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Effects of Plant Age and Root Damage on Internalization of Shiga Toxin-Producing *Escherichia coli* in Leafy Vegetables and Herbs

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Abstract: Our previous study reported that fresh produce grown in aquaponic and hydroponic systems can pose potential food safety hazards due to an accidental introduction of contaminated fish and cross-contamination between the systems. In this study, we examined the effects of plant species and age on the likelihood and level of internalization of Shiga toxin-producing *Escherichia coli* (STEC) in aquaponic and hydroponic systems. Four plant species, basil (*Ocimum basilicum* L. cv. Genovese), cilantro (*Coriandrum Sativum* L.), lettuce (*Lactuca sativa* cv. Cherokee), and kale (*Brassica oleracea* var. *sabellica*), received root damage treatment as seedlings before transplanting or mature plants at three weeks after transplanting by cutting off 1-cm tips of one-third of the roots. Enrichments and selective media were used for the isolation, and presumptive positive colonies were confirmed by PCR for the presence of *stx1* gene in plant tissues, recirculating water, and fish feces collected at four weeks after transplanting. In hydroponic systems, STEC was found neither in the solution nor in the roots and leaves of all four plant species, possibly through improved sanitation and hygiene practices. However, consistent with our previous findings, STEC was found in the water, on the plant roots, and in the fish feces in aquaponic systems, even after thorough sanitation prior to the study. Regardless of plant age, STEC was internalized in the roots of all plant species when the roots were damaged, but there was no difference in the degree of internalization with STEC among plant species. STEC was present in the leaves only when seedlings received root damage treatment and were grown to maturity, indicating that root damage allows STEC to internalize in the roots within a week, but a longer period is required for STEC to internalize into the leaves. We concluded that root damage on seedlings can cause the internalization of *E. coli* O157:H7 in the edible parts of leafy vegetables and herbs in soilless production systems.

Keywords: food safety; *E. coli*; aquaponics; hydroponics; agricultural water; PCR detection; indoor farming

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1. Introduction

The global food demand and security have been increased with the increasing population [1]. FAO (Food and Agriculture Organization of the United Nations) estimates that 10.8% of people worldwide suffered from undernourishment in 2018, and Sub-Saharan Africa experienced a sharp increase of undernourishment from 20.6% in 2015 to 22.8% in 2018 [2]. Facing the challenge of food demand, the urban farming fulfilled 37% of vegetable needs in Kathmandu, Nepal, 45% of local food needs in Hong Kong, and almost 60% of all Cuban vegetable demands [3–5]. Therefore, it is a potential way to feed urban residents in the world with fresh produce [6].

Meanwhile, nearly 48% of foodborne outbreaks are linked to the consumption of fresh fruits and vegetables due to the bacterial contamination of fresh produce [7,8]. The Foodborne Disease Outbreak Surveillance System (FDOSS) pointed out that 32 outbreaks

(14%), 919 illnesses (25%), and 205 hospitalizations (51%) reported in 2017 were associated with the consumption of raw vegetables, and 110 illnesses (11%) were associated with Shiga toxin-producing *E. coli* (STEC) [9]. In the United States, leafy greens and other vegetable crops are a major source of STEC infections, and 51 foodborne disease outbreaks linked to leafy greens were reported to the Centers for Disease Control and Prevention (CDC) from 2014 to 2018 [10]. Moreover, the UK and Germany reported 531 and 3785 cases of illness, respectively, in association with leafy vegetables between 2008 and 2010 [11,12].

Microbial contamination of produce can occur at any point from farm to fork. In field-grown vegetables, foodborne pathogens can be introduced from polluted irrigation or postharvest-washing water, soil, animal feces, and by handlers during harvest, post-harvest, or packing [8,13,14]. The multi-state outbreak of *E. coli* O145:H28 infections in southern Arizona was associated with STEC originated from stray dog and coyote feces in a major leafy green production field at the United States-Mexico border [15].

