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Isolation of AmpC- and extended spectrum β-lactamase-producing *Enterobacterales* from fresh vegetables in the United States

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Abstract

Vegetables may serve as a reservoir for antibiotic resistant bacteria and resistance genes. AmpC β-lactamases and extended spectrum beta-lactamases (ESBL) inactivate commonly used β-lactam antibiotics, including penicillins and cephalosporins. In this study, we determined the prevalence of AmpC and ESBL-producing *Enterobacterales* in retail vegetables in the United States. A total of 88 vegetable samples were collected for the screening of AmpC and ESBL-producing *Enterobacterales* using CHROMagar ESBL agar. These vegetables included washed ready-to-eat salad (23), microgreens/sprouts (13), lettuce (11), herbs (11), spinach (5), mushrooms (5), brussels sprouts (4), kale (3), and other vegetable samples (13). AmpC and ESBL activity in these isolates were determined using double disk combination tests. Two vegetable samples (2.27%), organic basil and brussels sprouts, were positive for AmpC-producing *Enterobacterales* and eight

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samples (9.09%), including bean sprouts, organic parsley, organic baby spinach, and several mixed salads, were positive for ESBL-producing *Enterobacterales.* Whole genome sequencing was used to identify the bacterial species and resistance genes in these isolates. Genes encoding AmpC β-lactamases were found in *Enterobacter hormaechei* strains S43-1 and 74-2, which were consistent with AmpC production phenotypes. Multidrug-resistant *E. hormaechei* strains S11-1, S17-1, and S45-4 possess an ESBL gene, *bla*$_{SHV66}$, whereas five *Serratia fonticola* isolates contain genes encoding a minor ESBL, FONA-5. In addition, we used shotgun metagenomic sequencing approach to examine the microbiome and resistome profiles of three spinach samples. We found that *Pseudomonas* was the most prevalent bacteria genus in the spinach samples. Within the *Enterobacteriaceae* family, *Enterobacter* was the most abundant genus in the spinach samples. Moreover, antibiotic resistance genes encoding 12 major classes of antibiotics, including β-lactam antibiotics, aminoglycoside, macrolide, fluoroquinolone, and others, were found in these spinach samples. Therefore, vegetables can serve as an important vehicle for transmitting antibiotic resistance. The study highlights the need for antibiotic resistance surveillance in vegetable products.

Keywords

vegetables; *Enterobacterales* ; ESBL; AmpC; microbiome; resistome

1. Introduction

The acquisition and rapid dissemination of antibiotic resistance in human pathogens is a global public health issue. Multidrug-resistant ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens are the leading cause of nosocomial infections and represent a serious threat to human health globally (de Oliveira et al., 2020). *Enterobacterales* is a newly recognized taxonomic order divided into *Enterobacteriaceae, Yersiniaceae* and other five families (Adeolu et al., 2016; McAdam, 2020). Infections caused by extended spectrum beta-lactamases (ESBL)-producing *Enterobacterales,* which are difficult to treat, are considered serious threats to public health by US Centers for Disease Control and Prevention (CDC, 2019). AmpC and ESBL enzymes destroy commonly used β-lactam antibiotics including penicillins and cephalosporins, conferring antibiotic resistance to bacteria that encode and express these enzymes (Thomson, 2010). AmpC enzymes produced by *Enterobacterales* are clinically important β-lactamases, which are inducible in many bacteria and high-level expression can occur by mutation, leading to the development of resistance upon therapy and poor clinical outcomes (Jacoby, 2009). ESBL-producing *Enterobacterales* caused 197,400 cases of estimated infections and 9,100 estimated deaths in the United States in 2017 (CDC, 2019). ESBL-producing *Enterobacterales* can be spread to humans through contaminated water and food outside the United States, but the role of water and food in the spread of these antibiotic-resistant bacteria is not clear in the United States (CDC, 2019).

Food is an important vehicle for transmitting foodborne microorganisms (Pires et al., 2021; Scallan et al., 2011). Fresh vegetables can be contaminated by foodborne pathogens

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and serve as vehicles for the transmission of human pathogens (Berger et al., 2010). Food products harboring antibiotic resistant bacteria, including pathogens and commensal bacteria, may contribute to antibiotic resistance in the food supply chains (Bengtsson-Palme, 2017). Unlike meat and poultry products, vegetables are often eaten raw or after they are minimally processed, which may increase consumers' exposure to antibiotic-resistant bacteria, including *Enterobacterales.* Vegetables could play a significant role in the dissemination of antibiotic resistance because they can carry antibiotic-resistant commensal bacteria. For example, bean sprout samples (25%) in the Netherlands were contaminated with ESBL-producing *Enterobacteriaceae* (Huizinga et al., 2018). Zurfluh et al. found that 25% vegetable samples imported to Switzerland from Dominican Republic, India, Thailand, and Vietnam yielded ESBL-producing *Enterobacteriaceae* (Zurfluh et al., 2015). AmpC- and ESBL-producing *Enterobacterales* were found in retail spinach samples (Richter et al., 2020). In contrast, ESBL-producing *Escherichia coli* was found absent from 400 fruit and vegetable samples in the United Kingdom (Randall et al., 2017). Moreover, leafy vegetables can be a source of transferable antibiotic resistance genes in plasmids, which could be transferred to human pathogens (Blau et al., 2018). Currently, there is limited research examining antibiotic resistance in fresh vegetables in the United States. Therefore, in this study, we used the combination of resistance phenotype screening, and genomics and metagenomics approaches to assess antibiotic resistance in retail vegetables in the United States. We screened for AmpC and ESBL-producing *Enterobacterales* in retail vegetables and used whole genome sequencing and bioinformatics to characterize antibiotic resistant isolates. Spinach is a leafy vegetable that is commonly eaten raw and has been associated with foodborne outbreak or product recall due to microbial contamination (Grant et al., 2008). Functional screening of metagenomic DNA for spinach microbiota identified novel antimicrobial genes (Jacoby, 2009). We also chose spinach samples and used a metagenomics approach to determine their microbiome and resistome profiles.

