore filter (Merck, Germany) and submitted to both plasmid extraction and total DNA extraction. ARGs were quantified from total DNA by qPCR as described above. Plasmid extraction was based on the previous protocols (Anderson and McKay, 1983; Kothari et al., 2019), with some adjustments. In brief, the filter was placed in a 50 mL Falcon tube and immersed in 7.6 mL of 6.7 % sucrose-50 mM Tris-1 mM EDTA (pH = 8), warmed for 10 min to 37 °C with gentle inversion, followed by vortexing for 5 min with 0.1 mm disrupter beads (Qiagen, Germany), and finally 2 mL lysozyme (10 mg/mL in 25 mM Tris, pH = 8.0) were added. After a 5 min incubation at 37 °C, 1 mL of 0.25 M EDTA-50 mM Tris (pH = 8.0) and 550 μL sodium dodecyl sulfate (20 % [wt/vol] in 50 mM Tris-20 mM EDTA, pH = 8.0) were added and mixed immediately and then incubated for 10 min at 37 °C to complete lysis. The lysate was vortexed for 30 s at the highest setting, 550 μL of fresh 3.0 M NaOH was added and mixed by inversion. Then, 1 mL 2 M Tris-hydrochloride (pH = 7.0) was added and gently mixed for 3 min. All liquid was then transferred to a MaXtract High Density phase lock tube (Qiagen, Germany). Then, 14.5 mL of 25:24:1 phenol-chloroform-isoamyl alcohol was added and mixed, followed by a 5 min centrifugation at 1500 ×*g*. The upper phase was transferred to a fresh phase lock tube and again, 14.5 mL of phenol-chloroform-isoamyl alcohol was added and mixed, followed by a 5 min centrifugation at 1500 ×*g*. The upper phase was transferred into a 50 mL falcon tube and the same volume of isopropanol was added and stored overnight on ice at 4 °C. The falcon was then centrifuged for 15 min at 5000 ×*g*, the supernatant discarded, the pellet suspended in 1 mL 70 % Ethanol and transferred into a 2 mL Eppendorf tube. This was then centrifuged for 10 min at max. Speed, the supernatant was discarded and pellet was dried in a thermoblock at 30 °C. The dried pellet was then resuspended in 50 μL of 10 mM Tris-1 mM EDTA (pH = 7). Linear chromosomal DNA was digested as described previously (Brown Kav et al., 2013; Kothari et al., 2019) by incubation with Plasmid-safe DNAse (Epicentre, Madison, WI, USA) for 24 h at 37 °C. Digestion was confirmed by negative 16S rRNA qPCR and plasmid-safe DNAs was inactivated by incubation at 70 °C for 30 min. The plasmid DNA was then amplified with phi29 DNA polymerase (New England Biolabs, USA) as described previously (Kothari et al., 2019).

2.7.2. Sequencing and bioinformatics

The enriched plasmid DNA was used to generate Illumina libraries using the TruSeqv2 reagent kit (Illumina, San Diege, CA, USA). Paired-end sequencing was performed on an Illumina MiSeq v3 flow cell with 600 cycles (2 × 300) resulting in 56′006'738 raw reads for the 4 samples. Quality control and filtering of raw reads was performed using multiQC version 1.9 (Ewels et al., 2016). Filtered reads were assembled into contigs using MEGAHIT version 1.2.9 (Li et al., 2016), with a minimum contig length of 500 nt. Taxonomic composition of assemblies were determined by Kraken 2 (Wood et al., 2019). PlasFlow version 1.1 (Krawczyk et al., 2018) was used to determine plasmid-borne contigs. Open reading frames on plasmid-borne contigs were detected by orfipy version 0.0.4 (Singh and Wurtele, 2021). The DeepARG model and database version 1.0.2 (Arango-Argoty et al., 2018) were used to detect plasmid contigs containing ARGs. To gain further insight into the plasmidome, MGEs in the contigs generated by PlasFlow were identified using Mobile Element Finder 1.0.3 (Johansson et al., 2021) at the Center for Genomic Epidemiology (https://cge.food.dtu.dk/services/PlasmidFinder/). All sequencing raw data has been deposited in the NCBI database under the BioProject number PRJNA954576 with the sample accession numbers SAMN34152379 (Carrot A), SAMN34152380 (Carrot B), SAMN34152397 (Coriander A) and SAMN34152402 (Coriander B).

3. Results

3.1. Culture-dependent approach

The enrichments of the bacterial eluates were plated on selective media as described above. With this method, ARB belonging to Enterobacterales (n = 78) such as *Enterobacter* spp., *Morganella morganii*, *Klebsiella* sp. or *Citrobacter* sp., were isolated from 37 % of the samples (27 Swiss and 29 imported samples) and *P. aeruginosa* (n = 13) from approximately 5 % of the samples (5 imported and 2 local samples, Fig. 1). Resistance profiles of these isolates were determined by broth dilution and their phenotypes were interpreted according to the EUCAST guidelines. To facilitate the interpretation of the results, resistant and intermediate phenotypes were considered non-susceptible and sensitive phenotypes as susceptible to an antibiotic. From the 78 isolates belonging the order of Enterobacterales, 64 were non-susceptible to at least one cephalosporine antibiotic, from which 15 were also non-susceptible to at least one carbapenem antibiotic. Sixteen of the Enterobacterales isolates resulted to be MDR, being non-susceptible to at least one antibiotic from 3 antibiotic classes or more. They were isolated from all investigated fresh produce types, except for strawberries: 7 from carrots, 6 from coriander, 2 from lettuce, and 1 from tomatoes. From those MDR isolates, 10 were non-susceptible to at least one carbapenem antibiotic and 9 were non-susceptible to the 4th generation cephalosporin cefepime (Table 1). All 13 *P. aeruginosa* isolates were non-susceptible to the anti-pseudomonal carbapenem imipenem, and one isolate to meropenem. Furthermore, all *P. aeruginosa* isolates were classified as MDR. Only a selection of 5 MDR *P. aeruginosa* is shown in Table 1, as several isolates showed identical resistant profile. The number of ABR isolates was too low to calculate statistical difference between imported and Swiss fresh produce samples.