Soilless culture is an applied technology of food production in a controlled environment, which has a higher water and land use efficiency compared with traditional field production [5,16–20] and can eliminate the risk of wild animal fecal contamination [21,22]. Hydroponics is one of the production methods in a controlled environment and known to produce safe and clean vegetables, and more than 404.7 ha of vegetables are produced hydroponically in the United States [23]. Aquaponics is another soilless production method, which contains major three organisms: aquatic animals, microorganisms, and plants [24–26]. While hydroponics uses chemical fertilizers containing high levels of nitrogen and phosphorus [27], aquaponics uses waste products generated from aquatic animals' digestion of fish feed. Contaminated seeds, growing media, irrigation water, worker health and hygiene, field and harvest sanitation, and sanitation of packing facilities can be the potential microbial contamination source in both systems [8,11,13,14,28,29]; however, aquatic animals can be an additional contamination source in aquaponics if an externally or internally contaminated aquatic animal is accidentally introduced and releases bacteria directly or indirectly via animal feces into the solution [13,25,30].

Contamination with enteric pathogens may occur at relatively low levels in aquaponics and hydroponic systems [31]. Nonetheless, our previous study showed that STEC was present in the water of both aquaponic and hydroponic systems, possibly due to the introduction of contaminated fish and cross-contamination between the systems, but not present in plant tissues [30]. Several studies suggested that mechanical or biological damage in the roots is associated with the internalization of enteric pathogens in roots but shoots. Moriarty et al. (2019) found that *E. coli* O157:H7 internalized in the roots of lettuce grown in hydroponic systems regardless of the degree of root damages [32]. Similarly, Hora et al. (2005) found that *E. coli* O157:H7 was present in the roots but not in the leaves of spinach grown in soil regardless of root damage treatment (mechanical or biological root damages) [33]. Meanwhile, *E. coli* O157:H7 was found in the leaves at 10 days after inoculation with *E. coli* O157:H7 when lettuce plants were physically damaged from being cracked along the central vein or infected with bacteria (*Xanthomonas campestris* pv. *vitians*) [34]. Although plant species and age have not been considered in most studies, these factors can affect the degree of internalization of human pathogens possibly due to the different levels of defense mechanisms. This aspect was demonstrated in a study by Jablasone et al. (2005), in which they inoculated seeds with *E. coli* O157:H7 and germinated on the solidified hydroponic medium and found that cress and spinach had a higher population of *E. coli* O157:H7 than did lettuce and radish [35]. A higher percentage of internalization was observed in 30-day-old green ice leaf lettuce (11%) than 12-day-old ones (7%) when they were grown in pots and irrigated with *E. coli* O157:H7-contaminated water [36].

Therefore, the objective of this study was to examine the likelihood and level of internalization of STEC into plant tissues caused by root damage in association with plant species and age. The results will help understand critical factors affecting the internalization of STEC in plants and minimize the risk of contamination in soilless culture systems.

