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Phylogroups, pathotypes, biofilm formation and antimicrobial resistance of *Escherichia coli* isolates in farms and packing facilities of tomato, jalapeño pepper and cantaloupe from Northern Mexico

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ABSTRACT

The most commonly used indicator of fecal contamination in fresh produce production and packing is *Escherichia coli*. In depth analysis of the prevalence and characteristics of naturally occurring *E. coli* strains in these environments is important because it can (1) serve as an indicator of sources of fecal contamination; and (2) provide information on strain pathogenicity, persistence, and other defining characteristics such as multidrug resistance. In this study, we analyzed 341 *E. coli* strains isolated from the jalapeño pepper, tomato and cantaloupe farm environments, in Northeast Mexico. Strains were isolated from produce, farmworkers' hands, soil and water. Pathotypes, genotypes, biofilm formation and antibiotic resistance were characterized. Phylogenetic subgroups and identification of diarrheagenic *E. coli* were determined by PCR; biofilm formation was quantified using a plate-based colorimetric method. Antibiotic resistance was analyzed by the Kirby Bauer diffusion disc method. Most isolates (N = 293, 86%) belonged to phylogenetic group A. Only four isolates (1.2%) were diarrheagenic: EPEC (N = 3) and ETEC (N = 1). Antibiotic resistance to tetracycline (23.2%) and ampicillin (19.9%) was high, and only 3.5% of the strains presented resistance to > 5 antibiotics. Biofilms were produced by most strains (76%), among which 34.4% were categorized as high producers. The presence of antibiotic resistant *E. coli* strains that may contain gene markers for pathogenicity and which can form biofilms suggests potential health risks for consumers.

1. Introduction

Escherichia coli is a normal inhabitant of the digestive tract of warm blooded animals, including humans (CDC, 2014). *E. coli* is one of the dominant enteric species in human feces and it has been used as an indicator of fecal contamination for close to a century. *E. coli* is often regarded as harmless; however, there are *E. coli* groups which have acquired virulence factors and have the ability to cause diarrheal disease in healthy humans (Kaper et al., 2004). A recent systematic review reported a low prevalence of pathogenic *E. coli* on farms (range 0–1.6%) and packing facilities (0–10%) from fourteen studies testing produce for pathogens. Only in the US, at least nine documented outbreaks of pathogenic *E. coli* have been linked to the consumption of fresh produce such as lettuce, spinach and sprouts from 2010 to 2017 (CDC, 2018). Produce can become contaminated in the field or from improper sanitation, handling or processing. The use of compost, sewage contaminated water for irrigation and droppings from wild and domesticated animals and birds are all considered sources of pathogen contamination on fresh produce (Liu et al., 2013).

The most commonly used indicator of fecal contamination in fresh produce production and packing is *Escherichia coli*. In depth analysis of the prevalence and characteristics of naturally occurring *E. coli* strains in these environments is important because it can (1) serve as an indicator of sources of fecal contamination through microbial source tracking (Carlos et al., 2010); (2) identify potentially pathogenic strains; (3) provide information about antimicrobial resistance profiles that can be used to understand strain emergence and clinical treatment of disease (Boehme et al., 2004); and (4) allow us to characterize of the

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Table 1
Phylogroups of *E. coli* strains isolated along the in-field production chain of jalapeño pepper, tomato and cantaloupe.

Sample type	Number of isolates (jalapeño, tomato, cantaloupe)		Jalapeño pepper	Tomato	Cantaloupe
		%	Phylogroup (%)		
Water	Source (22, 58, 5)	24.9	**A_0 (54.5)**[a], A_1 (22.7), D_1 (22.7)	**A_0 (58.6)**, A_1 (34.5), B_1 (5.2), $B2_2$ (2)	A_0 (20), **A_1 (80)**
	Irrigation hose (9, 44, 15)	19.9	**A_0 (66.7)**, A_1 (22.2), B_1 (11.1)	**A_0 (54.5)**, A_1 (27.3), B_1 (15.9), D_1 (2.3)	**A_0 (73.3)**, B_1 (6.7), D_1 (20)
Hands	Harvest (29, 8, 15)	15.2	**A_0 (69)**, A_1 (17.2), D_1 (13.8)	**A_0 (62.5)**, A_1 (25), B_1 (12.5)	**A_0 (66.7)**, A_1 (20), B_1 (13.3)
	Distribution (14, 7, 9)	8.8	**A_0 (71.4)**, A_1 (28.6)	**A_0 (42.9)**, A_1 (14.3), B_1 (28.6), D_1 (14.3)	A_0 (22.2), **A_1 (55.6)**, B_1 (11.1), D_1 (11.1)
	Packaging (5, 0, 4)	2.6	**A_0 (100)**	ND	**A_0 (75)**, B_1 (25)
Produce	Before harvest (19, 7, 6)	9.4	A_0 (47.4), **A_1 (52.6)**	**A_0 (71.4)**, B_1 (28.6)	**A_0 (83.3)**, B_1 (16.7)
	During harvest (3, 10, 3)	4.7	**A_0 (66.7)**, A_1 (33.3)	**A_0 (60)**, A_1 (10), D_1 (30)	**A_0 (66.7)**, A_1 (33.3)
	Distribution (6, 3, 5)	4.1	**A_0 (83.3)**, B_1 (16.7)	**A_1 (100)**	A_0 (20), A_1 (20), **B_1(40)**, $B2_3$ (20)
	Packaging (1, 10, 5)	4.7	**A_0 (100)**	**A_0 (70)**, A_1 (30)	A_0 (40), **A_1 (60)**
Soil	Around produce-plant sampled (9, 7, 3)	5.6	A_0 (11.1), **A_1 (77.8)**, $B2_3$ (11.1)	**A_0 (57.1)**, A_1 (14.3), D_1 (28.6)	**A_0 (100)**
Total	(117, 140, 70 = 341)	100 A0 (58.4), A1 (27.6), B1 (7.3), B22 (0.3), B23 (0.6), D1 (5.9)	**A_0 (60.7)**, A_1 (29.1), B1(1.7), $B2_3$ (0.9), D_1 (7.7)	**A_0 (57.1)**, A_1 (27.9), B1(9.7), $B2_2$ (0.6), D_1 (4.5)	**A_0 (57.1)**, A_1 (24.3), B1(11.4), $B2_3$ (1.4), D_1 (5.7)

[a] **Bold letter**: phylogroup with the highest percentage of isolates; ND: not detected.

propensity for biofilm formation to predict environmental persistence of this organism (Balcazar et al., 2015).

For microbial source tracking, a classification system has been developed based on phylogenetic cluster characteristics of this bacterium (Carlos et al., 2010; Lee, 2011). In this classification, A, B1, B2 and D constitute the main phylogroups, and the subgroups A0, A1, B1, $B2_2$, $B2_3$, D_1 and D_2, have been proposed to increase discrimination of *E. coli* strains (Carlos et al., 2010). Recently, Clermont et al. (2013) proposed a refined classification, adding four more phylogroups: C, E, F, and *Escherichia* cryptic clade I. The strains of all phylogroups differ in phenotypic and genotypic characteristics (Carlos et al., 2010; Gordon et al., 2008) such as their sugar metabolism, antibiotic resistance profiles, growth temperature ranges, ecological niches, and the presence and/or absence of select virulence factors (Carlos et al., 2010; Gordon et al., 2008). The ability to identify phylogroups has been useful in predicting human health risks. For example, diarrheal disease-causing *E. coli* are more likely of the B1 and E phylogroups and extraintestinal infection-causing *E. coli* strains are more likely of the B2 and E phylogroups (Nowrouzian et al., 2006).