3.2. Molecular approach

Of the 150 processed samples, 6 samples were excluded as 16S rRNA gene was not detected (4 tomato and 2 strawberry samples). Overall only in 7 samples (4.86 %, 4 lettuce, 2 strawberries, and 1 tomato, all Swiss produce) none of the 12 ARGs could be detected at all, despite being positive for 16S rRNA gene. This means that 95 % of investigated samples harbored at least 1 of the 12 investigated ARGs. The most frequently detected ARGs were *sul1*, bla_{TEM}, and *ermB,* being detected in 66 %, 63 %, and 48 % of Swiss samples, and 84 %, 75 %, and 70 % of imported samples, respectively. The least detected ARGs in Swiss produce samples were bla_{SHV}, bla_{OXA_48}, and *aadA* (in 8 %, 14 %, and 17 % of samples, respectively), and in imported produce $bla_{CTX\text{-}M}$ (18 %), bla_{OXA_48} (25 %), and bla_{SHV} (27 %). Genes conferring resistance to vancomycin (*vanA* and *vanB*) were detected in 66 samples (45 %), 42 (57 %) from imported sources and 24 (33 %) from Swiss agriculture. Relative abundance of ARGs in carrots and coriander were in most cases lower than in other produce types, probably because their bacterial load (determined by absolute quantification of 16S rRNA genes) were

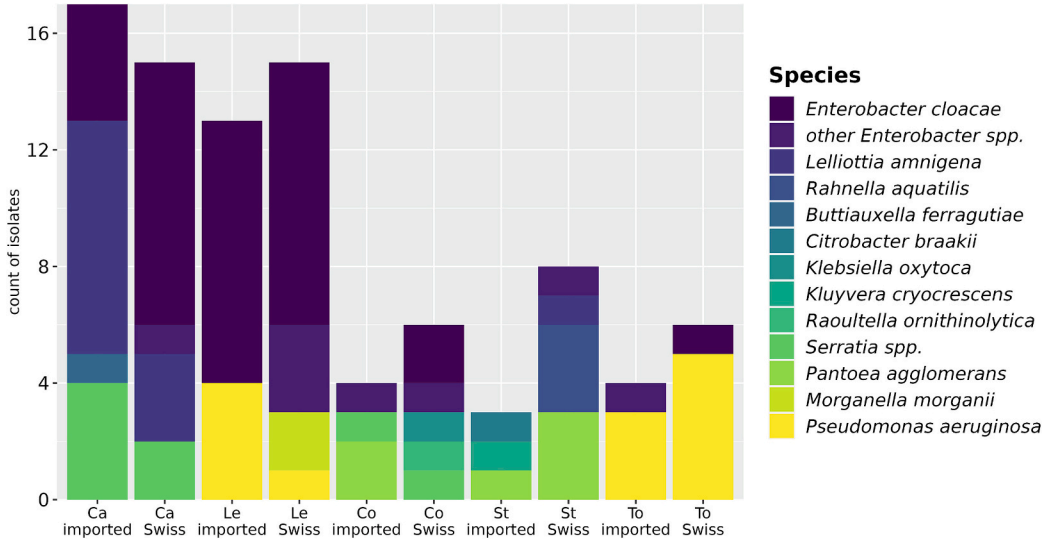


Fig. 1. Numbers and identification of ARB isolated from imported and Swiss produce. Ca: Carrots, Le: Lettuce, Co: Coriander, St: Strawberries, To: Tomatoes, imp: imported produce, CH: Swiss produce.

particularly high (Fig. S2). In fact, absolute quantification revealed that ARGs load for coriander and carrot samples were in a similar range as the other samples, between 10^4 and 10^6 gene copies per gram of sample.

Statistical difference of quantities of ARGs between local and imported fresh produce was determined by performing Wilcoxon rank-sum tests (Fig. 2 and supplemental material S3 for details). This showed that the mean of the cumulative relative abundance of the quantified ARGs only differed significantly between local and imported produce in coriander and in lettuce (both *p*-values <0.05). For both produce types quantities of ARGs were higher in imported samples. Similarly, the sum of the 5 quantified *bla* genes (bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{CMY}, bla_{OXA_48}), were significantly higher in imported coriander and lettuce. The gene *aadA*, despite being less prevalent, followed the same tendency and was significatively more detected in imported coriander, lettuce and strawberries. Quantification of the *sul1* gene was significantly higher in all imported produce, except for carrots. Quantification of *van* genes, *ermB*, *tetW*, and *qnrS* showed no difference between produce from imported and Swiss sources. To highlight the differences in the proportions of each ARG between imported and Swiss fresh produce, circular chord diagrams were constructed (Fig. 3). The difference in composition between Swiss and imported produce was particularly pronounced in *sul1*, which was found in all sample types but made up a much larger proportion in imported compared to Swiss produce. Overall imported produce samples showed a diversed and more complex network compared to Swiss produce.

