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Fate of *Salmonella enterica* and Enterohemorrhagic *Escherichia coli* Cells Artificially Internalized into Vegetable Seeds during Germination

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ABSTRACT Vegetable seeds contaminated with bacterial pathogens have been linked to fresh-produce-associated outbreaks of gastrointestinal infections. This study was undertaken to observe the physiological behavior of *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) cells artificially internalized into vegetable seeds during the germination process. Surface-decontaminated seeds of alfalfa, fenugreek, lettuce, and tomato were vacuum-infiltrated with four individual strains of *Salmonella* or EHEC. Contaminated seeds were germinated at 25°C for 9 days, and different sprout/seedling tissues were microbiologically analyzed every other day. The internalization of *Salmonella* and EHEC cells into vegetable seeds was confirmed by the absence of pathogens in seed-rinsing water and the presence of pathogens in seed homogenates after postinternalization seed surface decontamination. Results show that 317 (62%) and 343 (67%) of the 512 collected sprout/seedling tissue samples were positive for *Salmonella* and EHEC, respectively. The average *Salmonella* populations were significantly larger ($P < 0.05$) than the EHEC populations. Significantly larger *Salmonella* populations were recovered from the cotyledon and seed coat tissues, followed by the root tissues, but the mean EHEC populations from all sampled tissue sections were statistically similar, except in pregerminated seeds. Three *Salmonella* and two EHEC strains had significantly larger cell populations on sprout/seedling tissues than other strains used in the study. *Salmonella* and EHEC populations from fenugreek and alfalfa tissues were significantly larger than those from tomato and lettuce tissues. The study showed the fate of internalized human pathogens on germinating vegetable seeds and sprout/seedling tissues and emphasized the importance of using pathogen-free seeds for sprout production.

IMPORTANCE The internalization of microorganisms into vegetable seeds could occur naturally and represents a possible pathway of vegetable seed contamination by human pathogens. The present study investigated the ability of two important bacterial pathogens, *Salmonella* and enterohemorrhagic *Escherichia coli* (EHEC), when artificially internalized into vegetable seeds, to grow and disseminate along vegetable sprouts/seedlings during germination. The data from the study revealed that the pathogen cells artificially internalized into vegetable seeds caused the contamination of different tissues of sprouts/seedlings and that pathogen growth on germinating seeds is bacterial species and vegetable seed-type dependent. These results further stress the necessity of using pathogen-free vegetable seeds for edible sprout production.

KEYWORDS alfalfa, EHEC, fenugreek, lettuce, *Salmonella*, seedlings, sprouts, tomato, vegetable seeds

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The consumption of vegetable seed sprouts has become popular worldwide in recent decades due to the shift in consumers' preferences to more healthy and nutritious foods. However, the seed germination process has made sprouts highly susceptible to microbial contamination; thus, concerns have been raised about the safety of seeds used for sprout production (1). The contamination of sprout seeds by *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) has been confirmed as the cause of several sprout-associated outbreaks of infections (2). In 1995, an international outbreak in Finland and in 17 states in the United States was traced to alfalfa seeds contaminated with *S. enterica* subsp. *enterica* serovar Stanley (3). In 1997, simultaneous outbreaks of *E. coli* O157:H7 infections in Michigan and Virginia were linked to the consumption of alfalfa sprouts grown from the same lot of seeds (4). In 2003, two *E. coli* O157 outbreaks associated with alfalfa sprouts in Colorado and Minnesota were linked to a common seed source (5). In a 2009 sprouts-related multistate outbreak of *S. enterica* subsp. *enterica* serovar Saintpaul infections, seeds from a single grower were identified as the source of contamination (6).