E. coli, though part of the normal intestinal biota of animals and humans, has the potential to pose human health risks though acquired pathogenic virulence factors that induce diarrhea. These diarrheagenic *E. coli* (DEC) strains are classified into six different pathogenic types also known as pathotypes that include: enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) and diffuse adherent *E. coli* (DAEC) (Kaper et al., 2004; Russo and Johnson, 2000). DEC strains have been associated with outbreaks of severe disease, *i.e.*, bloody diarrhea and hemolytic uremic syndrome (HUS) as well as travelers' diarrhea in association with consumption of contaminated food and water (FDA, 2012). In Mexico for instance, the presence of EPEC, ETEC, STEC (*E. coli* producer of shiga toxin) and ETEC has been reported in ready-to-eat cooked vegetable salads (León et al., 2013), dairy, meat products, seafood, fish and prepared foods (Canizalez-Roman et al., 2013).

E. coli can also be an indicator of human health risks if strains are resistant to one or more antibiotics, and studies have identified isolates from agricultural foodstuff and vegetables in which strains were resistant to more than five antibiotics (Boehme et al., 2004; Schwaiger et al., 2011). The antibiotic resistance and susceptibility profiles of *E. coli* strains vary depending on geographic location, time of exposure to the antimicrobial compound, and other environmental factors (Dombek et al., 2000). Finally, the ability of *E. coli* to form biofilms may represent a strategy for strains to persist in produce and the production environment. Biofilms can protect bacteria from sanitizers, predation, desiccation and UV radiation (Costerton et al., 1995), providing an advantage for bacterial survival and persistence.

Mexico is one of the top producers of fresh produce and a significant trading partner with the U.S. and other countries. Eleven outbreaks have occurred in the US due to the consumption of contaminated produce originating from Mexico in the period 2006 to 2017 (CDC, 2018). The associated contaminated produce included sprouts, leafy greens, spinach and lettuce (FDA, 2012). Hence, there is a need for better understanding of the characteristics of naturally occurring *E. coli* isolates from this region of the world. The purpose of this study was to identify the phylogroups, pathotypes, antibiotic resistance profiles and biofilm formation ability of *E. coli* strains previously isolated and archived (Heredia et al., 2016) from a longitudinal study along the production chain of jalapeño pepper, tomato and cantaloupe from Nuevo Leon and Coahuila, Mexico. This information provides knowledge about the *E. coli* strains circulating in the farm environment, the possible sources of contamination in production, and the likelihood of pathogenic and antibiotic resistant strains that could provide a risk to public health.

2. Material and methods

2.1. Bacterial strains

In a previous study, 341 *E. coli* isolates (117 from jalapeño pepper farms, 154 from tomato farms and 70 from cantaloupe farms) were obtained from the production chain in the Nuevo Leon and Coahuila states in Mexico (Heredia et al., 2016). Strains were isolated from water [the intake before the irrigation hose (called source) (24.9%) and the in-field irrigation hose (19.9%)]; from farmworkers hands [during harvest (15.2%), at the distribution point (8.8%), or at packaging (2.6%)]; produce [before harvest (9.4%), during harvest (4.7%), at distribution (4.1%), and during packaging (4.7%)]; and from soil (5.6) (Table 1). Control strains included *E. coli* O157:H7 ATCC 43895 (EHEC, kindly provided by Dr. Lynne McLandsborough, University of Massachusetts, Amherst, MA, USA); *E. coli* ATCC 25922 (non-pathogenic, donated by Becton Dickinson Co., Mexico); and *E. coli* O111:NM ATCC 43887 (EPEC), *E. coli* O78:H11 ATCC 35401 (ETEC) (both commercially acquired); and *E. coli* 042 (EAEC) (donated by Dr. Fernando

Navarro-García, CINVESTAV, Mexico).

All strains were maintained in brain heart infusion broth (BHI, BD, Franklin Lakes, NJ, USA) with glycerol (20% v/v, Sigma) at −80 °C. Fresh cultures were made by inoculating an aliquot (10 µl) into 5 ml tryptone soy broth (TSB, BD, Franklin Lakes, NJ, USA), followed by incubation for 18–24 h at 37 °C. Working cultures were prepared by streaking an aliquot onto BHI agar tubes and incubating for 24 h at 37 °C. After the incubation, strains were kept at 4 °C until use. New cultures were prepared every three months.

2.2. Determination of E. coli phylogroups

A triplex PCR was used as described by Clermont et al. (2000) to determine phylogroups. Briefly, *E. coli* strains were grown in TSB and incubated overnight at 37 °C. An aliquot (500 µl) from the culture was used for DNA extraction (Wang et al., 1997), homogenized with 1 ml of phosphate buffer solution (PBS 0.05 mol/l, pH 7.4) and centrifuged at 9000 ×*g* for 3 min (Eppendorf Microcentrifuge 5415C). Pelleted bacteria were resuspended in 50 µl of ultrapure water (miliQ), diluted 1:10 with Triton X-100 (1% v/v, Sigma Aldrich Mexico) and heated (95–100 °C) for 10 min. Samples were cooled by immersion in ice water after centrifugation. An aliquot of 3 µl of DNA-containing supernatant was used as a template for PCR amplification. PCR was performed in a ThermoHybaid (Model HBPX110) thermocycler. Primers were used to amplify TSPE4.C2, *chuA*, and *yjaA* genes (Clermont et al., 2000). The final volume of the PCR mixture was 20 µl containing 2.5 U Taq polymerase (BIOLINE, Mexico), $MgCl_2$ 1.5 mM, 0.2 mM dNTP (BIOLINE, Mexico), 2 µl of 10× reaction buffer, 3 µl of DNA template, and 1 µM of each TSPE4.C2, chuA and yjaA oligonucleotides. The amplification conditions included an initial denaturation step of 4 min at 94 °C, followed by 30 cycles comprised 94 °C for 5 s, 59 °C for 10s and a final extension of 5 min was done at 72 °C.

Amplicons (10 µl) were separated in a 2% agarose gel and visualized after staining with ethidium bromide (50 µg/ml) under UV light (Gel Logic 200 Imaging System, Kodak). *E. coli* ATCC 25922 (non-pathogenic) was used as a control because it contains all three genes analyzed, does not produce biofilms, and belongs to phylogroup B2. Isolates belonging to phylogroup A0 were confirmed according to the PCR protocol of Higgins et al. (2007), in which a 365 bp fragment of the beta-galactosidase gene (lac-Z) was amplified. Amplicons were separated and visualized as described above.

2.3. Identification of pathotypes of E. coli (DEC)

Overnight TSB cultures were processed for DNA extraction as described by Wang et al. (1997). This was followed by application of the multiplex PCR of Vidal et al. (2005), using probes that amplified the genes *stx 1*, *stx 2*, *eae* (EHEC), *eae*, *bfp* (EPEC), *stII*, *lt* (ETEC), *virF*, *ipaH* (EIEC), *aafII* (EAEC) and *daaE* (DAEC). The amplification reaction consisted of 10 U Taq polymerase, 1.5 mM $MgCl_2$, 1 mM dNTP, 5 µl of reaction buffer 10 X, 3 µl of template DNA, and 0.5 µM of each set of primers (in the case of EHEC and EPEC primers, the concentration used was 0.1 µM) in a final volume of 50 µl. The amplification conditions consisted of 35 cycles of 94 °C for 1.5 min, 60 °C for 1.5 min, and 72 °C for 1.5 min. The amplification products were visualized as described above.