In order to detect correlations between the target genes and therefore, to determine whether any of these genes could be used as indicator of the total load of ARGs in a sample, Pearson's correlation coefficients were calculated. The sum of the quantified ARGs correlated strongly with both the *sul1* ($R = 0.74$, $p < 0.05$) and *intI1* gene ($R = 0.84$, $p < 0.05$). Furthermore, *sul1* and *intI1* showed a moderate positive correlation among them ($R = 0.56$, $p < 0.05$) (Fig. S4). By correlating the relative quantities of the fecal indicator *yccT* and *sul1* ($R = 0.83$, $p < 0.05$), as well as *yccT* and *intI1* ($R = 0.62$, $p < 0.05$), a link between the quantities of ARGs and the fecal contamination of the samples was demonstrated (Fig. 4).

3.3. Plasmidome

In order to characterize the plasmidome of 4 selected fresh produce samples, plasmids were extracted, enriched and sequenced using short-read technology. Each sample yielded between 6.4 and 7.7 M raw reads that were quality filtered and assembled, resulting in 247′341, 141′954, 10′520, and 16′790 raw contigs (Table 2). From these contigs 38 %, 40 %, 34 %, and 41 % were identified by PlasFlow as being plasmid sequences, respectively. Unfortunately, the number of plasmid-borne contigs was not sufficient to reconstruct and circularize the plasmids, but they were filtered for ARG content and size (> 2 kb). DeepARG revealed that plasmid-borne contigs harbouring ARGs were higher in coriander samples than in carrot samples (Table 2). The content of ARGs of each plasmidome is detailed in Table S5. Characterization of these contigs using CGE's Mobile Element Finder showed that some of these ARGs were located in the same contig with several IS that could be responsible for their transferrability. For instance, in both carrot samples IS belonging to the clinically important IS6 family (ISS1D, ISS1N, ISS1A) were detected on the same contig as genes conferring resistance to macrolides. The sample coriander B presented the higher number of contigs carrying ARGs, which harbored 4 detectable MGEs as well. IS-3 family member ISPsy24 was detected on the same contig as genes conferring resistance to vancomycin, macrolides, and a multidrug exporter induced by gold salts. Furthermore, this sample contained sequences belonging to an unclassified plasmid previously isolated from *A. baumanii* harbouring tetracycline resistance genes, as well as a *S. aureus* plasmid replicon (Rep7a) that was also linked to tetracycline resistance genes. Lastly, another staphylococcal replicon (rep13) was identified on the same contig as a gene conferring resistance to macrolides and lincosamides. Furthermore, in 3 out of the 4 plasmidomes, genes conferring resistance to disinfectants and antiseptics were detected, such as the genes conferring resistance to quaternary ammonium compounds from the *qac* family. Comparing the results obtained by qPCR versus the plasmidome analyses, not all the ARGs detected by qPCR were identified in the plasmidome contigs (Table S5).

4. Discussion

Following the One Health approach that recognizes food as a vector for the spread of AMR from the environment to humans, this study aimed to evaluate fresh produce at retail level for the presence of ARB, ARGs, as well as MGEs. The culture-dependent approach revealed that resistant strains belonging to Enterobacteriaceae were isolated from almost a third of the samples, including numerous MDR bacteria resistant to carbapenems and/or 3rd and 4th generation cephalosporins. Comparing our results to Nüesch-Inderbinen et al. (2015), who studied

Table 1
Resistance phenotypes of MDR bacteria isolated from fresh produce. Minimum inhibitory concentrations to different antibiotics were determined by broth dilution, phenotypes were interpreted by applying EUCAST breakpoint tables and expert rules (S: sensitive, I: intermediate, and R: resistant). ** indicates intrinsic resistance of a species to an antibiotic. + indicates produce of Swiss origin. AMK: amikacin; GEN: gentamicin, NET: netilmycin, TOB: tobramycin, CHL: chloramphenicol, ATM: aztreonam, PIP: piperacillin, AMP: ampicillin, TCC: ticarcillin-clavulanic acid, TZP: piperacillin-tazobactam, CXM: cefuroxime, CAZ: ceftazidime, CTX: cefotaxime, FEP: cefepime, DOR: doripenem, ETP: ertapenem, IPM: imipenem, MEM: meropenem, CST: colistin, CIP: ciprofloxacin, LVX: levofloxacin, TET: tetracycline, TGC: tigecycline, MIN: minocycline, SXT: trimethoprim-sulfamethoxazole, FOF: fosfomycin. Antibiotic categories are indicated in parentheses: (a) aminoglycosides, (b) phenicols, (c) monobactams, (d1) penicillins (d2) antipseudomonal penicillins + β-lactamse inhibitors, (e) 1st and 2nd generation cephalosporins, (f) 3rd and 4th generation cephalosporins (extended spectrum), (g) carbapenems, (h) polymyxins, (i) quinolones, (j) tetracyclines, (k) folate pathway inhibitors, (l) phosphonic acids.