The treatment of sprout seeds with 20,000 ppm calcium hypochlorite before germination is recommended by the U.S. Food and Drug Administration (FDA) to minimize the risk of microbial contamination (7). However, the efficacy of this sanitation protocol is highly variable due to the inability of chlorine to make contact with pathogen cells that are located in the internal spaces of vegetable seeds (8). Pathogenic bacteria can naturally infiltrate cracks, crevices, and intercellular spaces of seeds when a negative pressure is created across the seed coat by changes in environmental temperature and the hydraulic status of seed surfaces (9). The precise incidence of pathogen infiltration into vegetable seeds in the natural environment is unknown, but it is assumed to be extremely low. After reaching internal seed tissues, the fate of the pathogens during sprout and seedling production is largely unknown. Several studies have described the growth of *S. enterica* and *E. coli* during seed germination when the pathogen cells were inoculated onto the surfaces of seeds and seedlings (10–12). However, it is not yet clear if human pathogens internalized into vegetable seeds would behave in a similar fashion during germination, as the chemical and biological environments may differ within and outside the germinating seeds (13). This study was undertaken to observe the physiological behavior of selected bacterial pathogens artificially internalized into vegetable seeds during the germination process and to understand whether pathogen behaviors on various tissues of sprouting vegetable seeds is bacterial species, pathogen strain/serotype, and seed-type dependent.

RESULTS

Among the 512 sprout/seedling samples analyzed in the *Salmonella* or EHEC experiment, 317 (62%) tested positive for *Salmonella* and 343 (67%) tested positive for EHEC (detailed data not shown). Table 1 shows the mean populations of all four *Salmonella* or EHEC strains recovered from different sampling points, tissue sections, or vegetable seed types. The mean population of each *S. enterica* or EHEC strain is the mean value recovered from all four types of vegetable sprout/seedling tissues at all sampling points. It is evident that the mean *Salmonella* population recovered from all sprout/seedling tissue sections increased from the initial 1.45 log CFU/g to 3.20 log CFU/g at the end of a 9-day germination process, and the EHEC population increased from 0.88 log CFU/g to 2.30 log CFU/g. The average *Salmonella* population increased significantly from day 3 to day 5, and there was no significant change in the population before and after these two sample points. In contrast to that of *Salmonella*, a significant increase in the EHEC population was not observed until 7 to 9 days into the germination process. Cotyledon and seed coat tissues had the largest populations of *Salmonella* cells. On average, more *Salmonella* cells were recovered from the root tissues than from the stem tissues and pregerminated seeds. The *Salmonella* population from seed coat/cotyledon tissues was larger than that from pregerminated seeds but was not significantly different from the cell populations from the stem and root tissues. On average, the largest EHEC population was found on the cotyledon tissues, but this

TABLE 1 Overall mean populations of *Salmonella enterica* and EHEC recovered at different sampling points during germination and from different types and tissue sections of sprouts/seedlings

Sampling	Mean population (log CFU/g)[a]	
	BSA or NASMAC[b]	NATSA[c]
Salmonella		
Trial (n = 256 each)		
1	2.79 A	3.00 A
2	2.78 A	2.95 A
Germination day		
9 (n = 128)	3.20 A	3.57 A
7 (n = 128)	3.19 A	3.40 A
5 (n = 128)	3.13 A	3.30 A
3 (n = 96)	1.63 B	1.67 B
1 (n = 32)	1.45 B	1.48 B
Strain (n = 128 each)		
S. Stanley	3.09 A	3.52 A
S. Baildon	3.30 A	3.37 A
S. Cubana	3.16 A	3.24 A
S. Montevideo	1.55 B	1.75 B
Tissue sections of sprouts/seedlings		
Cotyledon (n = 96)	3.63 A	3.82 A
Seed coat (n = 96)	3.52 A	3.80 A
Root (n = 128)	2.69 B	2.96 B
Seed coat/cotyledon (n = 32)	2.11 B,C	2.13 C
Stem (n = 128)	2.17 C	2.30 C
Pregerminated seeds (n = 32)	1.45 D	1.48 D
Vegetable seed type (n = 128 each)		
Fenugreek	4.08 A	4.36 A
Alfalfa	4.21 A	4.30 A
Tomato	1.47 B	1.68 B
Lettuce	1.35 B	1.55 B
EHEC		
Trial (n = 256 each)		
1	1.60 A	1.93 A
2	1.54 A	1.89 A
Germination day		
9 (n = 128)	2.30 A	2.91 A
7 (n = 128)	1.76 B	2.27 B
5 (n = 128)	1.43 B	1.54 C
3 (n = 96)	1.11 B,C	1.15 C,D
1 (n = 32)	0.88 C	1.03 D
Strain (n = 128 each)		
E. coli K4492	1.70 A	1.91 A
E. coli H1730	1.72 A	1.93 A
E. coli BAA-2326	1.49 A,B	1.90 A
E. coli F4546	1.37 B	1.91 A
Tissue sections of sprouts/seedlings		
Cotyledon (n = 96)	1.83 A	2.19 A
Seed coat/cotyledon (n = 32)	1.66 A	2.18 A,B
Seed coat (n = 96)	1.67 A	1.95 A,B
Root (n = 128)	1.53 AB	1.85 A,B
Stem (n = 128)	1.44 AB	1.68 B
Pregerminated seeds (n = 32)	1.11 B	1.15 C
Vegetable seed type (n = 128 each)		
Alfalfa	3.31 A	3.70 A
Fenugreek	1.93 B	2.27 B
Lettuce	0.67 C	1.54 C
Tomato	0.38 C	1.15 D

