ECA 2021

sciforum

Conference Proceedings Paper

Prevalence of β-lactamase producing *Enterobacteriaceae* in fresh vegetables†

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† Presented at The 1st International Electronic Conference on Antibiotics—The Equal Power of Antibiotics And Antimicrobial Resistance, online, 08-17 May 2021.

Abstract: This study was conducted to determine the importance of vegetables consumed raw as vehicles of extended-spectrum β-lactamase (ESBL), AmpC and carbapenemase-producing *Enterobacteriaceae*. For this study we selected 112 vegetable and environmental samples, analyzing their microbial load of *Enterobacteriaceae* and *E. coli* through plate counting. The presence of β-lactam-resistant *Enterobacteriaceae* was investigated using selective-chromogenic media, and isolates obtained were characterized by PCR and antibiotic susceptibility testing. *Enterobacteriaceae* counts in vegetables ranged from 2.31 ±0.86 log CFU/g up to 5.50 ±0.7 log CFU/g. We detected a prevalence of 20.5 % of vegetable samples and 14.03 % of environmental samples with at least one AMR isolate.

Keywords: ESBL; AmpC; carbapenemases; *Enterobacteriaceae*; β-lactamase genes; fresh vegetables.

1. Introduction

Antimicrobial resistance is one of the main threats to public health nowadays. Food chain is related to this problem, enabling the propagation of antimicrobial resistant (AMR) bacteria and horizontal gene transfer of their genes. Consumption of fresh vegetables has particular importance because the lack of a bactericidal treatments allows AMR bacteria present in the food to colonize the gut during the intestinal passage or to disseminate through food handlers increasing the possibility of community dissemination of the AMR genes [1–3].

Members of the family *Enterobacteriaceae* are one major concern, due to the high prevalence of AMR isolates some of them with pathogenic potential as well. AMR members of the *Enterobacteriaceae* family can be found in the vegetable-production environment or can be transmitted from animal sources through manure or irrigation water contaminated by faeces and thus reach the vegetable surface.

The most prevalent and clinically relevant mechanism of AMR in *Enterobacteriaceae* is the production of β-lactamases. Enzymes like AmpC, extended spectrum β-lactamases (ESBL) and carbapenemases have been previously detected in fresh vegetables, but its prevalence has not been established with certainty [4]. Taking into account the scarcity of available data on fresh produce, this study was conducted to determine the importance of raw vegetables as vehicles of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*, along with other β-lactamases.

2. Materials and methods

2.1. Sample collection and processing

One hundred and twelve samples were collected from vegetable farms. Thirty-nine were enviromental samples from water (n=10), air (n=11), and soil (n=11) and also from the hands of farm

workers (n=8). The remaining 73 samples were taken from vegetables in three different farms (E1, E2 ,E3) and in a local street-market: tomato (Solanum lycopersicum, n=13), escarole (Cichorium endivia var. latifolium, n=3), parsley (Petroselinum crispum, n=3), carrot (Daucus carota subsp. sativus, n=15), cucumber (Cucumis sativus, n=13), pepper (Capsicum annuum, n=7) and lettuce (Lactuca sativa, n=19).

Vegetables were lightly cleaned to remove foreign matter and non-edible parts were cut. Ten grams of each vegetable and soil sample were homogenized with 90 mL of buffered peptone water (BPW). Water samples were processed by filtering 100 mL of the sample through a 0,45 μm filter, and then soaking the filter in 100 mL of BPW. A volume of 100 L air-sample was collected in farms using a microbial air sampler (Biotest Hycon, Dreieich, Germany) fitted with ChromAgar Enterobacteria plate (ChromAgar, Paris, France). One hand swab sample was taken from each farm worker which was then soaked in 10 mL of BPW.

2.2. Enterobacteriaceae counting and E. coli detection

The BPW-homogenates were diluted 1:10 in 0.1 % peptone and appropriate ten-fold dilutions were spread onto ChromAgar Enterobacteria plates (37 °C/24 h). Pink/reddish colonies were recorded as suspected *Enterobacteriaceae* and blue ones as suspected *E. coli*.