2.4. Biofilm formation assays

Biofilm formation assays were performed according to Naves et al. (2008), with some modifications (media used and temperature of incubation). Briefly, strains were grown in tubes containing 5 ml of Mueller Hinton (MH) broth (BD, Franklin Lakes, NJ, USA), and incubated overnight (18 h) at 37 °C. Aliquots (20 µl) were inoculated into 96 well flat-bottomed polystyrene microtiter plates (Nunc™, Thermo Scientific™, UK) containing 180 µl of Luria Bertani (LB) broth (EMD Millipore Corporation, Germany) supplemented with sodium citrate (1%) and glucose (0.2%). Plates were incubated overnight at 37 °C, and optical densities (ODs) were read at 630 nm (Bio-Tek Epoch multi-volume spectrophotometer, Bio-Tek Instrument, Winooski, VT, USA). Broth was removed and wells were rinsed once with 200 µl of distilled water, air dried, and stained with 200 µl of 0.1% crystal violet (CV, Sigma Aldrich, Mexico) for 15 min. The colorant was discarded and wells were rinsed five times with 200 µl of distilled water, and then air-dried. To extract the CV from the biofilm, 200 µl of 96% ethanol (Sigma Aldrich, Mexico) was added, and then incubated 5 min at room temperature. The ODs of the solutions were measured at 570 nm. Biofilm formation was quantitated by determining the biofilm formation index (BFI) obtained from the formula.

$$BFI = (AB - CW)/G,$$

where AB was the optical density of the stained attached microorganism, CW was the optical density of the stained control wells containing microorganism-free medium only, and G corresponded to the optical density of the cell growth in suspended culture (Teh et al., 2010). According to BFI values, biofilm formation was categorized as strong (> 1.10), moderate (0.70–1.09), weak (0.35–0.69), or no biofilm formed (< 0.35) (Garcia-Heredia et al., 2016).

2.5. Determination of antibiotic resistance profile of E. coli

The Kirby-Bauer disk diffusion method reported in the Clinical and Laboratory Standards Institute (CLSI, 2012) was used for these studies. Antibiotics tested were chosen on the basis of their use to treat infections of Gram-negative bacteria (Amabile-Cuevas, 2010) and included: nalidixic acid (NA, 30 µg), sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 µg), tetracycline (TE, 30 µg), gentamicin (CN, 10 µg), ciprofloxacin (CIP, 5 µg), ampicillin (AMP, 10 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg) and chloramphenicol (C, 30 µg) (Oxoid Company, Cambridge, UK).

Bacterial strains were streaked onto MH agar plates and incubated for 18–20 h at 37 °C. Isolated colonies were homogenized with sterile saline solution (0.85% w/v), adjusted to No. 0.5 McFarland standard at 600 nm absorbance (approximately 1.5×10^8 CFU/ml), and decimal serial dilutions were made with sterile saline solution. One hundred microliter of the 10^6 CFU/ml dilution was spread onto MH agar plates using a Drigalski spatula. After 15 min, the antibiotic discs were placed on the plates using sterile forceps. Plates were incubated at 37 °C for 16–18 h, and the inhibition halos were measured using a caliper. The zones of inhibition were interpreted according Clinical and Laboratory Standards Institute (CLSI, 2014) and the bacteria categorized as sensitive (S: ≥15 mm of diameter for CN and TE, ≥16 mm of diameter for SXT; ≥17 mm of diameter for AMP; ≥18 of diameter for C; ≥19 mm of diameter for NA; ≥21 mm of diameter for CAZ and CIP and ≥26 mm of diameter for CTX); intermediate (I: 11–15 mm of diameter for SXT; 12–14 mm of diameter for TE; 13–14 mm of diameter for CN; 13–17 mm of diameter for C; 14–16 mm of diameter for AMP; 14–18 mm of diameter for NA, 16–20 mm of diameter for CIP, 18–20 mm of diameter for CAZ and 23–25 mm of diameter for CTX); or resistant (R: ≤10 mm of diameter for SXT; ≤11 mm of diameter for TE; ≤12 mm of diameter for CN and C; ≤13 mm of diameter for AMP and NA; ≤15 mm of diameter for CIP; ≤17 mm of diameter for CAZ and ≤22 mm of diameter for CTX).

2.6. Statistical analyses

At least two separate experiments, each with triplicates, were conducted for each assay type. Statistical analyses were performed using SPSS software (version 22.0.0.0, SPSS Inc., Chicago, IL). Results were analyzed by the Chi-square (X^2) test to determine associations between phylogenetic groups, type of sample, biofilm formation and antibiotic resistance. Differences were considered significant if the P value

was < 0.05.

3. Results

3.1. Phylogroups of E. coli isolates

Phylogroup analyses of the 341 isolates indicated that 293 (86%) isolates belonged to phylogroup A; 25 (7.3%) to B1; 3 (0.9%) to B2; and 20 (5.9%) to D (Table 1). These groups were sub-classified into 7 subgroups as follow: 199 (58.4%) isolates belonged to phylogroup A0; 94 (27.6%) to A1; 25 (7.3%) to B1; 1 (0.3%) to $B2_2$; 2 (0.6%) to $B2_3$; 20 (5.9%) to D1; whereas none of the strains analyzed belonged to genotype D2 (Table 1). A0 was the phylogroup with highest prevalence (58.4%) whereas $B2_2$ showed the lowest prevalence (0.3%).

Phylogroups A0, A1, B1, and D1 were represented by isolates from all the three produce chains analyzed, while the subgroup $B2_2$ was detected in only one sample (0.3%, from water source of a tomato farm) and the subgroup $B2_3$ was detected in two samples (0.6%; from cantaloupe at distribution and soil from jalapeño farm). The most prevalent phylogroup was A0 with 60.7%, 57.1% and 57.1% of all isolates from jalapeño pepper, tomato and cantaloupe chains, respectively (Table 1). However, phylogroup A1 was predominant among isolates from produce (before harvest) and soil of jalapeño pepper, and from water (source), farmworkers hands (distribution), and produce at packaging point of cantaloupe. The phylogroup B1 was predominant in isolates of the cantaloupe at their distribution point (Table 1). In general, no statistically significant association (P > 0.05) was observed between phylogroup and origin of the isolates, or between phylogroup and type of produce. No differences among the collection points of the water source, produce at distribution, produce pre-harvest and soil were detected.

3.2. Pathotypes of E. coli isolates

From the 341 strains of *E. coli* analyzed, only 1.2% (4 isolates) were positive for at least one of the tested genes. Three isolates (0.9%, two from the source water from a tomato farm, and one from the soil from a jalapeño pepper farm) were positive for the *eae* gene and belonged to the EPEC pathotype. However, these strains were classified as atypical EPEC (aEPEC) since only the *eae* (intimin) gene was amplified, whereas the *bfpA* gene was not detected. Another isolate (0.3%, from the soil from a jalapeño pepper farm) was positive for the *lt* gene, and was classified as pathotype ETEC. Other pathotypes were not detected. The EPEC strains detected belonged to the A0, $B2_2$, and $B2_3$ phylogroups, whereas the ETEC strain was grouped as phylogroup A1.