[a]Mean populations of the same bacterial pathogen within a column and the same comparative category that are not followed by the same letter are significantly different ($P < 0.05$).
[b]BSA, bismuth sulfite agar; NASMAC, sorbitol MacConkey agar supplemented with nalidixic acid.
[c]NATSA, tryptic soy agar supplemented with nalidixic acid.

TABLE 2 Mean populations of individual *Salmonella* strains recovered from different types of sprouts/seedlings over the 9-day germination period

	Mean population (log CFU/g)[a]			
Seed type	***S.* Stanley**	***S.* Baildon**	***S.* Cubana**	***S.* Montevideo**
Fenugreek	4.44 A*	4.02 A*	4.12 A*	1.59 B*
Alfalfa	3.88 A*	4.14 A*	4.10 A*	2.01 B*
Tomato	1.49 A†	1.32 A†	1.18 A,B†	0.88 B†
Lettuce	0.90 B,C†	1.99 A†	1.27 B†	0.67 C†

[a]Mean values within a column that are not followed by the same symbol (* or †) are significantly different ($P < 0.05$). Mean values within a row that are not followed by the same uppercase letter are significantly different ($P < 0.05$).

population was not significantly different from that on other sprout/seedling tissues except on pregerminated seeds ($P < 0.05$). Among the four *Salmonella* strains included in the study, *S. enterica* subsp. *enterica* serovar Montevideo had the smallest cell population, and the populations of the other three *Salmonella* strains were not significantly different. The average population of *E. coli* F4546 was significantly smaller than the populations of *E. coli* 4492 and *E. coli* 1730. The mean populations of both *Salmonella* and EHEC recovered from fenugreek and alfalfa sprout tissues were significantly larger ($P < 0.05$) than those from tomato and lettuce seedling tissues.

The average cell populations of four individual *Salmonella* or EHEC strains from all tissue sections of each type of vegetable seeds over the 9-day germination period are summarized in Tables 2 and 3, respectively. Higher numbers of *Salmonella* and EHEC cells were recovered from fenugreek and alfalfa than from tomato and lettuce tissues, except *E. coli* F4546 (Tables 2 and 3).

The mean populations of *S.* Montevideo on fenugreek and alfalfa tissues were significantly smaller ($P < 0.05$) than the cell populations of the other three *Salmonella* strains (Table 2). On tomato seedling tissues, the average populations of *S. enterica* subsp. *enterica* serovars Stanley and Baildon were significantly larger than the population of *S.* Montevideo, and these three populations were not significantly different from the mean population of *S. enterica* subsp. *enterica* serovar Cubana. The average *S.* Baildon population on lettuce tissues was significantly larger than the populations of the other 3 *Salmonella* strains, and the mean population of *S.* Stanley was not significantly different from the average populations of *S.* Cubana and *S.* Montevideo.

E. coli BAA-2326 and F4546 had the smallest and largest cell populations, respectively, on alfalfa samples (Table 3), and the cell populations of the other two *E. coli* strains were not statistically different. In contrast to that from alfalfa samples, the average population of *E. coli* F4546 on fenugreek sprouts was significantly smaller ($P < 0.05$) than the other three *E. coli* strains. The *E. coli* BAA-2326 population on lettuce seedlings was significantly smaller than that of *E. coli* H1730, which was undetectable from tissues of tomato seedlings.