2. Materials and Methods

2.1. System Setup

Six experimental units consisting of three aquaponic and three hydroponic systems had been set up and operated in the greenhouse in West Lafayette, IN (lat. 40° N, long. 86° W), for nearly five years. Each unit was equipped with a fish tank or a nutrient reservoir (350 L), a clarifier (20 L), a two-stage biofilter (40 L) [20,26], and a deep-water hydroponic grow bed (350 L; 1.0 m^2). A month prior to the study, systems were thoroughly sanitized and disinfected except biofilters, and then they filled with reverse osmosis water. Nile tilapia (*Oreochromis niloticus* L.) fish were obtained from the Animal Sciences Research and Education Center at Purdue University, which had been cultivated in a conventional aquaculture system for 4-months. At least a month prior to the study, fish weight was measured (an average of 300 g per fish) and evenly distributed to three different fish tanks at a stocking density of 15 kg/m^3. The biofilter was connected to a peristaltic pump (Masterflex, Cole-Parmer, Chicago, IL, USA) to recirculate nutrient solution within the system. In each hydroponic system, the nutrient solution reservoir and hydroponic culture unit were filled with reverse osmosis water blended with the nutrient stock solution at 1:100 dilution rate which was used as initial and follow-up daily replenishment used for leafy vegetables and herbs (CropKing, Lodi, OH, USA). The electrical conductivity (EC) was maintained at 1.5 dS/m by adding and replenishing nutrition solutions daily. The pH of the aquaponic and hydroponic systems was automatically adjusted by a Bluelab pH controller (Walchem, Iwaki America Inc., Holliston, MA, USA) and maintained at around 6.5, using a combination of 1 M KOH and 200 mM $Ca(OH)_2$. The total water volume in both aquaponic and hydroponic systems was 700 L with a flow rate of 138 L/h (3.3 m^3/m^2/day), giving a water retention time ((surface area × water depth)/flow rate) of 6 h in a hydroponic culture unit of each system (Figure 1) [37]. Aquaponic solution or nutrient solution flowed through the hydroponic culture unit of each system and back to the fish tank or reservoir. Each aquaponic and hydroponic system had air stones to maintain dissolved oxygen (DO) concentrations at full saturation. Aquatic heaters were set in aquaponic and hydroponic systems to maintain the temperature in the ranges of 25 to 28 °C. Water temperature, pH, EC, and DO were measured daily using the HQ40d Portable Water Quality Lab Package (HACH Corp., Loveland, CO, USA).

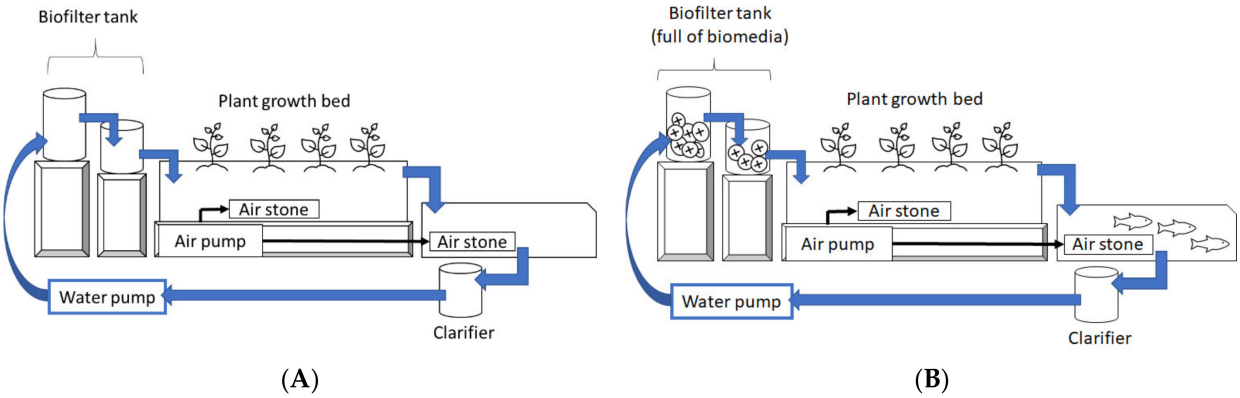

Figure 1. Schematic diagram of the experimental units, (**A**) aquaponic system and (**B**) hydroponic system.