3.3. Biofilm formation by strains

Biofilm formation was analyzed on 340 isolates. 117 (34.4%) formed strong, 80 (23.5%) moderate, and 61 (17.9%) weak biofilms; 82 (24.1%) did not form biofilms (Table 2). Most strains classified as strong biofilm formers (40.3%) were isolated from tomato farms, followed by those isolated from jalapeño and cantaloupe farms (31.9 and 25.7%, respectively). Strains that formed moderate and weak biofilms were mainly isolated from cantaloupe farms (24.3 and 20%, respectively) followed by tomato (24 and 16.9%, respectively) and jalapeño pepper farms (22.4 and 18.1%, respectively, Tables 2 and 3). Strains that did not form biofilms were mainly from cantaloupe farms (30%), followed by jalapeño pepper and tomato farms (27.6 and 18.8%, respectively) (Table 2). Strong biofilm forming isolates were present in water from tomato and jalapeño farms, whereas the water from cantaloupe farms contained mainly weak biofilm forming organisms. In the case of hands, produce, and soil isolates, the ability of isolates to form biofilms varied (Table 2). A similar pattern was observed between the degree of biofilm formation (strong, moderate, weak and non-forming) and phylogroup (Table 3): A0 was the most common for each biofilm-forming category, followed by A1. The relationships between pathotypes and biofilm formation were inconsistent. The EPEC isolates (one from source water of tomato farm, and one from soil of jalapeño farm) produced weak biofilms, whereas another isolate from the source water of a tomato farm produced moderate biofilms. The ETEC isolate from the soil of a jalapeño pepper farm formed a strong biofilm.

3.4. Antibiotic resistance profiles of E. coli isolates

Of the 341 strains analyzed, the greatest proportion of strains were resistant to tetracycline (79/341, 23.2%), followed by ampicillin (68/341,19.9%), ceftazidime (39/341,11.4%), chloramphenicol (31/341, 9.1%), sulfamethoxazole/trimethoprim (29/341, 8.5%), cefotaxime (27/341, 7.9%), nalidixic acid (24/341, 7%), gentamicin (23/341, 6.7%) and ciprofloxacin (4/341, 1.2%; Table 4). It is noteworthy that only 4 isolates (all from tomato at the packing point) were resistant to ciprofloxacin.

Isolates from tomato and jalapeño were more resistant to antibiotics than those from cantaloupe. Isolates from the tomato chain showed greater resistance to antibiotics, especially to ampicillin and tetracycline (29.9 and 28.6% of isolates, respectively). Almost 24% of isolates from jalapeño samples showed resistance against tetracycline, whereas strains isolated from the cantaloupe chain generally showed lower resistance against all antibiotics analyzed (Table 4).

Multidrug resistance, defined as resistance to two or more classes of antimicrobial agents, was observed mainly in strains isolated from jalapeño and tomato chains, whereas most isolates from cantaloupe chains showed resistance to only one or two antibiotics (Table 4). Bacterial isolates with multidrug resistance came from all points of the production chain; eight isolates from the tomato chain and four from jalapeño pepper chains showed resistance to six antibiotics. An interesting finding was that six strains isolated from tomato (3 at the distribution point and 3 at packaging) showed resistance against at least 5 of the antibiotics analyzed (Table 4).

Of the multidrug resistant isolates (6–7 antibiotics), seven corresponded to phylogroup A1, and five isolates to A0; these were isolated mainly from tomato and jalapeño pepper farms. The ETEC isolate from the soil of a jalapeño pepper farm (phylogroup A1) was resistant to four antibiotics, whereas the EPEC isolates were sensitive to all antibiotics tested (data not shown).

4. Discussion

Escherichia coli is an important inhabitant of the gastrointestinal tract of humans and warm-blooded animals; however, it also can be present, in a transient stage, outside the host and is capable of contaminating water and soil (Tenaillon et al., 2010).

Comparative genomic studies of *E. coli* strains shows that only around 2000 of the 4721 genes are conserved (called core genoma, Hendrickson, 2009). The rest of these genes show a high degree of genomic plasticity, readily gaining and losing genes (Rasko et al., 2008; Touchon et al., 2009). Because of this, a genomic classification of *E. coli* strains has been used as a simple and inexpensive method for assigning *E. coli* isolates to a specific phylogroup. Evidence suggests that organisms of the same phylogroup share phenotypic and genotypic characteristics, ecological niches, life history traits and disease causing ability (Tenaillon et al., 2010). Several phylogroups have been found in specific hosts and appear to display the same level of environmental adaptability (Bergholz et al., 2011).

In this study, of the 341 isolates analyzed, phylogroup A was the most prevalent (86%), whereas phylogroup B2 (5.9%) was the least commonly found. When the subgroups were analyzed, the most common were A0 and A1, while the least common subgroup was $B2_2$. These results differ from a previous report from the Mid-Atlantic region of the USA, where 445 *E. coli* strains isolated from water, animal bodies, and human and animal (pets and zoo) feces were analyzed, and 31%

Table 2
Biofilm formation by *E. coli* strains isolated along the in-field production chain of jalapeño pepper, tomato and cantaloupe.

Sample type	Origin of isolates (number of isolates from jalapeño, tomato and cantaloupe)	Jalapeño pepper	Tomato	Cantaloupe	Total per sample type
		% strains forming biofilm (S, M, W, NF)[a]			
Water	Source (22, 58, 5)	**S (45.5)**[b], M (22.7), W (18.2), NF (13.6)	**S (39.7)**, M (24.1), W (6.9), NF (29.3)	S (20), M (0), **W (60)**, NF (20)	**S (34/85, 40)**[c], M (19/85, 22.4), W (11/85, 12.9), NF (21/85, 24.7)
	Irrigation (9, 44, 15)	**S (44.4)**, M (11.1), W (22.2), NF (22.2)	**S (36.4)**, M (29.5), W (22.7), NF (11.4)	**S (26.7)**, **M (26.7)**, **W (26.7)**, NF (20)	**S (24/68, 35.3)**, M (18/68, 26.5), W (16/68, 23.5), NF (10/68, 14.7)
Hands	Harvest (28, 8, 15)	S (25), M (28.6), W (10.7), **NF (35.7)**	**S (37.5)**, M (25), W (25), NF (12.5)	S (26.7), **M (46.7)**, W (6.7), NF (20)	**S (14/51, 27.5)**, M (13/51, 33.3), W (6/51, 11.8), NF (14/51, 27.5)
	Distribution (14, 7, 9)	**S (28.6)**, **M (28.6)**, W (14.3), **NF (28.6)**	**S (42.9)**, M (42.9), W (14.3), NF (0)	S (33.3), M (0), W (11.1), **NF (55.6)**	**S (10/30, 33.3)**, M (7/30, 23.3), W (4/30, 13.3), NF (9/30, 30)
	Packaging (5, 0, 4)	S (20), **M (40)**, W (20), NF (20)	ND	S (25), M (0), W (25), **NF (50)**	S (2/9, 22.2), M (2/9, 22.2), W (2/9, 22.2), **NF (3/9, 33.3)**
Produce	Before harvest (19, 7, 6)	S (26.3), M (5.3), W (21.1), **NF (47.4)**	**S (71.4)**, M (14.3), W (14.3), NF (0)	S (0), M (33.3), **W (66.7)**, NF (0)	**S (10/32, 31.3)**, M (4/32, 12.5), W (9/32, 28.1), NF (9/32, 28.1)
	During harvest (3, 10, 3)	S (0), M (33.3), **W (66.7)**, NF (0)	**S (80)**, M (20), W (0), NF (0)	**S (33.3)**, **M (33.3)**, W (0), **NF (33.3)**	**S (9/16, 56.3)**, M (4/16, 25), W (2/16, 12.5), NF (1/16, 6.3)
	Distribution (6, 3, 5)	**S (33)**, M (16.7), **W (33)**, NF (16.7)	S (0), M (0), **W (66.7)**, NF (33.3)	**S (60)**, M (20), W (0), NF (20)	**S (5/14, 35.7)**, M (2/14, 14.3), W (4/14, 28.6), NF (3/14, 21.4)
	Packaging (1, 10, 5)	S (0), **M (100)**, W (0), NF (0)	S (0), **M (20)**, W (50), NF (30)	S (0), M (20), W (0), **NF (80)**	S (0), M (4/16, 25), W (5/16, 31.3), **NF (7/16, 43.8)**
Soil	Around plant sampled (9, 7, 3)	**S (44.4)**, M (22.2), W (11.1), NF (22.2)	S (57.1), M (0), W (14.3), **NF (28.6)**	**S (33.3)**, **M (33.3)**, W (0), **NF (33.3)**	**S (9/19, 47.4)**, M (3/19, 15.8), W (2/19, 10.5), NF (5/19, 26.3)
Total by produce	(116, 154, 70 = 340)	**S (37/116, 31.9)**[c], M (26/116, 22.4), W (21/116, 18.1), NF (32/116, 27.6)	**S (62/154, 40.3)**, M (37/154, 24), W (26/154, 16.9), NF (29/154, 18.8)	**S (18/70, 25.7)**, M (17/70, 24.3), W (14/70, 20), NF (21/70, 30)	**S (117/340, 34.4)**, M (80/340, 23.5), W (61/340, 17.9), N (82/340, 24.1)