2. Materials and Methods

2.1 Isolation of AmpC and ESBL-producing Enterobacterales

A total of 88 vegetable samples were purchased from seven grocery stores in 30-mile radius in central Arkansas in the United States between September-December 2020. Approximately 10 samples were collected in each shopping trip. We visited each store 1-3 times in the 3-month period depending on the variety of vegetables being sold in the stores. Individually packaged vegetable samples were placed in individual plastic bags and transported to the laboratory and subsequently stored in refrigerators (4 °C) and processed within 24 hr. The vegetables sampled in this study included washed ready-to-eat salad (23), microgreens/sprouts (13), lettuce (11), herbs (11), spinach (5), mushrooms (5), brussels sprouts (4), kale (3), and other vegetable samples (13) (Table S2). For each vegetable sample, 10 grams of sample was mixed with 90 ml of buffered peptone water (BPW, BD, Franklin Lakes, NJ) in a blender bag and processed in a stomacher at 300 rpm for 3 min to release bacterial cells from vegetable leaves. After 3-hr pre-enrichment in BPW at 37 °C and overnight selective enrichment in *Enterobacteriaceae* enrichment broth (BD) at 37 °C, each enriched culture was streaked on a CHROMagar ESBL agar plate (CHROMagar Microbiology, Paris, France) and incubated at 37 °C overnight for the screening of AmpC and ESBL-producing

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Enterobacterales (Richter et al., 2020). From each vegetable sample, up to four blue or pink colonies grown on CHROMagar ESBL agar were selected for AmpC and ESBL production assays. AmpC production was determined using the AmpC test kit (Liofilchem, Roseto, Italy) by evaluating the inhibition of AmpC activity by cloxacillin according to the manufacturer's instructions. Similarly, ESBL production was determined using double-disk combination test using antibiotic disks (BD) containing cefotaxime (CTX, 30 μg) with or without clavulanic acid (CLA, 10 μg) following standard procedures from Clinical and Laboratory Standards Institute (CLSI, 2020). Briefly, bacterial cell suspension was prepared to achieve a turbidity of 0.5 McFarland standard. A sterile cotton swab was used to inoculate the surface of Mueller Hinton Agar (BD). Cefotaxime disks with or without clavulanic acid were applied onto the surface of inoculated agar plate. After incubation at 37 °C for 18 hours, the size of inhibitory zones was measured. Clavulanic acid is an inhibitor of ESBL enzymes; a difference (5 mm) in the diameters of inhibition zone between CTX disk and CTX+CLA disk indicated the production of ESBL.

2.2 Antibiotic susceptibility profiling

Disk diffusion method was used to test the susceptibilities of AmpC and ESBL-producing isolates to 11 antibiotics, including ampicillin (AMP, 10 μg, Oxoid), amoxicillin/clavulanic acid (AMC, 30 μg, Oxoid), meropenem (MEM, 10 μg, BD), imipenem (IMI, 10 μg, BD), cefiderocol (FDC, 30 μg, Hardy Diagnostics), gentamicin (GEN, 10 μg, Oxoid), tetracycline (TET, 30 μg, Oxoid), chloramphenicol (CHL, 30 μg, Oxoid), ciprofloxacin (CIP, 5 μg, Oxoid), nalidixic acid (NAL, 30 μg, Oxoid), and trimethoprim/sulfamethoxazole (STX, 25 μg, Oxoid) following procedures from CLSI (CLSI, 2020). Cell suspension preparation, inoculation, and the testing conditions were described above in section 2.1. In addition, the susceptibility to polymyxin B was determined using broth microdilution method (CLSI, 2020).

2.3 Genetic characterization of resistant strains by whole genome sequencing

The genomes of 10 resistant isolates (Table 1) were sequenced and analyzed to determine potential antibiotic-resistance determinants. Genomic DNA was extracted using a Zymobiomics kit (Zymo Research, Irvine, CA) following the manufacturer's instructions (Zymo Research, 2021). The DNA sequencing library was prepared using NEBNext Ultra II DNA Library Prep kit (New England Biolabs, Ipswich, MA). DNA sequencing was carried out using Illumina NextSeq 500 with a mid-output kit (2 × 150 bp) (Illumina, San Diego, CA) at the Genomics Core Laboratory at University of Arkansas for Medical Sciences. Quality of pre-and post-processed paired end reads was assessed using FastQC v0.11.9 (de Sena Brandine & Smith, 2019). Once the quality of the raw reads was assessed, adapters were removed using fastp v.0.20.1 software using "detect_adapter_for_pe" option for paired-end reads (Chen et al., 2018). The high quality processed reads were examined using Kraken2 with standard database in order to perform bacterial species identification for each sample (Wood et al., 2019). *De novo* genome assembly was performed using SPAdes v.3.14.1 software with "error-correction" and "careful" options (k-mer sizes of 21, 33, 55, and 77, and a minimum contig size of 200 bp) (Bankevich et al., 2012). Antibiotic resistance genes of the final genome assemblies were identified using RGI (resistance gene identifier) v5.1.1 against CARD database v3.1.1 (Alcock et al., 2020). Multilocus sequence