The average cell populations of all 4 *Salmonella* and 4 EHEC strains from different tissue sections of each type of sprout/seedling over the 9-day germination period are shown in Tables 4 and 5, respectively. Similar to the result shown in Tables 2 and 3, the

TABLE 3 Mean populations of individual EHEC strains recovered from different types of sprouts/seedlings over the 9-day germination period

	Mean population (log CFU/g)[a]			
Seed type	***E. coli* K4492**	***E. coli* H1730**	***E. coli* BAA-2326**	***E. coli* F4546**
Alfalfa	2.52 B*	2.83 B*	1.59 C†	3.72 A*
Fenugreek	1.64 B†	1.74 A,B†	2.46 A*	0.21 C†
Lettuce	0.59 A,B‡	0.82 A‡	0.07 B‡	0.51 A,B†
Tomato	0.42 A‡	0.00 B‡	0.58 A‡	0.06 B†

[a]Mean values within a column that are not followed by the same symbol (*, †, or ‡) are significantly different ($P < 0.05$). Mean values within a row that are not followed by the same uppercase letter are significantly different ($P < 0.05$).

TABLE 4 Mean populations of all 4 *S. enterica* strains recovered from different tissue sections of each type of sprouts/seedlings over the 9-day germination period

Seed type	Mean population (log CFU/g)[a]					
	Cotyledon	Seed coat	Root	Seed coat/ cotyledon	Stem	Pregerminated seeds
Fenugreek	4.38 A*	4.24 A*	3.23 B*	2.52 B,C†	2.61 B*	1.59 C†
Alfalfa	4.21 A*	4.18 A*	2.99 B*	4.73 A*	2.68 B*	3.14 B*
Tomato	1.59 A†	1.21 A†	1.56 A†	1.14 A,B†‡	0.86 B†	0.00 B‡
Lettuce	1.75 A*†	1.44 A†	1.45 A†	0.00 B‡	0.85 B†	0.00 B‡

[a]Mean values within a column that are not followed by the same symbol (*, †, or ‡) are significantly different ($P < 0.05$). Mean values within a row that are not followed by the same uppercase letters are significantly different ($P < 0.05$).

average *Salmonella* and EHEC populations on individual tissue sections of alfalfa and fenugreek sprouts were significantly larger ($P < 0.05$) than those from tomato and lettuce seedlings except for the population from seed coats/cotyledons of fenugreek sprouts and tomato seedlings (Tables 4 and 5).

The cotyledons and seed coats of all 4 types of sprouts/seedlings had higher *Salmonella* counts (Table 4). The root tissues of tomato and lettuce seedlings and seed coat/cotyledon tissues of alfalfa sprouts also had higher *Salmonella* counts than other tissues of corresponding sprouts/seedlings (Table 4). Lower *Salmonella* counts were associated with stem tissues and pregerminated seeds. However, the growth trend of EHEC on tissues developed from each type of seeds was not as clear as that of *Salmonella* (Table 5).

Daily changes in *Salmonella* and EHEC populations on alfalfa, fenugreek, tomato, and lettuce sprout/seedling samples during the germination process are shown in Fig. 1 and 2, respectively. In general, cell populations of *Salmonella* and EHEC on all samples increased as the germination time increased.

DISCUSSION

The infiltration of human and plant pathogens into vegetable seeds can occur naturally at various stages during seed production (9). When the water pressure overcomes the internal gas pressure of seeds and the hydrophobic nature of seed surfaces, bacterial cells can infiltrate through cracks, crevices, and intercellular spaces of the vegetable seeds (14). Vacuum infiltration, first described by Boosalis (15), has been used to mimic this natural process and inoculate microorganisms into plant and seed tissues. Under vacuum, the air trapped in the intercellular spaces of seed tissue is removed to yield a lower internal gas pressure. A subsequent sudden release of the vacuum creates a positive pressure difference toward the internal seed tissues, which allows bacterial cells in a suspension to be drawn into the seeds (16). The efficacy of seed inoculation by vacuum infiltration was previously discussed by Prathuangwong and coworkers (17) who found that a 20-min vacuum at 20 lb/in.^{2} was as effective as direct micropyle injections in inoculating *Xanthomonas campestris* pv. glycines into soybean seeds. Thus, the technique has been used routinely to study seed-