Bold letter: biofilm formation with the highest percentage of isolates.
[a] S: strong, M: moderate, W: weak, NF: no forming.
[b] Type of biofilm(s) formed by the highest number of isolates.
[c] Number of positive samples/total of samples, percentage.

Table 3
Biofilm formation and phylogroup from *E. coli* isolates from the chain product of jalapeño pepper, tomato and cantaloupe and their genotypes.

Type of biofilm	Jalapeño pepper		Tomato		Cantaloupe		Total
	n (%)	Phylogroup	n (%)	Phylogroup	n (%)	Phylogroup	# (%)
Strong	37/116 (31.6)	A0 = 18 A1 = 17 B1 = 1 D1 = 1	62/154 (40.3)	A0 = 29 A1 = 20 B1 = 7 D1 = 6	18/70 (25.7)	A0 = 12 B1 = 5 D1 = 1	A0 (59/117, 50.4) A1 (37/117, 31.6) B1 (13/117, 11.1) D1(8/117, 6.8) 117 (34.4)
Moderate	26/116 (22.4)	A0 = 20 A1 = 1 B1 = 1 D1 = 4	37/154 (24)	A0 = 20 A1 = 12 B1 = 4 $B2_2$ = 1	17/70 (24.3)	A0 = 12 A1 = 3 B1 = 1 $B2_3$ = 1	A0 (52/80, 65) A1 (16/80, 20) B1 (6/80, 7.5) B22 (1/80, 1.3) B23 (1/80, 1.3) D1 (4/80, 5) 80 (23.5)
Weak	21/116 (18.1)	A0 = 16 A1 = 1 $B2_3$ = 1 D1 = 3	26/154 (16.9)	A0 = 14 A1 = 7 B1 = 4 D1 = 1	14/70 (20)	A0 = 6 A1 = 3 B1 = 2 D1 = 3	A0 (36/61, 59) A1 (11/61, 18) B1 (6/61, 9.8) B23 (1/61, 1.6) D1 (7/61, 11.5) 61 (17.9)
None	32/116 (27.6)	A0 = 16 A1 = 15 D1 = 1	29/154 (18.8)	A0 = 25 A1 = 4	21/70 (30)	A0 = 10 A1 = 11	A0 (51/82, 62.2) A1 (30/82, 36.6) D1 (1/82, 1.2) 82 (24.1)

n: Strains forming biofilm/number total of isolates by produce.
#: Strains that formed the specific biofilm.

Table 4
Antibiotic resistance of *E. coli* isolates from the chain product of jalapeño pepper, tomato and cantaloupe.

Product	Type of sample	No isolates (% resistance)									
		n	NA	TE	CN	CIP	AMP	CTX	CAZ	C	SXT
Jalapeño pepper	Source water	22		4 (18.2)			4 (18.2)	3 (13.6)	3 (13.6)		2 (9.1)
	Irrigation water	9		1 (11.1)					2 (22.2)		
	Preharvest product	19		8 (42.1)	3 (15.8)		2 (10.5)	3 (15.8)	2 (10.5)		
	Hands in harvest	29	2(6.9)	6 (20.7)	3 (10.3)		3 (10.3)	1 (3.4)	3 (10.3)	3 (10.3)	
	During harvest product	3		1 (33.3)			1 (33.3)		1 (33.3)	1 (33.3)	1 (33.3)
	Hands in distribution	14	3 (21.4)	4 (28.6)	3 (21.4)		6 (42.9)	6 (42.9)	5 (35.7)	3 (21.4)	
	Product in distribution	6	2 (33.3)	3 (50)				1 (16.7)		2 (33.3)	2 (33.3)
	Hands in packaging	5						1 (20)	2 (40)		
	Product in packaging	1									
	Soil	9		1 (11.1)	1 (11.1)		1 (11.1)				1 (11.1)
	Total	117	7 (5.9)	28 (23.9)	10 (8.5)	0	17 (14.5)	15 (12.8)	18 (15.4)	9 (7.7)	6 (5.1)
Tomato	Source water	58	1 (1.7)	9 (15.5)	1 (1.7)		12 (20.7)	6 (10.3)	9 (15.5)	1 (0.7)	3 (5.2)
	Irrigation water	44	2 (4.5)	11 (25)	1 (2.3)		9 (20.5)	2 (4.5)	6 (13.6)	7 (15.9)	11 (25)
	Preharvest product	7		5 (71.4)			1 (14.3)				
	Hands in harvest	8					2 (25)				
	During harvest product	10		5 (50)			6 (60)			1 (10)	4 (40)
	Hands in distribution	7	1 (14.3)	1 (14.3)	1 (14.3)		2 (28.6)	1 (14.3)	3 (42.9)	1 (14.3)	
	Product in distribution	3	3 (100)	3 (100)	3 (100)		3 (100)			3 (100)	
	Hands in packaging	0									
	Product in packaging	10	8 (80)	10 (100)	7 (70)	4 (40)	10 (100)	3 (30)	3 (30)	9 (90)	5 (50)
	Soil	7					1 (14.3)				
	Total	154	15(9.7)	44 (28.6)	13 (8.4)	4 (2.6)	46 (29.9)	12 (7.8)	21 (13.6)	22 (14.3)	23 (14.9)
Cantaloupe	Source water	5		3 (60)			3 (60)				
	Irrigation water	15		1 (6.7)			1 (6.7)				
	Preharvest product	6									
	Hands in harvest	15	1 (6.7)								
	During harvest product	3		1 (33.3)							
	Hands in distribution	9									
	Product in distribution	5	1 (20)				1 (20)				
	Hands in packaging	4		2 (50)							
	Product in packaging	5									
	Soil	3									
	Total	70	2 (2.9)	7 (10)	0	0	5 (7.1)	0	0	0	0
TOTAL		341	24 (7)	79 (23.2)	23 (6.7)	4 (1.2)	68 (19.9)	27 (7.9)	39 (11.4)	31 (9.1)	29 (8.5)

* % resistance of total samples by produce.
NA: nalidixic acid, TE: tetracycline, CN: gentamicin, AMP: ampicillin, CTX: cefotaxime, CAZ: ceftazidime, C: chloramphenicol, STX: sulfamethoxazole/trimethoprim.

belonged to phylogroup B1, 26% to A, 25% to D and 17% to B2 (Higgins et al., 2007).