2.2. Plant Materials and Growing Conditions

Basil (*Ocimum basilicum* L. cv. Genovese), cilantro (*Coriandrum sativum* L.), lettuce (*Lactuca sativa* cv. Cherokee), and kale (*Brassica oleracea var. sabellica*) were cultured in aquaponic and hydroponic systems for 30 days. Seeds were purchased from a commercial source (Johnny's Selected Seeds, Winslow, ME, USA) and sown in Agrifoam soilless plugs (SteadyGROWpro, Syndicate Sales, Kokomo, IN, USA) with a few day intervals to ensure uniform seedling size at the time of transplanting. Seeds were germinated

as described by Kim et al. (2018) and 2- to 3-week-old seedlings were transplanted to each system [38]. During the study period, the fish were fed daily (9:00 a.m.) with a 4.8-mm floating pellet (AquaMax Sport Fish 500, Purina Mills, St. Louis, MO, USA) containing a complete diet (41% protein, 1.1% phosphorus) at a constant weight of 60 g. The experiment was conducted between December 2018 and February 2019. The photoperiod was 14 h (8:00 a.m. to 10:00 p.m.), consisting of natural daylight with supplemental lighting using high-pressure sodium (HPS) lamps (600-W, P.L. Light Systems Inc., Beamsville, ON, Canada). A supplemental photosynthetic photon flux (PPF) of the greenhouse was measured using a quantum sensor (LI-250A light meter; LI-COR Biosciences, Lincoln, NE, USA), and maximum photosynthetically active radiation in the greenhouse was averaged at 168 μmol/m^2/s. Day (8:00 a.m. to 10:00 p.m.) and night (10:00 p.m. to 8:00 a.m.) temperatures were set at 24 and 18 °C, respectively.

2.3. Root Damage Treatment

After the third true leaf of the seedlings emerged, uniform healthy seedlings were randomly and equally divided into three groups: no root damage (Control), root damage at transplanting (T1), and root damage at preharvest (T2). Control plants were carefully removed from seedling trays, transplanted into mesh pots (diameter: 7.6 cm, height: 6.4 cm) each containing clay pebbles, then transferred to a hydroponic unit of aquaponic and hydroponic systems. The plants were grown for 4 weeks without disturbance. Meanwhile, T1 plants were removed from seedling trays, and one-third of the root system was cut off at 1-cm behind the root tips with alcohol sterilized scissors. After the treatment, T1 plants were transplanted into mesh pots and then transferred to aquaponic and hydroponic systems and grown for 4 weeks. The cut surfaces of the roots were fully submerged and maintained lower than the level of the solution. Likewise, T2 plants were transplanted into mesh pots and then transferred to and grown in the aquaponic and hydroponic systems. After 3 weeks, the whole plant was carefully removed from the systems, and one-third of the root system was cut off at 1-cm behind the root tips with sterile scissors. The plants were promptly transferred back to the systems and cultivated for another 7 days.

2.4. Plant Sample Collection and STEC E. coli Isolation

Leaf and root samples were collected at the end of the experiments. Six plant samples were blended with 45 mL buffered peptone water (PW; Difco, Detroit, MI, USA) for 15 s without surface-sterilization. Six plant samples were surface-sterilized to remove surface-located bacteria using 0.1% sodium hypochlorite for 10 s, followed by sterile water for 30 s [39]. A total of 5 g of plant tissue was blended with buffered peptone water in a 50 mL centrifuge tube, allowing the sample to be completely homogenized [40–42]. The homogenized samples were then incubated at 37 °C for 6 h to allow for the recovery of bacteria and potential enrichment. The samples were then serially diluted, and 0.1 mL was spread plated in duplicate on MacConkey agar with sorbitol, cefixime, and tellurite (CT-SMAC) agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and the plates were incubated for 24 h at 37 °C.

2.5. Microbial Detection in Water and Fish Feces Samples

Incoming water sources were tested for the bacterial pathogens before, during, and after the study, and the results were negative for STEC. Water samples from aquaponic and hydroponic systems were collected from six different locations immediately after harvest. Fish feces were collected from the clarifier tank where most solid waste was found, and excess water was carefully drained.