2.3. Isolation and identification of suspected β-lactam-resistant isolates

The remained homogenates were incubated 24 h at 37 °C. One loop (≈10 μL) of the enriched solution was streaked onto each of the following chromogenic media: ChromAgar ESBL, MacConkey Agar (Oxoid, Thermofisher Spain, Barcelona, Spain) supplemented with 16 mg/L of cefoxitin and ChromAgar KPC, for the isolation of ESBL, AmpC and carbapenemase-producing bacteria, respectively. Plates were incubated 24 hours at 37 °C and colonies with morphology associated with β-lactam resistance were selected for further characterization. Identification of the suspected AMR isolates was performed using Matrix laser desorption ionization - time of flight mass spectophotometry (MALDI-TOF MS, Bruker Daltonik gmbH, Bremen) technique.

2.4. PCR detection of β-lactam resistance associated genes

The suspected isolates were checked by PCR for the detection of genes associated with resistance: $bla_{cmy\text{-}2}$ [5] for AmpC; $bla_{ctx\text{-}M}$ [6], bla_{shv} [6] and bla_{tem} [6] for ESBL, and bla_{ndm} [7], bla_{kpc} [8] and bla_{OXA48} [9] for carbapenemase enzymes.

2.5. Antimicrobial Susceptibility Testing

The disk-diffusion technique was carried out to test the antimicrobial susceptibility of the isolates following the indications of EUCAST [10] for the screening of ESBL, AmpC and carbapenemase producers. MAST D72C AmpC and ESBL detection kit (MAST group Ltd., Bootle, UK) was used for ESBL and AmpC confirmation while carbapenemase production confirmation was performed following the EUCAST indications [10]. *E. coli* CECT 943 was used as a negative control, while *E. coli* M1L9c (from our collection), *E. coli* CCUG 58543, *Klebsiella pneumoniae* subsp. *pneumoniae* CCUG 56233 and *Klebsiella pneumoniae* subsp. *pneumoniae* CCUG 60138 were used as positive controls for ESBL, AmpC, and carbapenemase producers (KPC and NDM carbapenemases), respectively.

2.6. Statistical analysis

Data of bacterial counts were analyzed with SPSS 25 V (IBM SPSS, Chicago, IL, USA, 2017). The normality of the variable *Enterobacteriaceae* counts was checked through Kolmogorov-Smirnoff test. Mean bacterial counts of different sources were compared through a one-way ANOVA test, while differences between market and farm samples were assessed through t-student test.

3. Results and discussion

3.1. Enterobacteriaceae and E.coli counting

Table 1 shows bacterial counts in vegetable samples. Mean *Enterobacteriaceae* count was 3.57±1.39 log CFU/g. The highest *Enterobacteria*ceae counts were found on parsley and escarole (5.48±0,3 log CFU/g and 5.50±0,7 log CFU/g, respectively) and the lowest was found on cucumber (2.31±0.86 log CFU/g). Parsley and escarole also showed high AMR rates (table 3), suggesting the possibility of correlation between high carriage of *Enterobacteriaceae* and dissemination of AMR isolates. Market vegetable samples showed a mean *Enterobacteria*ceae count of 3.85±1.33 log CFU/g while vegetable samples collected in farms had a mean value of 3.49±1.39 log CFU/g, which were non statistically different (p<0.05). Data from other works indicate that *Enterobacteriaceae* counts in fresh produce differ depending on factors such as crops, agronomic practices and weather, ranging from values of 1.9 up to 7.2 log CFU/g [11,12], thus being difficult to compare among different studies.