Most extra-intestinal *E. coli* strains belongs to subgroup B2 and, to a lesser extent, to group D (Johnson and Russo, 2002), and these appear to have a greater propensity to carry more virulence factors than members of phylogroups A and B1, although some members of the latter have been classified as diarrheagenic strains (Clermont et al., 2000; Le Gall et al., 2007). Several reports indicate that strains from phylogroup B1 persist in the environment (soils and waters) (Julian et al., 2015; Walk et al., 2007).

The abundance of strains belonging to phylogroup A found in this study, is in agreement with Walk et al., 2007 which reported that phylogroups A and B are found in environmental samples. The phylogroups A and B1 are considered sister groups, whereas subgroup B2 is the most primitive taxon in terms of branching pattern (Lecointre et al., 1998). Humans can host all the phylogroups (majors and subgroups) with exception of the subgroup A0, with phylogroups A and B2 being those of higher prevalence. In fact, the presence of phylogroup $B2_3$ has been suggested as an indicator of contamination by human feces (Carlos et al., 2010). In our study, only two isolates belonged to the $B2_3$ phylogroup, one isolate originating from the distribution point of cantaloupe and the other from soil of jalapeño pepper (which corresponded to pathotype EPEC).

Detection of potentially pathogenic *E. coli* strains in the fresh produce production chain is very important. Contamination of water and soil could come from the feces of humans, wild and domestic animals. In our study, only 1.2% of the isolates were positive for an *E. coli* pathotype; three of them (two isolated from water of tomato farm and one from soil of a jalapeño pepper farm) belonged to the EPEC pathotype, and one (isolated from soil of a jalapeño farm) to the ETEC pathotype. The phylogroups of the EPEC isolates were A0, $B2_2$, and $B2_3$, whereas the ETEC strain belonged to phylogroup A1. Although in this study no pathogens were detected in final produce samples, the risk of contamination exists, since in-field vegetables can come into contact with soil and water (Franz and van Bruggen, 2008; García-Heredia et al., 2013). ETEC strains have been consistently found in surface waters, leafy vegetables, serrano and jalapeño pepper (Begum et al., 2005; Cerna-Cortes et al., 2012; Lothigius et al., 2008; Singh et al., 2010). Infection with ETEC is the leading cause of travelers' diarrhea and an important cause of diarrheal disease in lower-income countries, especially among children (CDC, 2014).

Biofilms are communities of microorganisms attached to surfaces by means of polysaccharides, proteins and nucleic acids (Sauer et al., 2007). Thirty-four percent of the strains analyzed in this study showed the ability to form strong biofilms, particularly isolates from jalapeño and tomato farms; however, 24% of the all isolates did not form biofilms at all. Most isolates from produce during harvest and from soil formed strong biofilm (56.3 and 47.4%, respectively). Since biofilm formation has been associated with tolerance to stress conditions and to increased pathogenicity (Naves et al., 2008), the ability of *E. coli* strains to form biofilms in the environment may represent a survivorship strategy, allowing them to persist longer and as a consequence, have greater likelihood of contaminating food (Méric et al., 2013). However, the *in-vitro* test used for biofilm formation does not directly predict environmental persistence of bacteria, since biofilm formation is not the only determinant of persistence. In produce, biofilms can protect against different stresses such as desiccation and bactericidal agents (Morris and Monier, 2003). It is recognized that soil represents a special reservoir for microorganisms, including those potentially pathogenic for humans (Opelt et al., 2007).

Many members of phylogroup B1 isolated from plants can form strong biofilms, produce high quantity of extracellular matrix and utilize sucrose, which can aid in colonization of surfaces (Méric et al., 2013). These usually form significantly more biofilm and extracellular matrix than mammalian-associated strains (Méric et al., 2013). In our study, many strains belonging to phylogroups A0, A1, B1 and D1 formed strong biofilms (50.4, 31.6, 11.1 and 6.8%, respectively). Agarwal et al. (2013) studied the phylogenetic background, virulence genotypes and biofilm formation of 172 *E. coli* strains, and found no significant differences in intensity and biofilm formation ability when comparing phylogroups or virulence scores. In agreement with these results, the EPEC isolates of this study showed weak and moderate biofilm production, whereas the ETEC strain formed a strong biofilm.

The evolution of antibiotic resistance among bacteria results from complex interactions with the environment where strains grow exposed to antibiotics and genomic constraints (Davies and Davies, 2010). Bacteria frequently are exposed to antibiotics due to their extensive use in human and veterinary medicine, providing a source of antibiotic resistance genes even to environmental bacteria (Walsh and Duffy, 2013). *E. coli* resistant to tetracycline, ampicillin and sulfonamide have been detected from water isolates of aquatic ecosystems in the USA (Hamelin et al., 2007). *E. coli* isolated from water and soil for agricultural use in Sinaloa, Mexico showed resistance to tetracycline, streptomycin and gentamicin (Lopez-Cuevas et al., 2009). Our study suggested that isolates from the fresh produce production chain were most often resistant to tetracycline (23.2%) and ampicillin (19.9%). Multidrug resistance was observed mainly in the isolates from jalapeño and tomato chains, whereas most isolates from the cantaloupe chain showed resistance to one or two antibiotics only. Differences observed in farms included less employee turnover rate in cantaloupe farms and a dryer region compared to tomato and pepper farms (data not shown).

Twelve strains isolated from tomato and jalapeño farms (six from produce packaging, four from farmworkers hands at the distribution point, one from source water, and one from irrigation water) belonged primarily to phylogroups A1 and A0 and showed resistance to six or seven antibiotics (mainly nalidixic acid, tetracycline, gentamicin, ampicillin and chloramphenicol). Our results agree with previous reports that indicated that phylogroup A is detected as the most prevalent in water samples, and could exhibit multidrug resistance against sulfamethoxazole, tetracycline, streptomycin and β-lactam antibiotics (Hamelin et al., 2007). *E. coli* isolates from ready-to-eat salads in Portugal, belonging to phylogroups A, B1 and D, showed resistance to tetracycline, streptomycin, sulfamethoxazole, trimethoprim, ampicillin, nalidixic acid, and less frequently to ciprofloxacin and chloramphenicol (Campos et al., 2013). Multi-drug resistant *E. coli* isolated from lettuce, irrigation water and soil from farms in Belgium also exhibited higher resistance to ampicillin and cephalothin and other antibiotics (Holvoet et al., 2013). It is important to recognize that antibiotic resistant bacteria in multispecies biofilms could protect other non-resistant strains in this environment, and that biofilms can serve as reservoirs or sources of antibiotic resistance genes to be transferred to other non-resistant bacteria (Balcazar et al., 2015).