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typing (MLST) was performed using PubMLST database (https://pubmlst.org/) and mist software (https://github.com/tseemann/mlst) with default settings. The genome sequences were submitted for annotation to the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). The genetic distances between isolates from vegetables in this study and all publicly available genomes for each species were examined using fastANI (Jain et al., 2018). There are currently 20 assemblies available for *Serratia fonticola*, and 891 assemblies for *Enterobacter hormaechei* for which a non-redundant dataset was generated according to the clustering approach described previously (Abram et al., 2021), which resulted in a set of 100 *E. homomaechei* assemblies.

2.4 Vegetable microbiome and resistome profiling by metagenomic sequencing

Twenty-five grams of each of three spinach samples was mixed with 225 ml diluent (0.1% peptone water and 0.1% Tween 80) in a stomacher bag. The following procedure was used to promote the release of bacterial cells from the vegetable surfaces: hand massage for 2 min, shaking at 220 rpm for 15 min at room temperature (25 °C), and hand massage again for 2 min. The suspension was passed through a 40 μm membrane filter (Nylon-Net Steriflip™ vacuum filter unit, Millipore Burlington, MA) to remove large leave debris, followed by membrane filtration using a 0.22 μm filter (Nalgene sterile analytical filter units, Thermo Scientific, Waltham, MA) to collect bacterial cells. After filtration, the filtration unit was disassembled and the membrane filter that carried bacterial cells was then removed from the filtration unit. The filter was cut using sterile scissors into small pieces and added to the lysis tube for DNA isolation. Metagenomic DNA was extracted from the filter using a Zymobiomics kit following the manufacturer's instructions (Zymo Research, 2021). Sequencing libraries were prepared using NEBNext Ultra II DNA Prep kit for Illumina. DNA sequencing was performed in Illumina NextSeq 500 using mid output kit (2 × 150bp) at the Genomics Core Laboratory at University of Arkansas for Medical Sciences.

About 8.7Gb to 9.8Gb data were obtained for each metagenomic sample. Raw reads were first trimmed using fastp (v0.20.1), and then were processed to remove host reads in sequencing data. Briefly, we mapped reads to the spinach genome (GCF_002007265) using bowtie2 (v2.4.1) and then extracted unmapped reads using samtools (v1.11). To build microbial community structure from our metagenomic data of spinach samples, we ran kraken2 (v2.1.1) which maps k-mers from reads against the kraken2 standard database. The number of reads assigned by kraken2 ranged from 27,400,000 to 33,600,000 across samples. To estimate abundance at the taxonomic ranks, we applied bracken (Bayesian reestimation of abundance with kraken) which uses the taxonomic assignment by kraken2 and a bracken-compatible database created from the kraken2 standard database. The ARG content of the samples was analyzed using beta version of RGI with metagenomic reads. For metagenomic data-based resistome profiling, we installed CARD'S resistomes & variants data additionally. Metagenomic short reads (trimmed and unmapped to the host genome) were aligned to CARD (Alcock et al., 2020) and WildCARD reference using kma (k-mer alignment) (Clausen et al., 2018). About 0.12% to 0.14% of reads (trimmed and unmapped to the host genome) were mapped to the ARGs. The number of reads mapped to the ARGs column in the gene mapping file was normalized using the all mapped reads to the ARGs and the reference length in kilobases long with the total number of reads available for

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mapping per million (RPKM) in each sample. We analyzed antibiotic resistome profiles at the antibiotic class level. Additionally, metagenome-based assembly and binning was performed using metawrap (Uritskiy et al., 2018) to construct draft metagenome assembled genomes (MAGs). Binned metagenome assemblies were analyzed using RGI against CARD with BLAST alignment tool which predicts ARGs from the contig-level input.

3. Results

3.1 Isolation of AmpC and ESBL-producing Enterobacterales from vegetables

Among 88 vegetable samples, two *E. hormaechei* isolates from two samples (2.27%), organic basil and brussels sprouts, showed AmpC activity, which was inhibited by cloxacillin as tested using the AmpC test kit. In addition, eight vegetable samples (9.09%), including bean sprouts, organic parsley, organic baby spinach, and several mixed salads, were positive for ESBL-producing *Enterobacterales* based on double-disk combination test using CTX with or without CLA. These included three *E. hormaechei* isolates and five *S. fonticola* isolates (Table 1).

3.2 Antibiotic susceptibility of AmpC and ESBL-producing Enterobacterales

AmpC-producing *E. hormaechei* S43-1 and S74-2, and five ESBL-producing *S. fonticola* isolates were resistant to some ß-lactam antibiotics but susceptible to other tested antibiotics (Table 2). All three ESBL-producing *E. hormaechei* strains, S11-1, S17-1, and S45-4, were susceptible to ciprofloxacin, and carbapenem antibiotics (meropenem and imipenem). However, these three ESBL-producing *E. hormaechei* isolates were nonsusceptible to other tested antibiotics, including ampicillin, amoxicillin/clavulanic acid, cefiderocol, chloramphenicol, gentamicin, nalidixic acid, trimethoprim/sulfamethoxazole, and tetracycline (Table 2).