Sample	Species	Antibiotic resistant phenotypes																									
		AMK (a)	GEN (a)	NET (a)	TOB (a)	CHL (b)	ATM (c)	PIP (d1)	AMP (d1)	TCC (d2)	TZP (d2)	CXM (e)	CAZ (f)	CTX (f)	FEP (f)	DOR (g)	ETP (g)	IPM (g)	MEM (g)	CST (h)	CIP (i)	LVX (i)	TET (j)	TGC (j)	MIN (j)	SXT (k)	FOF (l)
Carrot	*Enterobacter cloacae*	S	S		S		R	R	**		S	**	R	R	S	S	R	S	S	S	S	S	S	S	S	S	R
Carrot+	*Enterobacter cloacae*	S	S		S		R	R	**		R	**	R	R	R	R	R	R	I	R		S	I	R	S	S	R
Coriander	*Enterobacter cloacae*	R	R		R		R	R	**		R	**	R	R	R	R	R	R	I	R	R	S	I	S	S	S	R
Coriander	*Enterobacter cloacae*	R	R		R		R	R	**		R	**	R	R	R	R	R	R	I		S	S	S	S	S	S	S
Coriander	*Enterobacter cloacae*	S	S		S		R	R	**		R	**	R	R	R	S	R	S	S	R	S	S	S	S	S	S	S
Lettuce	*Enterobacter ludwigii*	S	R		S		R	S	**		S	**	S		I	R	R	R	I	R	S	S	S	S	S	S	S
Coriander+	*Enterobacter asburiae*	S	S		S		R	S	**		S	**	R	R	I	S	R	S	S	S		S	S	S	S	R	S
Tomatoe	*Enterobacter kobei*	S	S		S	S	R	S	**		S	I	R		S	S	S	S	S	R	S	S	S	S	S	S	R
Carrot	*Lelliottia amnigena*	S	S		S		S	R	R		R	I	I	S	S	R	R	R	I	S	S	S	S	S	S	S	R
Carrot+	*Lelliottia amnigena*	S	S		S		S	R	R		S	R	I	I	S	S	S	S	S	S	S	S	I	S		S	R
Coriander+	*Morganella morganii*	S	S		S		I	**	**		S	**	R		S	S	S	I	S	**	S	S	R	S	R	S	R
Coriander+	*Morganella morganii*	S	S		S		S	**	**		S	**	I		S	S	S	I	S	**	S	S	R	S	R	S	S
Carrot	*Serratia fonticola*	S	S		S	S	R	R	R		S	R	I		R	S	S	S	S	R	S	S	S	S	S	S	S
Carrot	*Serratia fonticola*	S	S		S	S	R	R	R		S	R	S		I	S	S	S	S	R	S	S	S	S	S	S	S
Carrot+	*Serratia fonticola*	S	S		S	S	R	R	R		S	R	R		R	S	S	S	S	R	S	S	S	S	S	S	R
Lettuce	*Serratia odorifera*	S	S		S	R	S	S	R		S	R	I		S	S	S	S	S	R	S	S	S	S	S	S	S
Tomato	*Pseudomonas aeruginosa*	S	S	R	S	**	I	I	**	I	I	**	I	**	I		**	I	S	S	I	I					
Tomato	*Pseudomonas aeruginosa*	S	R	R	S	**	I	I	**	R	I	**	I	**	I		**	R	I	R	I	I					
Tomato+	*Pseudomonas aeruginosa*	S	S	I	S	**	I	I	**	I	I	**	I	**	I		**	I	S	R	I	I					
Coriander	*Pseudomonas aeruginosa*	S	S	S	S	**	I	I	**	I	I	**	I	**	I		**	I	S	S	I	I					
Coriander +	*Pseudomonas aeruginosa*	S	S	I	S	**	I	R	**	R	R	**	R	**	I		**	I	S	S	I	I					