TABLE 5 Mean populations of all 4 EHEC strains recovered from different types of sprouts/seedlings over the 9-day germination period

Seed type	Mean population (log CFU/g)[a]					
	Cotyledon	Seed coat/ cotyledon	Seed coat	Root	Stem	Pregerminated seed
Alfalfa	3.18 A,B*	3.14 A,B*	3.03 A*	2.56 A,B*	2.01 B*	3.36 A*
Fenugreek	1.87 A†	2.29 A*	1.58 A†	1.55 A†	1.38 A,B†	0.30 B†
Lettuce	0.51 A,B‡	0.00 B†	0.50 A,B‡	0.68 A‡	0.49 A,B‡	0.00 B‡
Tomato	0.34 A,B‡	0.15 A,B†	0.06 A,B‡	0.46 A‡	0.27 A,B‡	0.00 B‡

[a]Mean value within a column that are not followed by the same symbol (*, †, or ‡) are significantly different ($P < 0.05$). Mean values within a row that are not followed by the same uppercase letter are significantly different ($P < 0.05$).

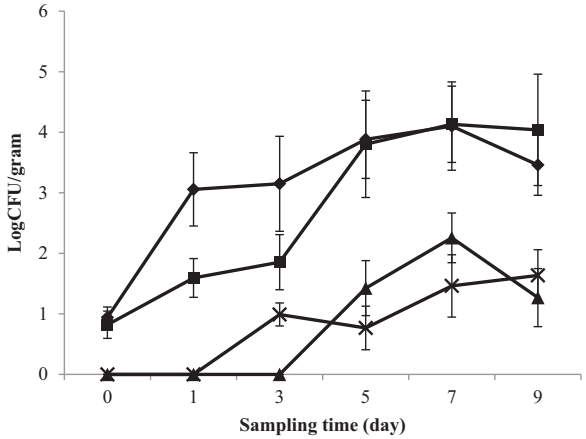

FIG 1 Growth of *S. enterica* on sprouting seeds of alfalfa (◆), fenugreek (■), tomato (×) and lettuce (▲). Values are averages from *S.* Montevideo, *S.* Stanley, *S.* Cubana, and *S.* Baildon populations recovered from various tissues of different types of germinating seeds.

microorganism interactions (11, 16, 18–20). In the present study, vacuum infiltration was used to introduce *Salmonella* and EHEC cells into alfalfa, fenugreek, lettuce, and tomato seeds.

During vacuum infiltration, the surfaces of vegetable seeds might be simultaneously contaminated. As a control measure, seed surface decontamination was performed after the vacuum infiltration process in the present study. The treatment of vacuum-infiltrated vegetable seeds with sodium hypochlorite successfully inactivated *Salmonella* or EHEC cells on seed surfaces, as no pathogen cells were detected from the seed-rinsing water. However, a low number (0.26 to 0.95 log CFU) of pathogen cells was recovered from the homogenates of alfalfa and fenugreek seeds. These results indicate that vacuum infiltration successfully introduced *S. enterica* and EHEC cells into the vegetable seeds used in the study.

The growth and dissemination of internalized *S. enterica* and EHEC cells to different tissue sections of sprouts/seedlings were demonstrated in the present study (Table 1). The precise mechanism for the observed phenomenon is not known. However, Cooley et al. (21) reported that *Salmonella* and *E. coli* could migrate along plant surfaces by either diffusion or active movement. Kroupitski et al. (22) found that cells of some *Salmonella* strains sensed and moved toward specific chemoattractants, such as sucrose, on plant surfaces. Since cotyledon and root tissues and seed coats are closely