A 20 mg fish feces sample from each system was mixed with 180 μL water and vortexed for 15 s. Water samples and fish feces samples were plated after collection and enrichment for 6 h. The water samples and fish feces samples were serially diluted and spread plated as described above in duplicate on CT-SMAC agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for the selection of *E. coli* O157. Samples were

cultured on MacConkey agar with sorbitol, cefixime, and tellurite (CT-SMAC) agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 24 h at 37 °C, and the colorless colonies were confirmed after incubation at 37 °C for 20 h and detected by PCR targeting of the *stx1* gene.

2.6. PCR Assay for Detection of Virulence Genes

A total of 90 isolated colonies each from presumptive positive STEC were picked into 20 µL of distilled water and then inactivated at 90 °C using a dry heat bath (Benchmark Scientific Inc., Edison, NJ, USA) for 5 min. PCR was then performed to amplify the *stx1* gene from *E. coli* O157 [43] (Table 1).

Table 1. PCR primers for Shiga toxin-producing *Escherichia coli* (STEC).

	Sequence	PCR Program	Reference
Shiga Toxin-Producing *E. coli* (STEC)			
stx1-F	CAGTTAATGTGGTGGCGAAG	95 °C for 3 min, 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, repeat steps 2–4 30 times, 72 °C for 10 min	[43]
stx1-R	CACCAGACAATGTAACCGCTG		

2.7. Experimental Design and Data Analysis

Treatments consisted of two production systems, four plant species, and three root treatments. Each system had three replicates based on a split-plot design. Plant species and root treatments were arranged in a completely randomized design within the system. Six plants per system were randomly chosen as biological replicates, totaling 18 replicates per system. Three subsamples (technical replicates) per plant were taken for the analysis of bacterial pathogens. The statistical analysis used post-hoc pairwise comparisons in R 3.6.1 (R, Comprehensive R Archive Network, USA, http://cran.us.r-project.org/; Last accessed on 15 December 2020) at a significance level of 0.05.

3. Results and Discussion

3.1. Water Conditions for Aquaponic and Hydroponic Systems

The average values of water quality parameters in the aquaponic and hydroponic systems are shown in Table 2. It is well-documented that environmental factors, such as DO, pH, water temperature, and EC, affect bacterial populations [44,45]. We controlled and maintained these factors similar between aquaponic and hydroponic systems (Table 2) to minimize environmental variations that may affect the STEC population in the recycling nutrient solution. For example, enteric pathogens can survive for a longer time at cold and freezing temperatures than at warmer (20–30 °C) temperatures [46], and STEC can survive in water for 12 weeks at 25 °C [47]. *E. coli* and *Salmonella* sv. Typhimurium can grow better at pH 4.7 than 5.2 [48]. EC is a common indicator of soluble salts dissolved in a nutrient solution and a key factor affecting the survival of bacteria in the environment [8]. A high level of nutrients is ideal for bacterial growth; therefore, the viability of *E. coli* O157:H7 increases in nutrient-rich soils and hydroponics [49–51]. The measured DO, pH, water temperature, and EC in our study were sufficient to support the growth of STEC. This implies that pathogenic bacteria can grow in greenhouse-based aquaponic and hydroponic systems if they are accidently introduced.

Table 2. Water quality parameters in aquaponic and hydroponic systems during the experimental period.

Production System	DO [z] (mg/L)	pH	Temperature (°C)	EC (dS/m)
Aquaponics	7.81 ± 0.03 [y] (7.73–8.09)	6.5 ± 0.0 [x]	22.9 ± 0.1 (22.7–23.1)	1.52 ± 0.04 (1.48–1.58)
Hydroponics	7.78 ± 0.04 (7.70–7.84)	6.5 ± 0.0 [x]	23.0 ± 0.1 (22.7–23.3)	1.54 ± 0.02 (1.50–1.56)
Significance	ns	ns	ns	ns

Abbreviations: [z] dissolved oxygen (DO); electrical conductivity (EC). [y] Each value is the mean of four replicates (2 sample replicates per system) ± SE (standard error). [x] means that pH was automatically adjusted by pH controllers in this study. ns means no significant difference.