3.3 Whole genome sequencing and identification of resistance genes

Two AmpC-producing *E. hormaechei* strains, S43-1 and S74-2, were assigned a sequence type of ST550. The ESBL-producing *E. hormaechei* strains, S11-1, S17-1, and S45-4, belong to the sequence type ST145. *S. fonticola* isolates were not assigned to any known sequence type. The AmpC-producing *E. hormaechei* strains, S43-1 and S74-2, formed a clade in the genomic distance tree. Genomic distance analyses between the representative set of strains for the species and our isolates indicated that *E. hormaechei* strains, S11-1, S17-1, and S45-4, from different vegetable samples were highly related to each other genetically (Figure 1A). Similarly, five FONA-5-producing *S. fonticola* isolates formed a clade in the genomic distance tree (Figure 1B).

Resistance genes in 10 AmpC or ESBL-producing isolates were identified by analyzing the genomes (Table 1 and Table S1). Genes encoding AmpC ß-lactamases were found in *E. hormaechei* strains S43-1 and 74-2, which was consistent with the AmpC production phenotypes. ESBL genes were identified in eight ESBL-producing isolates. *E. hormaechei* strains, S11-1, S17-1, and S45-4, possess genes encoding SHV66 whereas five *S. fonticola* isolates contain genes encoding a minor ESBL, FONA-5 (Table 1).

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3.4 Microbiome and resistome in retail spinach samples

Pseudomonas was the most prevalent bacterial genus in all three retail spinach samples, which on average accounted for 57% of all bacteria (Figure 2A). *Enterobacteriaceae* accounted for 0.4% of all bacteria in the samples with *Enterobacter* being the most abundant genus (0.11%) in the family (Figure 2B). *Serratia* spp. in the family of *Yersiniaceae* on average made up 0.11% of all bacteria in three spinach samples. The relative abundance of antibiotic resistance genes in three spinach samples is shown in Figure 2C. Resistance genes detected in spinach samples encode 12 major classes of antibiotics, including ß-lactam antibiotics (subclass: cephalosporin, penam, cephamycin, and penem), aminoglycoside, triclosan, macrolide, fluoroquinolone, tetracycline, phenicol, rifamycin, diaminopyrimidine, glycylcycline, aminocoumarin, and peptide antibiotics. To infer which organisms might be responsible for the resistome profiles, we calculated Pearson correlation scores between genus-level abundance profiles and resistance gene abundance profiles. Figure 2D represents only strong associations with correlation >0.8. *Enterobacter, Escherichia, Salmonella, Klebsiella* showed strong correlation with various antibiotics. We identified antibiotic resistance genes on metagenome-assembled genomes (MAGs) reconstructed by the metagenome-assembled genome binning approach which resulted in 7 MAGs with 80% of completion and 20% of contamination cutoffs for each spinach sample. However, no bins were annotated as a member of *Enterobacteriaceae* in Figure 2B.

4. Discussion

The prevalence rate of AmpC and ESBL-producing *Enterobacterales* in this current study was 2.27% and 9.09%, respectively. The prevalence rate in this study was lower than that reported in a study conducted in South Africa in which AmpC/ESBL-producing *Enterobacterales* were found in 25% retail spinach samples (Richter et al., 2020). ESBL-producing *Enterobacteriaceae* were isolated in 13.3% fresh vegetable samples in China (Ye et al., 2018). In contrast, in a study conducted in the Netherlands, 3.4% and 1.6% retail vegetables were positive for AmpC and ESBL-producing *Enterobacteriaceae,* respectively (van Hoek et al., 2015). In our study, we did not identify AmpC or ESBL-producing *E. coli* or *K. pneumoniae* among 88 vegetable samples. Similarly, in a recent study, only one out of 399 vegetable samples collected in Germany yielded cefotaxime-resistant *E. coli* (Kaesbohrer et al., 2019). However, the prevalence of ESBL-producing *E. coli* and *K. pneumoniae* in retail ready-to-eat vegetables in South Korea was 10.1% (Kim et al., 2015).

Members of *Enterobacter* species are environmental microorganisms and become one of the leading causes of nosocomial infections (Davin-Regli et al., 2019). *E. hormaechei* is closely related to *Enterobacter cloacae* in the family *Enterobacteriaceae* (O'Hara et al., 1989). *E. hormaechei* is a nosocomial pathogen that resulted in hospital outbreaks in the United States (Wenger et al., 1997) and in the Netherlands (Leverstein-Van Hall et al., 2006). Multidrug-resistant ESBL-producing *E. hormaechei* strains were isolated from fresh vegetable samples in this study (Table 2). These antibiotic-resistant strains in vegetables may pose potential health risks to vulnerable consumers. Similarly, ESBL-producing *E. hormaechei* was isolated in the vegetable supply chains in Tunisia (ben Said et al., 2015). *S. fonticola* was first described as a species in the family of *Enterobacteriaceae* (Gavini

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et al., 1979) and was recently reclassified in the family of *Yersiniaceae* (Adeolu et al., 2016). The bacterium is an opportunistic pathogen and occasionally caused infections (Aljorayid et al., 2016; Bollet et al., 1991) and even death (Hai et al., 2020) in humans. Five ESBL-producing *S. fonticola* strains were isolated from vegetables in this study (Table 1). Similarly, ESBL-producing *S. fonticola* was isolated from the vegetable supply chains in South Africa (Richter et al., 2020). FNOA-producing *S. fonticola* strains were isolated from retail vegetables in the Netherlands (van Hoek et al., 2015) and from imported chicken meat in Japan (Tanimoto et al., 2021).