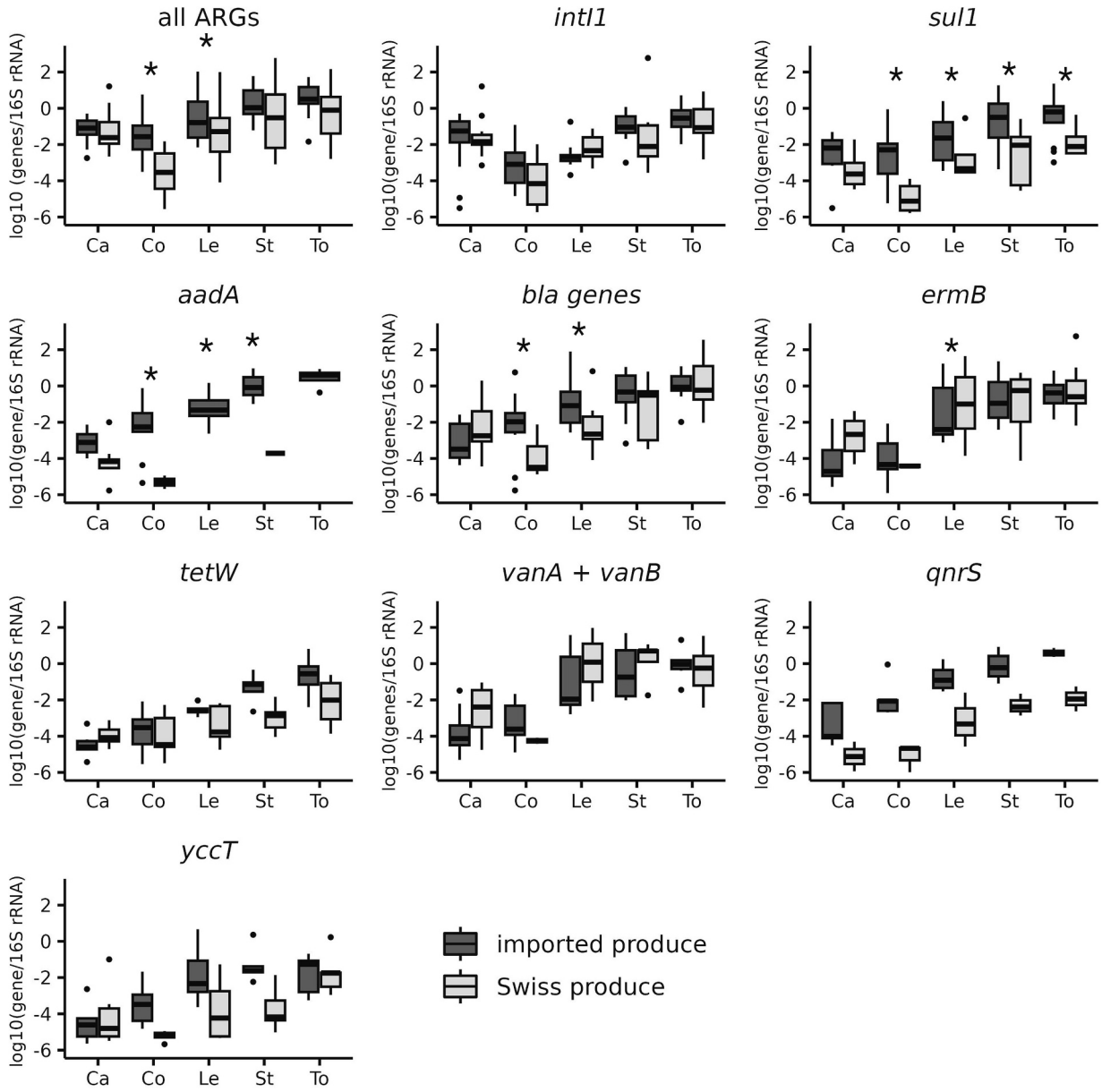


Fig. 2. Boxplots of relative abundances of quantified ARGs, *intI1* gene and *yccT* gene. Bars extending outside the box show the variability of the data, the bar across the box indicates the mean, and black dots represent outliers. Note that data is log10 transformed and 0 values were removed for visualization purposes only. Statistical difference between imported and local produce ($p < 0.05$) is indicated with an asterisk (*). Ca: carrots, Co: coriander, Le: lettuce, St: strawberries, To: tomatoes.

antibiotic resistant Enterobacteriaceae in ready-to-eat produce in Switzerland, we can add that not only these processed products can be a reservoir of ARB, but also non- or minimally processed fresh produce can harbor MDR opportunistic pathogens. In particular, 11 out of 29 MDR isolates from our study were obtained from coriander, supporting previous works stating that coriander can be heavily contaminated with ARB (Blau et al., 2018; Díaz-Gavidia et al., 2021; Reid et al., 2020). Fresh herbs undergo multiple processing steps and hence, are exposed to numerous sources of contamination that can introduce bacteria to the edible parts before reaching the store and ultimately the consumer. This microbial contamination could also explain their load of ARB and ARGs. It is also noteworthy that the opportunistic pathogen *P. aeruginosa* was isolated from roughly 5 % of fresh produce samples and all 13 isolates were non-susceptible to at least one carbapenem antibiotic. Carbapenem-resistant *P. aeruginosa* is listed by the WHO as health threat of critical priority (WHO, 2017) as it can cause severe infections, especially in cystic fibrosis patients, for which carbapenems are considered a last resort for treatment. From our results we cannot determine if ARB are more likely to be present in imported or non-imported produce, as the low numbers of isolates do not allow for statistical analysis. However, the presence of MDR and WHO-listed isolates in both imported and Swiss produce is unsettling and highlights the need to consider fresh produce as a player in the dissemination of AMR and MDR opportunistic pathogens in the future.

Quantifiable amounts of ARGs in 95 % of the samples stresses the importance of fresh produce as a reservoir of ARGs. When comparing imported and Swiss fresh produce we found, that overall, quantified ARGs were on average higher in all imported produce types, despite being only statistically significant in coriander and lettuce. The higher quantities of *bla* genes, *aadA,* and *sulI* in imported coriander and lettuce also support this finding. In fact, the quantities of *sul1*, conferring resistance to sulfonamides, were significantly higher in most imported