3.2. The Effects of Growing System and Plant Species on the Occurrence of STEC

We assessed the presence of bacterial pathogens and the likelihood of contamination in aquaponic and hydroponic systems. Unlike our previous study, in which STEC was found in recycling water of both systems due to the cross-contamination between adjacent aquaponic and hydroponic systems [30], STEC was absent in recycling water of hydroponic systems in this study but present in recycling water and fish feces of aquaponic systems (Table 3). The discrepancy could be attributed to the differences in sanitation and management practices between these studies. Recognizing the presence of STEC in these systems, we thoroughly sanitized the systems before this study and avoided cross-contamination through improved management practices. It turned out to be that these practices were effective in controlling STEC for hydroponic systems but not for aquaponic systems. Although plant growth beds and fish tanks were thoroughly sanitized, the fish had not been cleaned or changed since the last experiments [30]; therefore, the preexisting condition of the fish should be attributable to the current results. As we discussed previously [52], tilapia are considered filter feeders and can efficiently harvest filamentous and planktonic algae [53]. It is likely that a fish ingested STEC from filamentous or planktonic algae, and the fish feces was released into water contaminating the system (M.B. Timmons, personal communication, 9 September 2020). If a fish is grown in polluted water, STEC can survive on fish skin and internal organs (e.g., kidney, liver, and digestive tract) [47,54]. When tilapia, catfish, common carp, and silver carp were grown in the wastewater infested with a high level of *E. coli* (10^6 g^{-1}), the concentration of *E. coli* as high as 10^8–10^9/g was recovered in the digestive tract [54]. Especially if a contaminated fish is introduced to a recirculating aquaponic system like this, it will pollute the water and subsequently contaminate the entire system, making it difficult to eliminate the foodborne pathogens from the system. Therefore, these results indicate that improved sanitation and management practices can ensure producing safe foods in hydroponics, but different strategies may be required in aquaponics to reduce foodborne illness. For example, if the system is contaminated with foodborne pathogens, the existing fish should be removed, the entire system needs to be thoroughly sanitized, and clean fish stock should be introduced. Adding UV-radiation is another efficient way to inactivate STEC in an aquaponic system, as it can decrease 10 to 100 times coliform bacteria in aquaponic solution [55].

Table 3. Occurrence of *STEC* in recirculating water, fish feces, and the roots and leaves of basil, cilantro, lettuce, and kale grown in aquaponic and hydroponic systems.

Vegetable	Tissue Type	*Control*		*Root Damage at Transplanting (T1)*		*Root Damage at Preharvest (T2)*	
		Aquaponics	**Hydroponics**	**Aquaponics**	**Hydroponics**	**Aquaponics**	**Hydroponics**
Basil	Internal leaf	–[z]	–	+(18/18)	–	–	–
	Internal root	–	–	+(18/18)	–	+(18/18)	–
	Root surface	+(18/18)	–	+(18/18)	–	+(18/18)	–
	Water	+(18/18)	–	+(18/18)	–	+(18/18)	–
	Fish feces	+(18/18)	NA	+(18/18)	NA	+(18/18)	NA
Cilantro	Internal leaf	–	–	+(18/18)	–	–	–
	Internal root	–	–	+(18/18)	–	+(18/18)	–
	Root surface	+(18/18)	–	+(18/18)	–	+(18/18)	–
	Water	+(18/18)	–	+(18/18)	–	+(18/18)	–
	Fish feces	+(18/18)	NA	+(18/18)	NA	+(18/18)	NA
Lettuce	Internal leaf	–	–	+(18/18)	–	–	–
	Internal root	–	–	+(18/18)	–	+(18/18)	–
	Root surface	+(18/18)	–	+(18/18)	–	+(18/18)	–
	Water	+(18/18)	–	+(18/18)	–	+(18/18)	–
	Fish feces	+(18/18)	NA	+(18/18)	NA	+(18/18)	NA
Fort Smith, Arkansas Kale	Internal leaf	–	–	+(18/18)	–	–	–
	Internal root	–	–	+(18/18)	–	+(18/18)	–
	Root surface	+(18/18)	–	+(18/18)	–	+(18/18)	–
	Water	+(18/18)	–	+(18/18)	–	+(18/18)	–
	Fish feces	+(18/18)	NA	+(18/18)	NA	+(18/18)	NA