In general, our study showed no real association between *E. coli* pathotype or phylogroup with respect to the origin of the strains, their patterns of resistance to antibiotics, and their ability to form biofilms. However, multidrug resistance was observed, with > 3.5% of the strains resistant to six or seven antibiotics. Although in low proportion, potential pathogenic *E. coli* strains were detected, and a relatively high percentage of the isolates were able to form strong biofilms. The presence in the fresh produce production chain of *E. coli* strains having multi-drug resistance, their ability to form biofilms, and the potential pathogenicity of various isolates, could present significant opportunities for contamination that results in health risks for consumers. Efforts to keep *E. coli* out of the human food chain continue to be necessary throughout the farm-to-fork continuum.

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Conflict of interest

The authors declare no conflicts of interest.

References

Agarwal, J., Mishra, B., Srivastava, S., Srivastava, R., 2013. Genotypic characteristics and biofilm formation among *Escherichia coli* isolates from Indian women with acute cystitis. Trans. R. Soc. Trop. Med. Hyg. 107, 183–187.

Amabile-Cuevas, C., 2010. Antibiotic resistance in Mexico: a brief overview of the current status and its causes. J. Infect. Dev. Ctries. 4, 126–131.

Balcazar, J.L., Subirats, J., Borrego, C.M., 2015. The role of biofilms as environmental reservoirs of antibiotic resistance. Front. Microbiol. 6, 1–9.

Begum, Y.A., Talukder, K.A., Nair, G.B., Qadri, F., Sack, R.B., Svennerholm, A.M., 2005. Enterotoxigenic *Escherichia coli* isolated from surface water in urban and rural areas of Bangladesh. J. Clin. Microbiol. 43, 3582–3583.

Bergholz, P.W., Noar, J.D., Buckley, D.H., 2011. Environmental patterns are imposed on the population structure of *Escherichia coli* after fecal deposition. Appl. Environ. Microbiol. 77, 211–219.

Boehme, S., Werner, G., Klare, I., Reissbrodt, R., Witte, W., 2004. Occurrence of antibiotic-resistant enterobacteria in agricultural foodstuffs. Mol. Nutr. Food Res. 48, 522–531.

Campos, J., Mourão, J., Pestana, N., Peixe, L., Novais, C., Antunes, P., 2013. Microbiological quality of ready-to-eat salads: an underestimated vehicle of bacteria and clinically relevant antibiotic resistance genes. Int. J. Food Microbiol. 166, 464–470.

Canizalez-Roman, A., Gonzalez-Nuñez, E., Vidal, J.E., Flores-Villaseñor, H., León-Sicairos, N., 2013. Prevalence and antibiotic resistance profiles of diarrheagenic *Escherichia coli* strains isolated from food items in northwestern Mexico. Int. J. Food Microbiol. 164, 36–45.

Carlos, C., Pires, M.M., Stoppe, N.C., Hachich, E.M., Sato, M.I., Gomes, T., Amaral, L., Ottoboni, L.M., 2010. *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of fecal contamination. BMC Microbiol. 10, 161.

CDC, 2014. Center for Disease Control and Prevention. Enterotoxigenic *E. coli* (ETEC). CDC ([www Document] URL). https://www.cdc.gov/ecoli/etec.html, Accessed date: 16 June 2016.

CDC, 2018. Center for Disease Control and Prevention. *E.coli* (*Escherichia coli*). Reports of Selected E. coli Outbreak Investigations. Available at: https://www.cdc.gov/ecoli/outbreaks.html. , Accessed date: 2 July 2018.

Cerna-Cortes, J.F., Gómez-Aldapa, C.A., Rangel-Vargas, E., del Refugio Torres-Vitela, M., Villarruel-López, A., Castro-Rosas, J., 2012. Presence of some indicator bacteria and diarrheagenic *E. coli* pathotypes on jalapeno and serrano peppers from popular markets in Pachuca City, Mexico. Food Microbiol. 32, 444–447.

Clermont, O., Bonacorsi, S., Bingen, E., 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl. Environ. Microbiol. 66, 4555–4558.

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ. Microbiol. Rep. 5, 58–65.

Clinical and Laboratory Standards Institute (CLSI), 2012. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Eleventh Edition. CLSI document M02-A11. (Wayne, PA).

Clinical and Laboratory Standards Institute (CLSI), 2014. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. CLSI document M100-S24. (Wayne, PA).

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M., 1995. Microbial biofilms. Annu. Rev. Microbiol. 49, 711–745.

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev. 74, 417–433.

Dombek, P.E., Johnson, L.K., Zimmerley, S.T., Sadowsky, M.J., 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Appl. Environ. Microbiol. 66, 2572–2577.

FDA, 2012. Food and Drug Administration. Bad Bug Book: Foodborne Pathogenic Microorganisms and Natural Toxins. Chapter: Pathogenic *E. coli* Group. 2012. FDA ([WWW Document] URL). https://www.fda.gov/downloads/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/ucm297627.pdf, Accessed date: 16 June 2016.

Franz, E., van Bruggen, A.H., 2008. Ecology of *E. coli* O157: H7 and *Salmonella enterica* in the primary vegetable production chain. CRC Crit. Rev. Microbiol. 34, 143–161.

García-Heredia, A., Orue, N., Heredia, N., García, S., 2013. Efficacy of citrus-based disinfectants to inhibit growth, swarming, and biofilm formation of *Salmonella* and decontaminate parsley. J. Food Agric. Environ. 11, 1295–1299.

Garcia-Heredia, A., Garcia, S., Merino-Mascorro, J.A., Feng, P., Heredia, N., 2016. Natural plant products inhibits growth and alters the swarming motility, biofilm formation, and expression of virulence genes in enteroaggregative and enterohemorrhagic *Escherichia coli*. Food Microbiol. 59, 124–132.

Gordon, D.M., Clermont, O., Tolley, H., Denamur, E., 2008. Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. Environ. Microbiol. 10, 2484–2496.

Hamelin, K., Bruant, G., El-Shaarawi, A., Hill, S., Edge, T.A., Fairbrother, J., Harel, C., Maynard, L., Masson, Brousseau, R., 2007. Occurrence of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from different aquatic ecosystems within the St. Clair River and Detroit River areas. Appl. Environ. Microbiol. 73, 477–484.

Hendrickson, H., 2009. Order and disorder during *Escherichia coli* divergence. PLoS Genet. 5 (1), e1000335.

Heredia, N., Caballero, C., Cardenas, C., Molina, K., Garcia, R., Solis, L., Burrowes, V., Bartz, F., Fabiszewski de Aceituno, A., Jaykus, L.A., Garcia, S.León.J., 2016. Microbial indicator profiling of fresh produce and environmental samples from farms and packing facilities in northern Mexico. J. Food Prot. 79, 1197–1209.

Higgins, J., Hohn, C., Hornor, S., Frana, M., Denver, M., Joerger, R., 2007. Genotyping of *Escherichia coli* from environmental and animal samples. J. Microbiol. Methods 70, 227–235.