Precise species identification and subspecies typing through genome sequencing is needed to differentiate resistant isolates from other closely related strains. In this study, genomic distance trees using whole genome sequences were constructed to determine the relationships among the isolates from vegetables and other related strains in the same species in publicly available database (Figure 1). We found that five *E. hormaechei* isolates in this study formed two clades in the genomic distance tree (Figure 1A), which suggested that *E. hormaechei* strains in each of two clades may derive from a common bacterial lineage or from a common source of contamination in the vegetable supply chains. *E. hormaechei* strains in this study were isolated from different types of vegetable products (Table 1) and the environmental sources of these *Enterobacter* strains remained unknown. *Enterobacter* species are ubiquitous bacteria in natural environments, which are found in habitats such as water, sewage, soil, and plants (Rogers et al., 2015). The AmpC-producing isolates, S43-1 and S74-2, showed the closest genetic relatedness to antibiotic-resistant clinical isolates collected in Japan (Aoki et al., 2018). Similarly, FastANI analysis also revealed that five ESBL-producing *S. fonticola* isolates from different vegetable products (Table 1) formed a clade in the genomic distance tree (Figure 1B). *S. fonticola* S18-2 was genetically related to an ESBL-producing strain *S. fonticola* BWK15, which was isolated from feces of migratory birds in Japan (Kenzaka & Tani, 2017). Other researchers also reported the isolation of ESBL-producing *E. hormaechei* and *S. fonticola* from the vegetable supply chains in Africa (ben Said et al., 2015; Richter et al., 2020), but the genomes of these isolates are not currently available in public databases for genomic comparison with our isolates.

E. hormaechei strains, S11-1, S17-1, and S45-4, were resistant to multiple clinically significant antibiotics (Table 2). The resistance phenotypes were largely consistent with the resistance genotypes. For example, ß-lactamase genes, *catII, aac(3)-IIg, dfrA19,* and *tet(B)* and *tet(D)* were found in the genomes of *E. hormaechei* strains, S11-1, S17-1, and S45-4 (Table S1). In addition, *E. hormaechei* strains, S11-1, S17-1, and S45-4, carried a polymyxin-resistant gene, *mcr-9,* but these strains were susceptible to polymyxin B with an MIC <0.5 μg/ml, which suggested that the *mcr-9* gene was not expressed constitutively to confer polymyxin B resistance. The results were consistent with previous reports in which *mcr-9*-carrying strains were susceptible to polymyxin and prior exposure to the polymyxin may induce the expression of the resistance gene (Kieffer et al., 2019).

Shotgun metagenomic sequencing approach revealed a diverse microbiome and resistome in spinach samples in this study. Microbiome analysis indicated that *Pseudomonas* was the dominant bacterial genus in three spinach samples. Similarly, *Pseudomonas* was one of

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the dominant genera in unwashed spinach leaves using 16S rDNA amplicon sequencing approach (Gu et al., 2018). *Enterobacter* and *Serratia* accounted for 0.11% bacteria in spinach samples in this study. This was coincident with the observation that multiple AmpC/ESBL-producing *Enterobacter* and *Serratia* strains were isolated from vegetable samples in this study. Antibiotic resistomes in soil and water have been studied extensively but only limited studies are available for plant resistomes (Chen et al., 2019). Shotgun metagenomic sequencing is a comprehensive approach to profile resistomes associated with the surface of vegetables. In our study, resistance genes encoding almost all major classes of antibiotics were identified in the spinach samples.

Farming practices, such as the selection of organic fertilizers, types of irrigation water, and sanitation of vegetable processing environments, may affect the composition of microbiome and antibiotic resistome in vegetables. Vegetables harvested from compost-amended soil may carry an additional burden of antibiotic resistance genes. For instance, in a greenhouse pot experiment, antibiotic resistance genes in lettuce microbiome overlapped with the resistomes in the compost-amended soil (Zhang et al., 2019). Lettuce grown in compost-amended soil carried over 40-fold *sul1* gene copies than those grown in soil with chemical fertilizers (Fogler et al., 2019). Irrigation water quality may also affect antimicrobial-resistant bacteria in vegetable products. In a greenhouse experiment, microbial source tracking identified clonal *E. coli* carrying the same transmissible multidrug-resistant plasmid along the irrigation water chain from an open-top reservoir and in harvested fresh chive (Gekenidis et al., 2018). Understanding the various potential pathways for antibiotic resistome dissemination through vegetable microbiomes is critical for the mitigation of antibiotic resistance.

5. Conclusions

We used the combination of microbiology, genomics and metagenomics approaches to assess antibiotic resistance in retail vegetables in the United States. ESBL-producing *E. hormaechei* and *S. fonticola*, and AmpC-producing *E. hormaechei* were isolated from retail vegetables. Multidrug-resistant ESBL-producing *E. hormaechei* carrying *mcr-9* resistance gene was isolated for the first time in vegetables. The study highlights the need for antibiotic resistance surveillance in vegetable products. It is also important to determine consumers' concerns and raise their awareness of antibiotic resistance in fresh produce. One limitation of the current study is the relatively small number of vegetable samples collected for screening. A large-scale sampling of different types of fresh vegetables would benefit the assessment of the prevalence of antibiotic resistance in retail vegetables in the Unites States. Sampling vegetable products and associated environments across different growing seasons in the vegetable supply chains from farm to fork would provide complete assessment of antibiotic resistance and its potential impact to public health. Future studies that identify the critical factors contributing to antibiotic resistance during vegetable production would help mitigate antibiotic resistance in fresh produce.