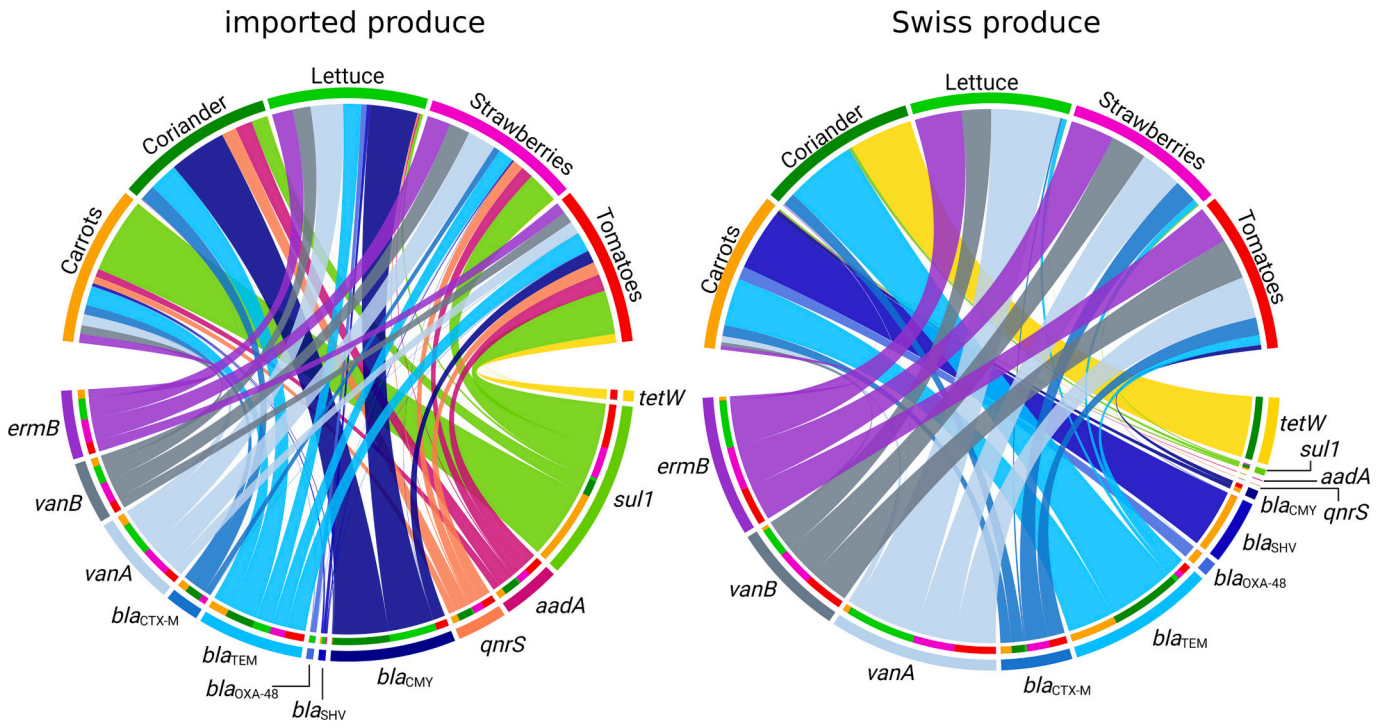


Fig. 3. Chord diagram showing the proportions of ARGs in the samples. Produce and ARGs are color-coded as indicated by the outer circle. The left diagram shows the composition of imported produce, the right diagram the composition of Swiss produce. The total amount of ARGs per produce are scaled to 100 %. The thickness of the connection from the sample to an ARG represents its relative abundance in that sample. The inner circles in the lower half of the diagrams represent the breakdown of that ARG into the produce types.

produce types. Sulfonamides were among the first antibiotics used to treat infections systemically and hence, were widely used in the clinic. Sulfonamide resistance spread quickly thereafter among gram-negative bacteria, due to often being located on plasmids. In particular, *sul1* is usually found in the Tn21 type integron linked to other ARGs (Sköld, 2000). This gene has been suggested as an indicator of anthropogenic contamination in waters (Koczura et al., 2016; Ying et al., 2018), soils, and sediments (Berendonk et al., 2015; Chen et al., 2013). Similarly to *sul1*, *intI1* has also been linked to human contamination and it has been proposed as a marker for anthropogenic impacts on environmental microbiomes (Gillings et al., 2015). This study demonstrated that *intI1* and *sul1* correlated positively in fresh produce, in agreement with the previous findings showing that sulfonamide genes are prevalent in class 1 integrons (Jiang et al., 2019). The abundances of *intI1* as well as *sul1* were positively correlated to the overall abundance of ARGs, suggesting that these genes might serve as an indicator for overall abundance of ARGs in fresh produce samples, just as it has been shown for the above mentioned other environments. Cerqueira et al. (2019) even interpreted the results of their study that *intI1* can serve as an indicator of the presence of MDR bacteria in agricultural fields.

Origins of imported products varied greatly during the seasons and the produce types and included Denmark, France, Israel, Italy, Morocco, Netherlands, Portugal, South Africa, and Spain. Therefore, despite all of them being grouped in the same category of "imported" in this study, there could be major differences between them regarding their agricultural practices and post-harvest treatments. As for contamination sources, soil, especially when treated with manure (Byrne-Bailey et al., 2011; Jechalke et al., 2014; Marti et al., 2013), and irrigation water (Díaz-Gavidia et al., 2021; Gekenidis et al., 2021; Piña et al., 2020) have been recognized as sources of ARB and ARG contamination in agricultural settings. In our study, the strong correlation between the *E. coli* specific fecal indicator *yccT* and *sul1*, and a moderate correlation between *yccT* and *intI1*, suggest that fecal contamination might play a considerable role as a source of ARGs in fresh produce. However, in some samples with quantifiable amounts of ARGs, *yccT* was not detected or below the LOQ, suggesting that ARG contamination does not stem from fecal contamination alone. Despite most of studies focusing on the pre-harvest dissemination of AMR, post-harvest cross-contamination might also pose a considerable risk for the spread of AMR. Post-harvest treatments, such as washing with sanitizing solutions are often applied to fresh produce to minimize the presence of human pathogens (Gurtler et al., 2022; López-Gálvez et al., 2009), although they also harbor a major risk of cross-contamination with pathogens (in detailed reviewed in Possas and Pérez-Rodríguez, 2023). Moreover, it has been suggested that post-harvest treatments could contribute in shaping the resistome in apples (Wassermann et al., 2022), so this might also be the case for other fresh produce. In fact, previous work demonstrated that the pressure exerted by biocides used in post-harvest treatments could promote the co-selection of ARGs (Pal et al., 2015). The presence of *qac* genes, for instance, which provide a selective advantage in the presence of quaternary ammonium compounds, might be linked to the use of disinfectants and biocides in the post-harvest food processing industry (Gerba, 2015). These genes are commonly carried by class 1 integrons and linked to other ARGs (Gaze et al., 2005; Gillings et al., 2015; Tezel and Pavlostathis, 2015). Our findings, that *qac* genes are located on the same contigs as other genes conferring resistance to antibiotics in the plasmidome of fresh produce, suggests that co-selection of biocide and antimicrobial resistances might play a role in these matrices.