Holvoet, K., Sampers, I., Callens, B., Dewulf, J., Uyttendaele, M., 2013. Moderate prevalence of antimicrobial resistance in *Escherichia coli* isolates from lettuce, irrigation water, and soil. Appl. Environ. Microbiol. 79, 6677–6683.

Johnson, J.R., Russo, T.A., 2002. Extraintestinal pathogenic *Escherichia coli*: "the other bad *E. coli*". J. Lab. Clin. Med. 139, 155–162.

Julian, T.R., Islam, M.A., Pickering, A.J., Roy, S., Fuhrmeister, E.R., Ercumen, A., Harris, A., Bisahi, J., Schwab, K.J., 2015. Genotypic and phenotypic characterization of *Escherichia coli* isolates from feces, hands, and soils in rural Bangladesh via the Colilert Quanti-Tray system. Appl. Environ. Microbiol. 81, 1735–1743.

Kaper, J.B., Nataro, J.P., Mobley, H.L., 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2, 123–140.

Le Gall, T., Clermont, O., Gouriou, S., Picard, B., Nassif, X., Denamur, E., Tenaillon, O., 2007. Extraintestinal virulence is a coincidental by-product of commensalism in B2 phylogenetic group *Escherichia coli* strains. Mol. Biol. Evol. 24, 2373–2384.

Lecointre, G., Rachdi, L., Darlu, P., Denamur, E., 1998. *Escherichia coli* molecular phylogeny using the incongruence length difference test. Mol. Biol. Evol. 15, 1685–1695.

Lee, C., 2011. Genotyping *Escherichia coli* isolates from duck, goose, and gull fecal samples with phylogenetic markers using multiplex polymerase chain reaction for application in microbial source tracking. JEMI 15, 130–135.

León, B.D., Gómez-Aldapa, C.A., Rangel-Vargas, E., Vázquez-Barrios, E., Castro-Rosas, J., 2013. Frequency of indicator bacteria, *Salmonella* and diarrhoeagenic *Escherichia coli* pathotypes on ready-to-eat cooked vegetable salads from Mexican restaurants. Lett. Appl. Microbiol. 56, 414–420.

Liu, C., Hofstra, N., Franz, E., 2013. Impacts of climate change on the microbial safety of pre-harvest leafy green vegetables as indicated by *Escherichia coli* O157 and *Salmonella* spp. Int. J. Food Microbiol. 163, 119–128.

Lopez-Cuevas, O., León Félix, J., Jimenez Edeza, M., Chaidez Quiroz, C., 2009. Detection and antibiotic resistance of *Escherichia coli* and *Salmonella* in water and agricultural soil. Rev. Fitotec. Mex. 32, 119–126.

Lothigius, Å., Janzon, A., Begum, Y., Sjöling, Å., Qadri, F., Svennerholm, A.M., Bölin, I., 2008. Enterotoxigenic *Escherichia coli* is detectable in water samples from an endemic area by real-time PCR. J. Appl. Microbiol. 104, 1128–1136.

Méric, G., Kemsley, E.K., Falush, D., Saggers, E.J., Lucchini, S., 2013. Phylogenetic distribution of traits associated with plant colonization in *Escherichia coli*. Environ. Microbiol. 15, 487–501.

Morris, C.E., Monier, J.M., 2003. The ecological significance of biofilm formation by plant-associated bacteria. Annu. Rev. Phytopathol. 41, 429–453.

Naves, P., del Prado, G., Huelves, L., Gracia, M., Ruiz, V., Blanco, J., Dahbi, G., Blanco, M., Ponte, M., Soriano, F., 2008. Correlation between virulence factors and in vitro biofilm formation by *Escherichia coli* strains. Microb. Pathog. 45, 86–91.

Nowrouzian, F.L., Adlerberth, I., Wold, A.E., 2006. Enhanced persistence in the colonic microbiota of *Escherichia coli* strains belonging to phylogenetic group B2: role of virulence factors and adherence to colonic cells. Microbes Infect. 8, 834–840.

Opelt, K., Berg, C., Berg, G., 2007. The bryophyte genus *Sphagnum* is a reservoir for powerful and extraordinary antagonists and potentially facultative human pathogens. FEMS Microbiol. Ecol. 61, 38–53.

Rasko, D.A., Rosovitz, M.J., Myers, G.S., Mongodin, E.F., Fricke, W.F., Gajer, P., Crabtree, J., Sebaihia, M., Thomson, N.R., Chaudhuri, R., Henderson, I.R., Sperandio, V., Ravel, J., 2008. The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. J. Bacteriol. 190, 6881–6893.

Russo, T.A., Johnson, J.R., 2000. Proposal for a new inclusive designation for extra-intestinal pathogenic isolates of *Escherichia coli*: ExPEC. J. Infect. Dis. 181, 1753–1754.

Sauer, K., Rickard, A.H., Davies, D.G., 2007. Biofilms and biocomplexity. Microbe 2, 347–353.

Schwaiger, K., Helmke, K., Hölzel, C.S., Bauer, J., 2011. Antibiotic resistance in bacteria isolated from vegetables with regards to the marketing stage (farm vs. supermarket). Int. J. Food Microbiol. 148, 191–196.

Singh, G., Vajpayee, P., Ram, S., Shanker, R., 2010. Environmental reservoirs for enterotoxigenic *Escherichia coli* in south Asian Gangetic riverine system. Environ. Sci. Technol. 44, 6475–6480.

Teh, K.H., Flint, S., French, N., 2010. Biofilm formation by *Campylobacter jejuni* in controlled mixed-microbial populations. Int. J. Food Microbiol. 143, 118–124.

Tenaillon, O., Skurnik, D., Picard, B., Denamur, E., 2010. The population genetics of commensal *Escherichia coli*. Nat. Rev. Microbiol. 8, 207–217.

Touchon, M., Hoede, C., Tenaillon, O., Barbe, V., Baeriswyl, S., Bidet, P., Bingen, E., Bonacorsi, S., Bouchier, C., Bouvet, O., Calteau, A., Chiapello, H., Clermont, O., Cruveiller, S., Danchin, A., Diard, M., Dossat, C., Karoui, M., Frapy, E., Garry, L., Marc Ghigo, J., Gilles, A.M., Johnson, J., Le Bouguénec, C., Lescat, M., Mangenot, S., Martinez-Jéhanne, V., Matic, I., Nassif, X., Oztas, S., Agnès Petit, M., Pichon, Z.R., Saint Ruf, C., Schneider, D., Tourret, J., Vacherie, B., Vallenet, D., Médigue, C., Rocha, E.P.C., Denamur, E., 2009. Organized genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. PLoS Genet. 5 (1), e1000344.

Vidal, M., Kruger, E., Durán, C., Lagos, R., Levine, M., Prado, V., Toro, C., Vidal, R., 2005.

Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. J. Clin. Microbiol. 43, 5362–5365.
Walk, S.T., Alm, E.W., Calhoun, L.M., Mladonicky, J.M., Whittam, T.S., 2007. Genetic diversity and population structure of *Escherichia coli* isolated from freshwater beaches. Environ. Microbiol. 9, 2274–2288.
Walsh, F., Duffy, B., 2013. The culturable soil antibiotic resistome: a community of multi-drug resistant bacteria. PLoS One 8, e65567.
Wang, R.F., Cao, W.W., Cerniglia, C.E., 1997. A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. J. Appl. Microbiol. 83, 727–736.