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Nucleotide sequences

The whole-genome and metagenome shotgun project has been deposited at GenBank under the accession no. PRJNA705736.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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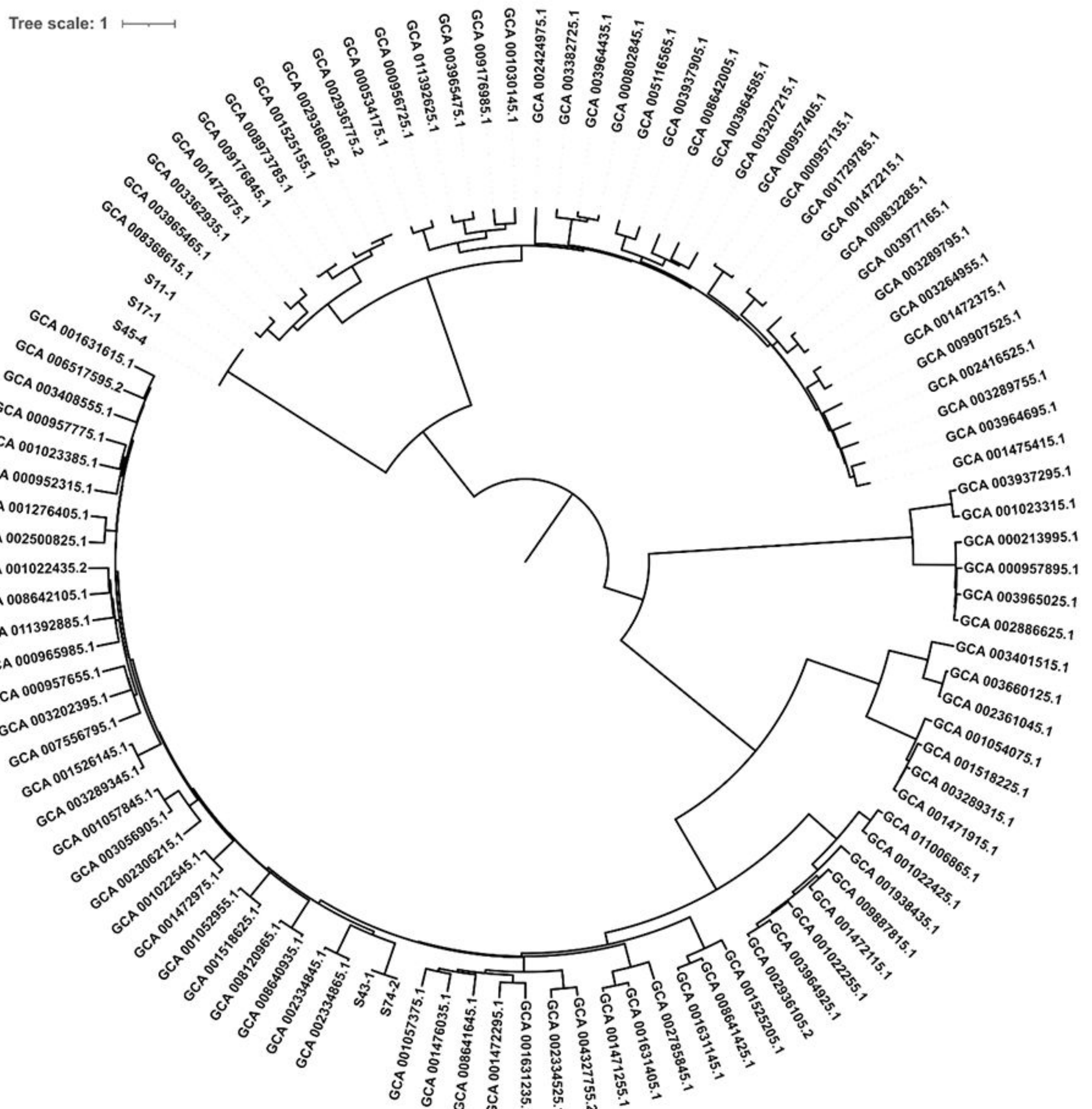
Highlights

- Multidrug-resistant ESBL-producing *E. hormaechei* carrying *mcr-9* from vegetables
- Shotgun metagenomic sequencing revealed a diverse resistome in spinach samples.
- Antibiotic resistance surveillance is needed for vegetable products.