The presence of ARGs alone without association to a viable pathogen host is not yet considered a direct public health risk. However, ARGs are now classified as emerging environmental contaminants (Pruden et al., 2006). The fact that many ARGs remain on fresh produce until the end of the supply chain, as demonstrated in this current study, is concerning no matter their source, since the spread of ARG among Enterobacteriaceae by HGT (Blau et al., 2018; Gekenidis et al., 2020) as well as direct uptake of extracellular DNA from the environment by bacteria (Dong et al., 2019) have both been documented. In addition, studies have also shown that nutrient load coming from sewage or manure may favor HGT

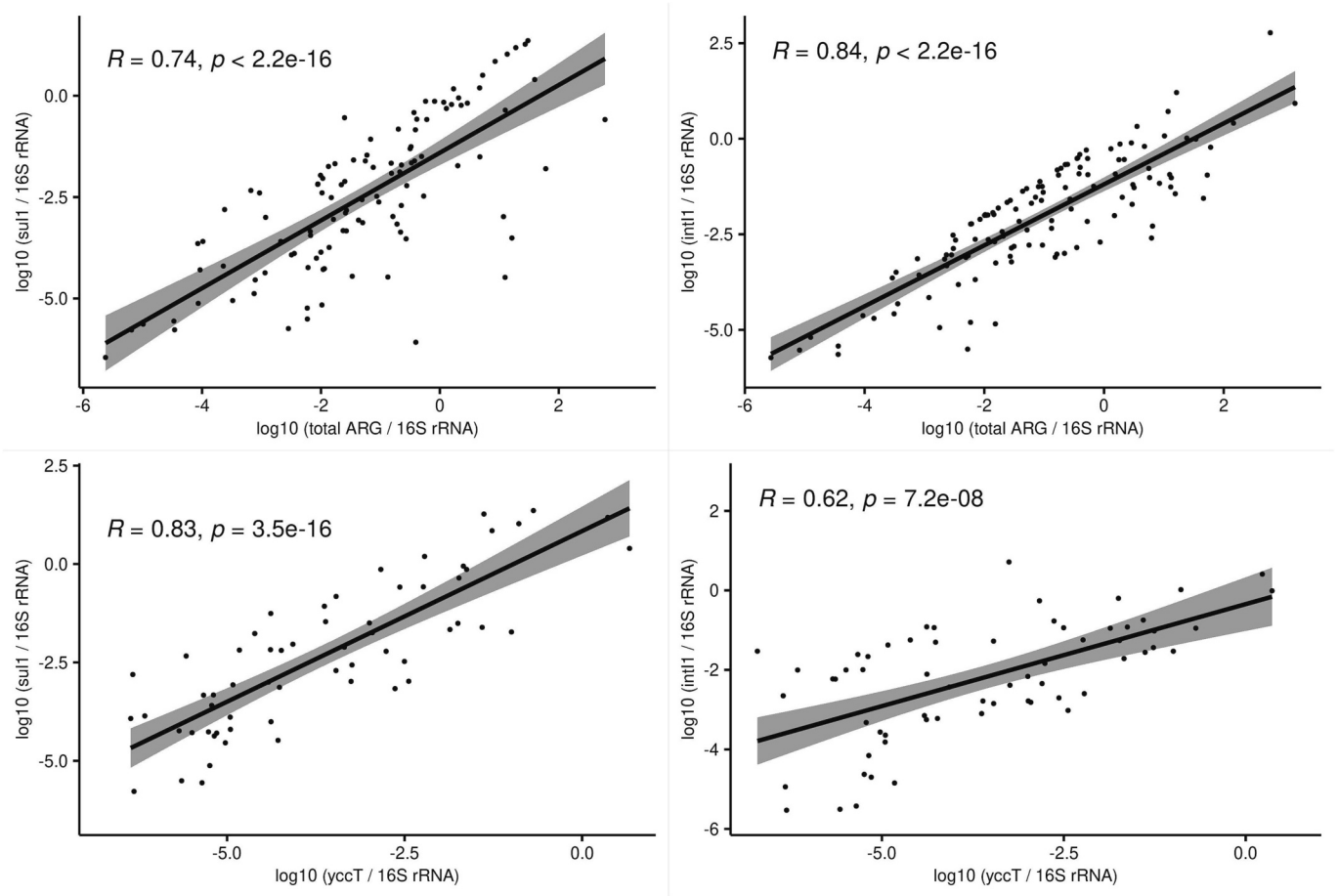
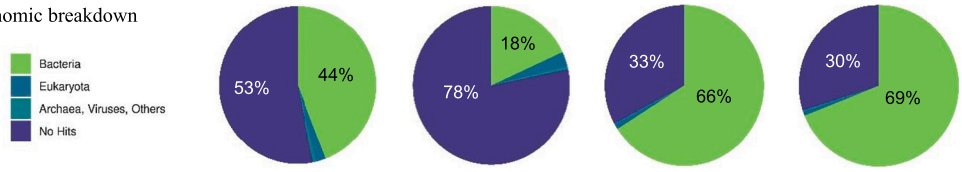


Fig. 4. Scatter plots showing positive correlations between quantified genes. Target gene copy numbers were normalized by the corresponding 16S rRNA copy number and log-transformed. R: Pearson's correlation coefficient.