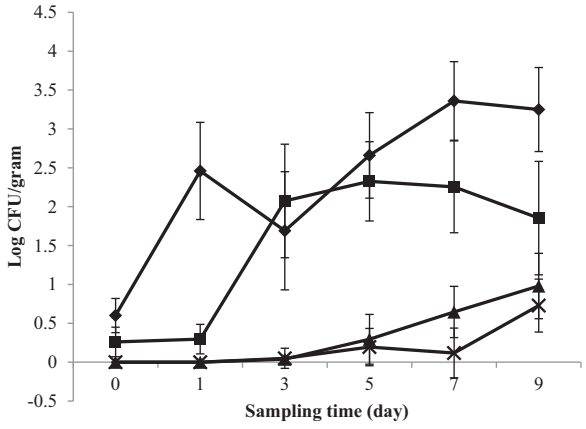

FIG 2 Growth of EHEC on sprouting seeds of alfalfa (◆), fenugreek (■), tomato (×), and lettuce (▲). Values are averages from *E. coli* F4546, *E. coli* K4492, *E. coli* H1730, and *E. coli* ATCC BAA-2326 populations recovered from various tissues of different types of germinating seeds.

related to seed/seedling exudation, abundant nutrients and water exuded from these tissues can support the growth of microorganisms (23). Klerks et al. (24) observed the active movement of *S. enterica* subsp. *enterica* serovar Dublin toward root exudates of lettuce seedlings in soil and glass capillary tubes. Effective chemoattractants of *E. coli*, such as D-glucose, fructose, and maltose (25), are frequently present in vegetable seed and root exudates (26). As *Salmonella* populations in seed coats and roots and *Salmonella* and *E. coli* populations in cotyledon were larger than those in other tissue sections according to the results of the present study (Table 1), it is likely that chemotaxis is one of the forces that drove *Salmonella* and *E. coli* cells into these locations.

It was observed that pathogen growth was less significant on lettuce and tomato seedling tissues compared to those on alfalfa and fenugreek sprouts (Table 1). The reason for this difference is currently unknown. However, differences in the initial bacterial population (data not shown) on various types of infiltrated vegetable seeds might be partially responsible for the observed phenomenon. Vegetable seeds used in the study varied in mass and size, as well as in their chemical and physical surface properties, which may have influenced the efficacy of vacuum infiltration.

The chemical compositions of vegetable seeds and seed exudates might also affect the growth of bacterial cells on sprouts/seedlings during germination (27–29). Generally, seeds and seed exudates that contain more essential nutrients and fewer growth inhibitors better support the growth of microorganisms (30). Previous studies have shown that alfalfa and fenugreek seeds contain arabinose (31, 32), which is absent in tomato (33) and lettuce seeds (34). Arabinose may serve as a carbon source to support the growth of *Salmonella* and EHEC, as it can be utilized by the cells of most *S. enterica* and EHEC strains (35, 36). Furthermore, L-arabinose is an active regulator of *Salmonella* pathogenicity island 1, which has been suggested to play a role in the interactions between *Salmonella* and plant hosts (37). In addition to arabinose, the abundance of threonine differs in the four types of vegetable seeds used in the study (38). The amounts of threonine per unit weight of alfalfa and fenugreek seeds are higher than those in tomato and lettuce seeds (31–34). Although arabinose could be a good carbon source and threonine is an essential amino acid for bacterial growth, it is not known whether they have contributed to the differential growth of *Salmonella* and EHEC on tissues of alfalfa and fenugreek sprouts versus on lettuce and tomato seedlings in the present study.

Tu (39) compared the seed and early root exudates of 19 crop species and reported significantly varied abundances in amino acids, amines, amides, and reducing sugars. The researchers recovered only trace amounts of reducing sugars and amino acids that could be utilized by microorganisms from tomato seedlings after 5 days of germination. In addition, the exudates of tomato seeds and seedlings contained one order of magnitude more organic acids than sugars, and had a weakly acidic pH of 5.5 (40, 41). Neumann et al. (42) reported the presence of benzoic and lauric acids, which were natural antimicrobial agents, in the root exudates of lettuce seedlings.