Nine vegetable samples and one sample taken from a farm worker were positive for the presence of *E.coli*. In seven of this samples, *E. coli* count was just above the detection limit (1,69 logCFU/g), whereas a sample from a farm worker, an escarole sample (both from the same farm) and sample of lettuce presented *E. coli* counts of 3.70, 2.81 and 3.04 log CFU/g respectively. These results suggest that *E. coli* contamination in the studied samples was low and may be associated with sporadic contaminations from farm workers.

Table 1. Bacterial counts in vegetable samples (mean ± standard deviation).

Type of sample	***Enterobacteriaceae* Mean (log CFU/g)**	**N° *E. coli* positive samples**
Lettuce	3.91^{bc} ±1.33	2
Tomato	2.71^{ab} ±1.01	1
Cucumber	2.31^{a} ±0.86	0
Carrot	4.29^{cd} ±1.05	1
Pepper	3.36^{abc} ±0.53	2
Parsley	5.48^{d} ±0.65	2
Escarole	5.50^{d} ±0.34	1
Total	3.57±1.39	10

$^{a-d}$ Means bearing different lowercase letter differ statistically (p<0.05).

Table 2 shows bacterial counts obtained on environmental samples. *Enterobacteriaceae* counts were lower in environmental samples than in vegetables (p<0.05); except in the case of soil samples, which did not show any significant difference (p<0.05) with the mean vegetable count.

Table 2. Bacterial counts on samples on environmental samples (mean ± standard deviation).

Type of sample	***Enterobacteriaceae* Mean count (log CFU/g)**	**N° *E. coli* positive samples**
Soil	2.89	0
Water	0.42*	0
Air	0.22**	0
Farm workers	2.34	1

*Bacterial count expressed as log CFU/mL of water **Bacterial count expressed as log CFU/100L of Air.

3.2. Phenotypic and genotypic characterization of the isolates

AMR rates among the different vegetables analyzed are shown in table 3. Highest AMR rates were found in escarole with 66.7 % of samples positive for one resistant isolate, followed by pepper and parsley. Overall AMR rate obtained in vegetables in this study is 20.5 %, which is higher than the rates obtained by Ben Said *et al*. [1] and van Hoek *et al*. [4], which obtained rates of 8.51 % and 5.2 % of samples containing ESBL and/or AmpC positive isolates, respectively. Blaak *et al* [3] reported a higher rate (61.9 %) but their study included only supermarket samples, in which we observed higher AMR rate (33.0 %) when compared the farm-collected ones (24.1 %). AMR resistant *Enterobacteria*ceae were isolated from all studied farms, which suggests a wide dissemination in the vegetable-production environment.

Table 3. AMR rates among the different vegetables analyzed. "n": number of samples analyzed; "N>1": number of samples in which at least one resistant isolate could be detected.

Type of sample	n	N>1	ESBL	AMPc
Lettuce	19	3 (15.78 %)	0	3 (15.78 %)
Tomato	13	3 (23.10 %)	0	3 (23.10 %)
Cucumber	13	2 (15.30 %)	0	2 (15.30 %)
Carrot	15	1 (6.67 %)	1 (7.14 %)	0 (7.14 %)
Pepper	7	3 (42.80 %)	0	3 (42.80 %)
Parsley	3	1 (33.30 %)	1 (33.30 %)	0
Escarole	3	2 (66.70 %)	1 (33.30 %)	1 (33.30 %)
Total	73	15 (20.50 %)	3 (4.10 %)	12 (16.40 %)

Out of 28 environmental samples, four water samples were positive for one AMR isolate. All of these isolates shown an AmpC β-lactam-resistance mechanism. High AMR rates on water samples from vegetable-production environment were also observed by van Hoek *et al*. [4] with a 100 % (3/3) of positive samples. Aquatic niches has been previously reported as key sources of AMR in which bacterial from human and animal faecal sources can be incorporated, providing optimal conditions for interaction and gene exchange [13]. This fact supports the "One Health" approach for prevention of human disease. In this regard, prevention of the dissemination of AMR isolates should start at the beginning of the food chain, developing actions targeting factors like irrigation water and fertilization to limit contamination from these sources [14].