[z] The symbols, + and –, indicate presence and absence, respectively. NA means no fish feces in hydroponics. Each symbol in the table is the result of 18 samples (six biological replicates × three technical replicates). The numbers in the parentheses mean the number of STEC-positive samples/the number of total samples. Ten isolates per positive plate were examined for PCR confirmation.

Although STEC was found on the root surface of all four plant species grown in aquaponic systems, plant species had no effect on the degree of internalization (Table 3). There is very little information on the influence of plant species on internalization and persistence of STEC in hydroponic or aquaponic systems. Most studies conducted in the soil-based systems reported that STEC colonization and internalization were varied by plant species [56,57]. Wright et al. (2013) found that *E coli* can colonize on the roots and translocate to the aerial parts in a similar process as endophytic bacteria [56]. The root exudates, which contain sugars, protein, or other nutrients, can provide a rich environment for the growth of not only plant pathogens but also human pathogens [58,59]. Merget et al. (2018) examined four plant species, fenugreek, alfalfa, spinach, and lettuce in soil after inoculation with STEC and found that the internalization of STEC in spinach and lettuce was 10 times higher than that in fenugreek and alfalfa, and that spinach extracts supported a higher level of biofilm formation compared to lettuce extracts [57]. Wright et al. (2017) demonstrated the variations in the level of internalization with *E. coli* O157:H7 Sakai among plant species and that the bacterial growth was restricted in spinach and lettuce at the internal boundary of the epidermal cell but not in *Nicotiana benthamiana*, in which a 400-fold increase in the number of bacteria was found in their leaves compared to lettuce and spinach after 20 days [60]. They also reported that the internal population of *E. coli* O157:H7 Sakai was affected only in low dose (10^3 CFU/mL; colony forming unit per mL) but not in high dose (10^7 CFU/mL).

This study, however, did not find plant species effects on the degree of internalization with STEC (Table 3). The contradictory results may be due to the different concentrations of human pathogens, application methods, and culture systems. It should be noted that the level of STEC in our systems was extremely low to a level that requires enrichment for the detection, unlike the above studies. Moreover, we grew all plant species in soilless systems, which allow exudates from damaged roots to release easily into the recycling

water, attracting various microbes including foodborne pathogens to the roots, if present. Besides, unlike soil-based systems, STEC can freely flow through the water-based systems, attach, and form biofilms on the roots, and detect entry points in the roots. In supporting our view, a different level of internalization with *E. coli* O157:H7 was found in spinach, where both the roots and shoots were internalized in hydroponic medium, but only in the roots when grown in soil [61]. Therefore, unlike soil-based systems, the impact of root exudates in water-based systems is considered significant for STEC in finding the point of entry even with low-density contamination, due to no spatial boundaries of root spread and dynamic water movement.

3.3. The Effects of Plant Age and Root Damage on the Degree of Internalization of STEC in Plants

STEC was present in the roots of both T1 and T2 plants in aquaponics, while it was present in the leaves of T1 plants only (Table 3). The results demonstrate the interaction between plant age and root damage, which together play important roles in determining the degree of internalization of STEC in plants. STEC can colonize and internalize the roots and rhizosphere of plants, and the growth of STEC can be supported by root metabolites [58,59]. In our study, four plant species were subjected to root damage treatments either at transplanting or at preharvest. The seedlings were 14-day-old after the seeds were germinated at the time of transplanting for both T1 and T2 plants. In T1 treatment, following the root damage treatment, the seedlings were grown in contaminated water for 30 days. In T2 treatment, plants were 20 days old at the time of root injury and grown for additional 7 days in contaminated water until harvest. Root damage can occur during transplanting or handling; therefore, this approach suggests a potential mechanism and provides practical implications to develop strategies by targeting and minimizing the potential risk of food pathogen internalization in soilless culture systems.