Table 2
Summary of bioinformatic outputs after Illumina sequencing of 4 plasmidomes from fresh produce.

	Carrot A	Carrot B	Coriander A	Coriander B
Raw reads	6'480'302	6'456'064	7'384'028	7'682'975
Quality filtered reads	6'077'878	6'161'509	6'936'465	7'154'101
No. contigs	247'341	141'954	10'520	16'799
Taxonomic breakdown				
No. of contigs > 2kb	7'331	3'271	960	2'009
No. plasmid contigs	94'347	68'739	3'586	6'951
No. plasmid contigs > 2kb	1'978	1'420	194	627
Largest plasmid contig (nt)	31'469	6'621	77'474	19'446
No. plasmid contigs with ARG	38	12	11	31
No. plasmid contigs with ARG > 2kb	4	5	6	18

among microorganisms (van Elsas et al., 2003; Lima et al., 2020) and more recently, a study suggested that the vegetable surface itself can capture and enrich clinically relevant and mobile ARGs during the preharvest period (Keenum et al., 2022b). Under this aspect, it seems particularly important to study transferable ARGs in fresh produce, such the ones located in plasmids. Most reports characterizing plasmids carrying ARGs from fresh produce rely on the previous isolation of bacteria which does not represent the large diversity and composition of the produce-associated bacterial communities (Leff and Fierer, 2013). On the other hand, high-throughput sequencing techniques to study the plasmidome have been predominantly applied in matrices containing high bacterial abundances such as sludge (Sentchilo et al., 2013), soil (Luo et al., 2016), the gastrointestinal tract of animals (Brown Kav et al., 2013), or hospital wastewater (Hennequin et al., 2022). In low cell density environments these reports are few, and to our knowledge, this study is the first to study the plasmidome in fresh produce. In spite of technical challenges, mostly the generation of large quantities of non-plasmid data that did not allow the reconstruction of circular plasmids, it was demonstrated that fresh produce is a reservoir of MGEs containing diverse resistances to antibiotics and disinfectants. From our sequencing data a direct link between *intI1* and *qac* genes could not be established, however, the presence of *intI1* by qPCR did demonstrate the presence of this integron in fresh produce. In addition, it has been found that certain antibacterial biocides can stimulate HGT (Jutkina et al., 2018), making the post-harvest treatment of fresh produce a favorable environment for the selection, enrichment, and ultimately the spread of ARB and ARGs. Furthermore, agricultural soils can be heavily contaminated with heavy metals and other pollutants that can be a driver of co-selection of antibiotic resistances (Heydari et al., 2022; Seiler and Berendonk, 2012). This highlights the importance of taking mobile ARGs in fresh produce into consideration when studying the dissemination of AMR.

In summary, different methods were applied in this study to examine the role of retail-level fresh produce in the dissemination of AMR, contributing to gain knowledge about the identification of new type of environments relevant for AMR monitoring, as outlined by AMR experts in a recent report (Bengtsson-Palme et al., 2023). Our results are in accordance with the literature concluding that cultivation-based approaches remain the gold-standard in many diagnostics and monitoring applications, despite them not always being useful to isolate environmental bacteria due to their limited ability to grow under laboratory conditions (Franklin et al., 2021). As these overlooked cells may can still play a role in the spread of AMR, either directly by infection or indirectly through HGT of ARGs, it is crucial to include molecular techniques for surveillance of AMR. The high prevalence of certain ARGs in fresh produce samples in this study highlights the usefulness of applying qPCR-based techniques for monitoring purposes. Once established, qPCR is cost-effective, quicker than culture-based techniques, both highly specific and sensitive, and allows for quantification of the target genes. However, while there is a priority list for pathogenic ARB (WHO, 2017), ranking ARGs in terms of public health importance is more challenging due to their extremely high diversity (EFSA et al., 2021). Recently, qPCR has been embedded in a framework to standardize the quantification monitoring of ARGs in surface water, recycled water, and wastewater (Keenum et al., 2022a). Based on the results of our study, evaluation of the feasibility of such a framework for the monitoring of ARG in fresh produce should be taken into consideration in the future. Regarding the application of sequencing techniques, they are inevitable to gain insight into the genetic context of a broader diversity of ARGs located on MGEs. Despite the sequencing of the plasmidome was only applied to a limited number of samples and the plasmid extraction and enrichment protocol should be improved, this is the first report tackling the plasmidome of fresh produce. Similarly to other environmental studies, plasmidome analyses showed the potential of metagenomics to provide a higher coverage of the diversity of ARGs compared to qPCR (Ferreira et al., 2023). Overall, more research is needed to provide more data on the dissemination of ARB and ARGs through fresh produce and thus understand the magnitude of the associated public health risk.

CRediT authorship contribution statement

Anita Kläui: Software, Data curation, Formal analysis, Investigation, Resources, Visualization, Writing - original draft, Writing - review & editing. **Ueli Bütikofer:** Formal analysis, Writing – review & editing. **Javorka Naskova:** Investigation. **Elvira Wagner:** Investigation. **Elisabet Marti:** Conceptualization, Methodology, Validation, Investigation, Resources, Funding acquisition, Supervision, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.167671.

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