The most prevalent mechanism of β-lactam resistance was production of AmpC-like β-lactamases with 20 confirmed isolates detected from 16 samples. ESBL producing strains were detected in 2 samples, with 3 confirmed isolates. Conversely, studies carried out by van Hoek *et al.* [4] and Blaak *et al.* [3] detect a higher prevalence of ESBL than AmpC isolates. No isolates of carbapenemase-producing *Enterobacteriaceae* were detected.

Seventeen of the 20 AmpC confirmed isolates showed an inducible AmpC pattern and were identified as *Citrobacter spp*. (13), *Enterobacter aerogenes* (3) and *Enterobacter cloacae* (1); while 3 of them were constitutive AmpC producers and were identified as *Enterobacter cloacae*. The presence of bla_{cmy-2} was detected in 11 isolates, all of them showing an inducible-type AmpC mechanism and were identified as *Citrobacter spp*. The remaining inducible type isolates (TO04E, LE1021, LE10E22, PE05E2 and PE07E) and the constitutive AmpC producers (TO01E, TO02E, and TO03E) were negative for the presence of bla_{cmy-2}.

AmpC sequences are commonly present in *Enterobacter* and *Citrobacter* species as chromosomic inducible sequences. The main concern about inducible AmpC enzymes is the fact that these species can show an *in vitro* susceptibility in the absence of an inductor compound and develop a resistant phenotype when exposed to one, leading to a possible therapeutical failure. β lactam like ampicillin, benzyl penicillin, cephazolin, cephalothin, amoxicillin, imipenem and cefoxitin are strong inducers of AmpC production [15].

The profile shown by TO01E, TO02E and TO03E is associated with a constitutive production of AmpC β-lactamases. This mechanism of resistance is associated with mutations on the regulation genes or can also be associated with an acquired plasmid which usually lacks the *AmpD* gene leading to a non-inducible mechanism [15,16]. These isolates were negative to the presence of bla_{cmy-2} gene, nevertheless there has been described up to 43 different allelic variants of the bla_{cmy-2} gene, as well as other AmpC genes which could be present in these strains [16,17].

Four ESBL positive isolates were detected in three different samples one sample of parsley (isolate PJ07E), one sample of escarole (isolate ES09E) and one sample of carrot (isolates ZA07E1, ZA07E2), belonging to the species *Serratia fonticola* (n=2) and *Rahnella aquatillis* (n=2). The presence of bla_{CTX-M} was detected in isolates ZA07E1 and ZA07E2, while PJ07E and ES09E were negative to all of the ESBL genes tested *(bla_{CTX}, bla_{shv} and bla_{tem}).* LE18E, a *Serratia fonticola* strain isolated from lettuce, was found to be positive to the presence of bla_{CTX-M} gene, but did not show a phenotypic pattern associated with ESBL resistance mechanism.

All of the AMR isolates detected in the study (24) are of minor importance as foodborne pathogens. *Citrobacter* (65.0 %) and *Enterobacter* (37.5 %) species are faecal bacteria which can be opportunistic pathogens while *Rahnella* (8.33 %) and *Serratia* (8.33 %) are usually identified as environmental and mostly harmless bacteria. However, these isolates could represent a concern due to their importance in disseminating antibiotic resistance factors through horizontal gene transfer and the colonization of gut microbiota[1–3**]**. Other studies also observed that these species represent the majority of the AMR microbiota of fresh produce and vegetable-production environment[3,4].

4. Conclusions

According to our data, fresh produce could be a source of ESBL and AmpC-producing *Enterobacteriaceae* and disseminate them along the food chain. Potential risks for public health of this transmission should be assessed in future studies.

Funding: This research was funded by SPANISH R&D PROGRAM -"Agencia Estatal de Investigación"- (project PID2019-107870RB-I00/AEI/10.13039/5011000111033) and UNIVERSITY OF LEÓN (research program) (Alberto Pintor Cora).

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