Although in lower numbers, *Salmonella* and EHEC cells disseminated from contaminated seeds to lettuce and tomato seedlings. However, whether the presence of the pathogen on the seedlings would impose any health risks to fresh produce safety largely depends on the fate of the pathogens at later stages of plant development. Deering et al. (43) recovered *E. coli* O157:H7 cells in tomato fruits grown from seeds artificially inoculated with the pathogen. Gu et al. (44) examined tomato fruits grown from seeds extracted from tomato fruits infested with *Salmonella* but did not recover the pathogen from the second-generation fruits. Cooley et al. (21) observed a temporal reduction in the contamination frequency of plants grown from contaminated *Arabidopsis thaliana* seeds during 30 days of cultivation.

Howard and Hutcheson (18) previously reported that the growth of *S. enterica* on germinating alfalfa seeds is serotype independent. The researchers inoculated alfalfa seeds with nine different serotypes of *S. enterica* and observed no difference in growth among the tested strains after 24 and 48 h of germination. However, the present study

reveals that the average population of *S.* Montevideo recovered from different sprout/seedling tissues was significantly smaller than the other *Salmonella* strains used in the study (Table 1 and 2). This indicates that the *S.* Montevideo strain might be poorly adapted for growth in germinating seeds. Interestingly, *S.* Montevideo was reported in early studies as better adapted to growth on tomato plants than nine other *S. enterica* serotypes, such as *S. enterica* subsp. *enterica* serovars Newport, Dublin, and Typhimurium (45, 46). In contrast to previous studies that used mature tomato plants (47), germinating tomato seeds were used in the present study. Furthermore, only four *S. enterica* strains were tested in the current study, whereas approximately 100 documented pathogenic *Salmonella* serotypes are frequently associated with human infections (48). More strains/serotypes should be tested before a conclusion can be drawn with respect to the serovar dependency of *Salmonella* growth on germinating vegetable seeds.

We observed, in the present study, that the population of EHEC recovered from sprouts/seedling tissues was significantly smaller than that of *Salmonella*. Similar results were reported by Charkowski et al. (11), who compared the growth of five *S. enterica* strains and six *E. coli* O157:H7 strains on germinating alfalfa seeds for 2 days. The population of *S. enterica* was 0.5 to 2.0 log CFU/sprout higher than that of *E. coli* O157:H7. The authors ascribed the lower growth rate of *E. coli* on alfalfa sprouts to poor adaptation to alfalfa seed exudates and/or the inability of the pathogen to firmly attach to sprout surfaces. Roy et al. (49) reported that relative to *S. enterica*, *E. coli* O157:H7 triggered a stronger stomatal immunity and elevated the expression of plant defense-related marker genes, such as *PR1* in *A. thaliana* and lettuce plants.

In conclusion, this study found that *Salmonella* and *E. coli* artificially internalized into vegetable seeds caused the contamination of different tissues of sprouts/seedlings and that pathogen growth on germinating seeds is bacterial species and vegetable seed-type dependent. The average *Salmonella* populations recovered from different tissue sections of sprouts/seedlings were larger than the *E. coli* populations. Alfalfa and fenugreek sprout tissues had, on average, larger pathogen populations than lettuce and tomato seedling tissues. Whether pathogen cells on vegetable seedlings, such as those of lettuce and tomato, will impose any risk to fresh produce safety largely depends on the fate of the pathogens at later stages of plant development. However, sprouts of alfalfa and fenugreek seeds are often consumed as raw or minimally processed products and have the potential to impose health risks to the consumers once they are contaminated with bacterial pathogens.

MATERIALS AND METHODS

Bacterial strains. Four *Salmonella* strains (*S.* Baildon, *S.* Cubana, *S.* Montevideo, and *S.* Stanley), three *E. coli* O157:H7 strains (F4546, H1730, and K4492) and one *E. coli* O104:H4 strain (ATCC BAA-2326) that were isolated from fresh produce-associated outbreaks of human gastrointestinal infections were used in this study (50). Nalidixic acid (NA)-resistant mutants of each bacterial strain were selected on tryptic soy agar (Becton, Dickinson and Company, Sparks, MD) supplemented with 50 μg/ml of NA (NATSA). Bacterial inocula were prepared by transferring a loop of each overnight culture to Luria-Bertani no-salt broth supplemented with 50 μg/ml of NA and incubated for 16 to 18 h at 37°C. The resulting bacterial cultures were diluted in sterile water to approximate cell concentrations of 10^4 CFU/ml. The exact cell populations in the inocula were determined using a standard plate count assay on NATSA.