Most research on the internalization of human pathogens focused on the effect of injury on the internalization and reported that damaged roots caused by mechanical or biological damages were associated with the internalization of enteric pathogens [14,62–64]. Internalization of *E. coli* to roots was detected in lettuce grown in hydroponics after root cutting and in spinach after exposure to the northern root-knot nematode [33,55]. Ward and Mahler (1982), inoculated phage f2 at the midpoint of corn and bean plants in hydroponic systems and found a large number of phage were taken from cut roots within 1 day, but no phage uptake through intact roots [65]. Bernstein et al (2006) also indicated that the plants with damaged roots had 27.8 times higher number of *Salmonella enterica* serovar Newport in roots than the plants with intact roots at 2 days after inoculation [63].

Our study showed that plant age plays a critical role in the internalization of STEC in water-based systems and that infection during the early stage of plant development can increase the persistence of human pathogens in the plant. STEC was found in the leave of T1 plants but not in the leaves of control or T2 plants (Table 3), suggesting that it takes more than 7 days for enteric pathogens to translocate to edible parts of mature lettuce plants through damaged root tissues. Root damage allows STEC to attach and internalize in the roots within a week, but a longer period is needed for STEC to internalize into the shoots. When plants are young, their root systems are small and more susceptible to plant pathogens or human pathogens [66]. As plants mature, they can develop additional defense mechanisms against plant or human pathogens. For example, Islam et al. (2010) evaluated the effect of plant age on anti-human pathogenic activity of endophytes in balloon flower roots and found the highest anti-human pathogenic activity (70%) in 3-year-old roots [67]. Due to the differences in plant maturity at the time of root damage and the growing period afterward, the two-time points may allow attachment, internalization, and persistence of STEC to occur differently. The differences in the internalization in these age groups may be also related to the differences in the level of reactive oxygen species and callose deposition against *E. coli* O157:H7 [68].

However, some studies have demonstrated that the internalization of STEC is negatively associated with the age of plants [36,64]. Bernstein et al (2007) reported that *Salmonella*

was present in 33-day-old lettuce but not in 17-day-old lettuce at 2 days after inoculation of 17- and 33-day-old lettuce with 10^6–10^8 CFU/g *Salmonella* in soil [64]. Mootian et al. (2009) observed a higher number of *E. coli* O157:H7 internalization in 30-day-old lettuce compared to 12-day-old lettuce in soil culture system after exposure to 10^1–10^4 CFU/g *E. coli* O157:H 7 [36]. As discussed earlier, the discrepancy among the studies may be related to the differences in the concentration of human pathogens, application method, and culture systems, and this aspect needs further clarification.

4. Conclusions

Sanitation and good hygiene intervention is effective in interrupting STEC contamination and internalization in hydroponics. Contamination with STEC can be eliminated in hydroponic systems if the entire system is thoroughly sanitized before each use. However, such intervention may not be possible in aquaponics as the biofilters should not be disturbed for the microbiome and may not be effective once polluted fish is introduced to the system. Therefore, obtaining a clean fish stock is of prime importance in aquaponic operation. STEC may be present at an undetectable level without enrichment but can colonize roots even at low density.

Enteric pathogens can internalize in the roots by mechanical injury, and the internalization of STEC in the leaves can occur, especially when the roots are damaged at transplanting, allowing sufficient time for STEC to transmit to shoots. Since plant age at the time of root injury is critical for the degree of transmission of STEC in plant tissues, it is essential to prevent root damages during transplanting to minimize the risk of contamination of fresh vegetables and herbs grown in soilless production systems.

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