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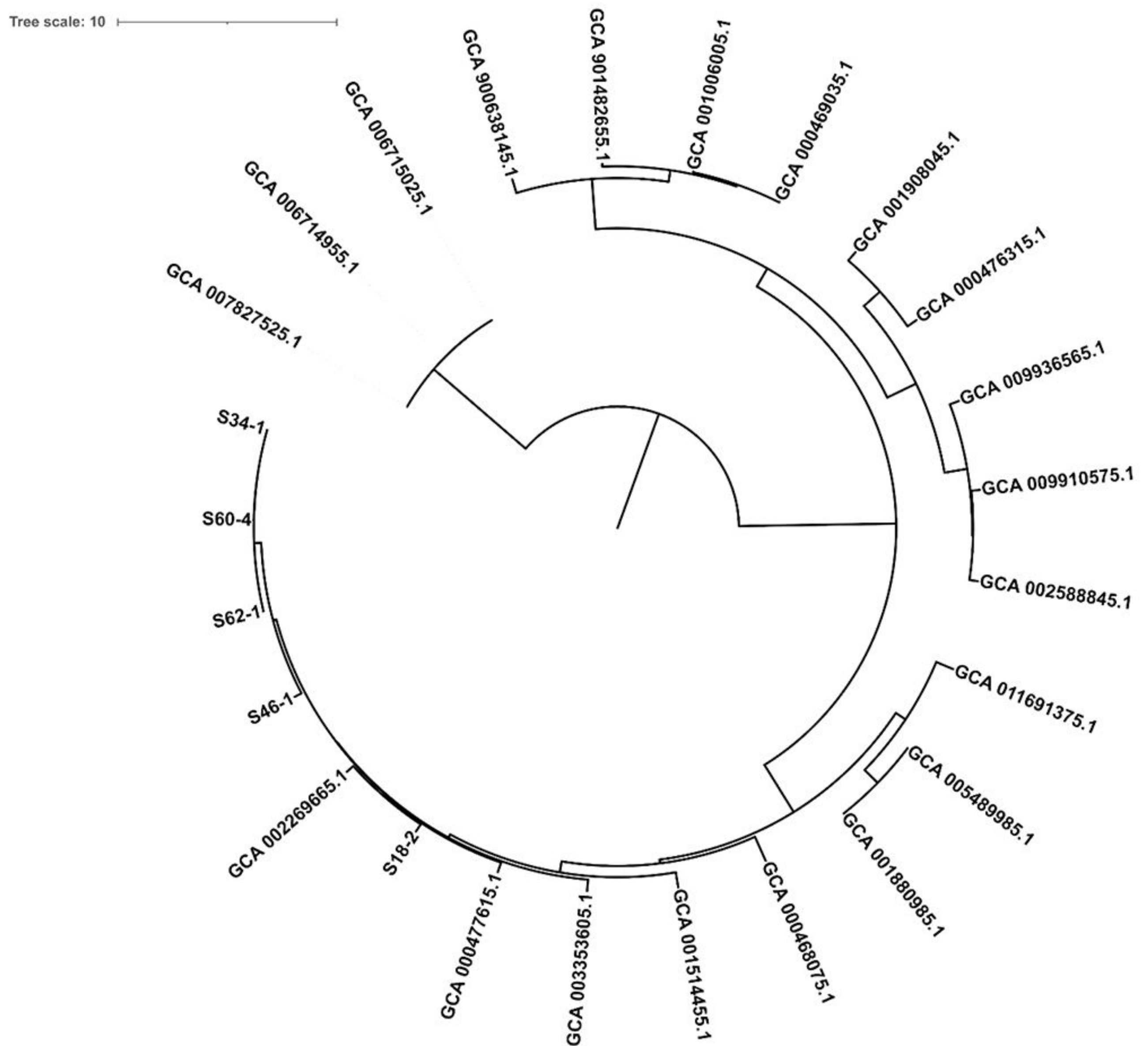

Figure 1.

Genomic distance trees of antibiotic resistant isolates from vegetables and assemblies publicly available within the species. **(A)** *Enterobacter hormaechei* isolates (S43-1, S74-2, S11-1, S17-1, and S45-4) from vegetables and 100 assemblies; **(B)** *Serratia fonticola* isolates (S18-2, S34-1, S46-1, S60-4, and S62-1) from vegetables and 20 assemblies within the species.