Inoculation of vegetable seeds. Alfalfa (*Medicago sativa*), fenugreek (*Trigonella foenum-graecum*), tomato (*Solanum lycopersicum* [Roma]), and lettuce (*Lactuca sativa* [iceberg]) seeds were purchased from Twilley seed company (Hodges, SC) and stored at 10°C as instructed by the distributer. These seed species were selected based on the fact that alfalfa and fenugreek sprouts and tomato (fruit) and lettuce (leafy green) are commonly consumed fresh produce, which have all been previously linked to outbreaks of human gastrointestinal infections. Before pathogen inoculation, each vegetable seed type (2 g) was surface disinfected in 20 ml of 20,000 ppm sodium hypochlorite at room temperature for 10 min with agitation at 120 rpm on a platform shaker (Orbit shake; Lab-Line Instruments, Inc.). Residual chlorine on vegetable seeds was neutralized with 20 ml Dey-Engley neutralization broth (BD) for 10 min. Disinfected seeds were rinsed twice, each with 20 ml sterilized distilled water, and were vigorously vortexed for 20 s. Vacuum infiltration was performed according to a procedure previously described by Darrasse et al. (51) with modifications. Specifically, the seeds were placed in 20 ml of *Salmonella* or *E. coli* inoculum at room temperature for 30 min before being exposed to a vacuum of 25 in. Hg for 10 min. The vacuum was then broken to create a negative pressure. The draw and release of vacuum were repeated three times (52), and inoculated vegetable seeds were collected and dried overnight in a biological safety

cabinet (class II type A/B 3; Nuaire, Plymouth, MN). *Salmonella* or *E. coli* cells on seed surfaces were inactivated using another sodium hypochlorite treatment as described above. Successful inactivation of *Salmonella* or *E. coli* cells on seed surfaces was confirmed by plating the final seed rinse water on bismuth sulfate agar (BSA; BD) or sorbitol MacConkey agar (SMAC; BD) supplemented with 50 μg/ml NA (NASMAC), respectively. Both types of samples were also plated on NATSA plates. The detection limit of the plating assay was 10 CFU/ml. Twenty seeds of each inoculated and disinfected seed type were individually homogenized in 0.5 ml of phosphate-buffered saline (PBS) and plated on NATSA and BSA or NASMAC in duplicates, as described above, to determine the initial bacterial loads.

Germination of vegetable seeds. For seed germination, 1.0% (wt/vol) water agar was prepared in sterile 100 mm $\times$ 100 mm square plates (Fisher Scientific, CA). Twenty seeds of each type inoculated with an individual bacterial strain were placed on a water agar plate with moderate spacing. The plates were then placed in germination boxes at 25°C in the dark, and samples were taken every other day for microbiological analysis. The experiment was conducted twice.

Sampling and microbiological analyses. On each sampling day, 10 contaminated seeds or sprouts/seedlings were aseptically removed from water agar plates using sterilized forceps. Intact vegetable seeds were collected on day 1, and on the rest of the sampling days, sprouts/seedlings were dissected into multiple tissue sections, including seed coat, cotyledon, stem, and root, and each tissue section was sampled individually for *Salmonella* and EHEC populations. At the 3rd day of germination, seed coats and cotyledons were sampled as "seed coat/cotyledon" due to the difficulty of separating the two tissue sections. Samples were homogenized in 2.0 ml PBS (pH 7.4), and the resulting homogenates were serially diluted as necessary. Homogenates obtained from *Salmonella*-contaminated samples were plated on BSA and NATSA and those from *E. coli*-contaminated samples were plated on NASMAC and NATSA. All plates were spread plated in duplicates and incubated at 37°C for 24 h before colonies were enumerated. The enrichment of samples with negative plating results was performed according to the FDA's bacteriological analytical manual (53, 54).

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test using the R 3.2.2 software. Cell populations of *Salmonella* or EHEC strains recovered at different sampling points and from different seed types and sprout/seedling tissue sections were compared. For all comparisons, *P* values of less than 0.05 were considered significant.

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