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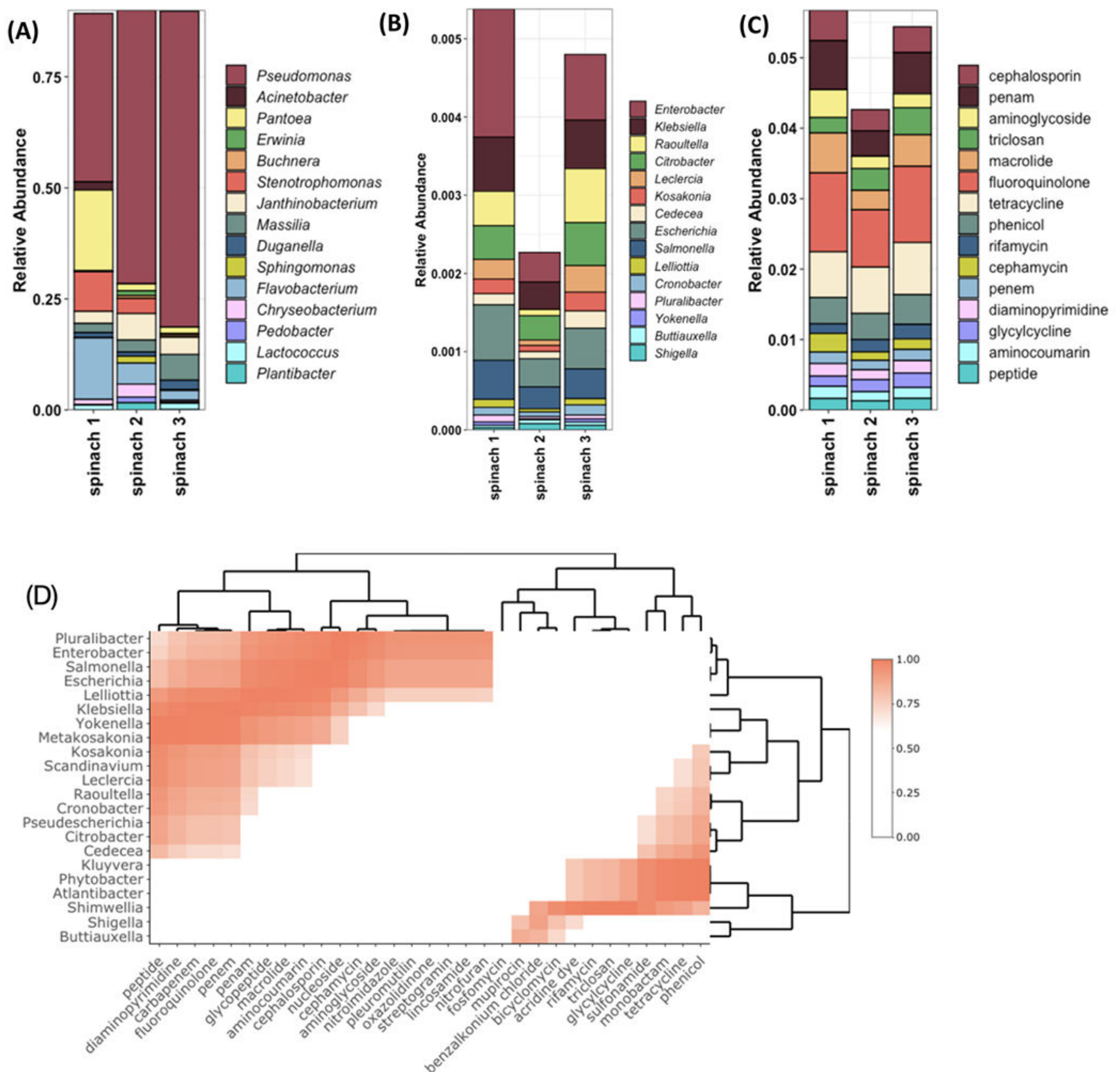

Figure 2.

Microbiome and resistome analyses of three spinach samples. **(A)** Relative abundance of genera in all bacteria; **(B)** Relative abundance of genera in the family of *Enterobaceraiacae*; **(C)** Relative abundance of antibiotic resistance genes. **(D)** Correlation heatmap where correlation scores > 0.8 are visualized between genus-level abundance profiles and resistance gene abundance profiles.

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Table 1.

AmpC and ESBL-producing *Enterobacterales* isolated from retail vegetables in the United States.

Isolates	Vegetables	AmpC/ESBL production	AmpC/ESBL genes
E. hormaechei S43-1	Organic basil	AmpC	bla_{AmpC}
E. hormaechei S74-2	Brussels sprouts	AmpC	bla_{AmpC}
E. hormaechei S11-1	Bean sprouts	ESBL	bla_{SHV66}
E. hormaechei S17-1	Organic parsley	ESBL	bla_{SHV66}
E. hormaechei S45-4	Organic baby spinach	ESBL	bla_{SHV66}
S. fonticola S18-2	Asian vegetable	ESBL	bla_{FONA-5}
S. fonticola S34-1	Butter lettuce and red leaf lettuce mix	ESBL	bla_{FONA-5}
S. fonticola S46-1	Organic lettuce mix	ESBL	bla_{FONA-5}
S. fonticola S60-4	Organic baby spinach, and baby lettuce blend	ESBL	bla_{FONA-5}
S. fonticola S62-1	Broccoli, carrots and red cabbage mix	ESBL	bla_{FONA-5}

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Table 2.

Antibiotic susceptibility[a] of AmpC and ESBL-producing *Enterobacterales* isolates

Isolates	AMP	AMC	MEM	IMI	FDC	GEN	TET	CHL	STX	NAL	CIP
E. hormaechei S43-1	R	R	S	S	S	S	S	S	S	S	S
E. hormaechei S74-2	R	R	S	S	S	S	S	S	S	S	S
E. hormaechei S11-1	R	R	S	S	I	R	R	R	R	I	S
E. hormaechei S17-1	R	R	S	S	I	R	R	R	R	I	S
E. hormaechei S45-4	R	R	S	S	I	R	R	R	R	I	S
S. fonticola S18-2	R	S	S	S	S	S	S	S	S	S	S
S. fonticola S34-1	R	S	S	S	S	S	S	S	S	S	S
S. fonticola S46-1	R	S	S	S	S	S	S	S	S	S	S
S. fonticola S60-4	R	S	S	S	S	S	S	S	S	S	S
S. fonticola S62-1	R	S	S	S	S	S	S	S	S	S	S

[a:] Results were interpreted using CLSI breakpoints.

S, susceptible; **I**, intermediate; **R**, resistant. **AMP**, ampicillin; **AMC**, amoxicillin/clavulanic acid; **MEM**, meropenem; **IMI**, imipenem; **FDC**, cefiderocol; **GEN**, gentamicin; **TET**, tetracycline; **CHL**, chloramphenicol; **STX**, trimethoprim/sulfamethoxazole; **NAL**, nalidixic acid; and **CIP**, ciprofloxacin.