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Fate of various *Salmonella enterica* and enterohemorrhagic *Escherichia coli* cells attached to alfalfa, fenugreek, lettuce, and tomato seeds during germination

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ABSTRACT

Contaminated vegetable seeds have been identified as a potential source of foodborne pathogens. This study was undertaken to observe the physiological behavior of various *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) cells attached to different types of vegetable seeds during germination. Surface-sanitized seeds (2 g) of alfalfa, fenugreek, lettuce, and tomato were exposed to four individual strains of *Salmonella* (Baildon, Cubana, Montevideo, and Stanley) or EHEC (three O157:H7 and one O104:H4 strain[s]) at 20 °C for 1 h. Vegetable seeds with attached bacterial cells were germinated on 1% water agar at 25 °C in the dark. Populations of *Salmonella* and EHEC on various tissues of sprouts/seedlings (seed coat, root, cotyledon, stem, *etc.*) were determined every other day over a 9-day germination period. The mean populations of *Salmonella* and EHEC on sprout/seedling tissues increased as the prolongation of germination time. Overall, 93.0% and 92.3% of collected tissue samples (n = 544) tested positive for *Salmonella* and EHEC, respectively. Seed coats had significantly higher ($P < 0.05$) bacterial counts (7.8 log CFU/g) compared to the root, cotyledon, and stem tissues (7.1–6.2 log CFU/g). On average, lettuce seedlings and alfalfa sprouts had significantly higher ($P < 0.05$) numbers of *Salmonella* and EHEC cells, followed by fenugreek sprouts, and tomato seedlings. The *E. coli* O104:H4 strain established the lowest ($P < 0.05$) cell population (5.2 log CFU/g) on sprout/seedling tissues among all EHEC strains used in the study. Among the three *E. coli* O157:H7 strains, F4546 (7.2 log CFU/g) and H1730 (7.0 log CFU/g) had significantly ($P < 0.05$) higher mean cell population than K4499 (6.8 log CFU/g). The mean population of *S.* Cubana (7.0 log CFU/g) was significantly higher ($P < 0.05$) than the populations of the other 3 *Salmonella* strains (6.5–6.6 log CFU/ g) used in this study. These data suggested that the growth and dissemination of *Salmonella* and EHEC cells on alfalfa, fenugreek, lettuce, and tomato sprouts/seedlings were influenced by the bacterial strains, type of sprouts/seedlings, and specific sprout/seedling tissues involved.

1. Introduction

Fresh produce is recognized by consumers as an important source of nutrients, particularly vitamins, minerals, and dietary fibers (Rickman, Bruhn, & Barrett, 2007). As a result, fresh produce consumption in the United States increased significantly in the last few decades (Olaimat & Holley, 2012). As fresh produce consumption increased, a parallel increase in fresh produce-associated outbreaks of human gastrointestinal infection was noticed (Warriner, Huber, Namvar, Fan, & Dunfield, 2009). Fresh produce was the most common vehicle for transmitting foodborne illnesses in the United States during the period of 2004–2013 and overall, 19% of the foodborne outbreaks and 24% of the total number of foodborne illnesses reported to the CDC were associated with fresh produce consumption (Fischer, Bourne, & Plunkett, 2015). Fresh produce that has been linked to the outbreaks of human gastrointestinal infections included lettuce, tomato, as well as alfalfa and fenugreek sprouts (Beutin & Martin, 2012; CDC, 2007; Cummings et al., 2001; Slayton et al., 2013).

Fresh produce contamination by bacterial pathogens such as *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) could occur at both pre- and post-harvest stages in the production

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chain (Jiang, Chen, & Dharmasena, 2014). Vegetable seeds are prone to microbial contamination, and un-sanitized vegetable seeds tainted with human enteric pathogens could lead to the contamination of fresh produce, especially sprouts (Hanning, Nutt, & Ricke, 2009). According to the U.S. Food and Drug Administration (1999), most sprout-associated outbreaks have been caused by seeds contaminated with bacterial pathogens before the sprouting process begins because currently available seed sanitation treatments are not always effective in eliminating bacterial pathogens associated with sprout seeds (Ding, Fu, & Smith, 2013; Fu, Reineke, Chirtel, & VanPelt, 2008; Hong & Kang, 2016). Contamination of sprout seeds by *Salmonella* and EHEC has been confirmed as the cause of several sprout-associated outbreaks of infections (Breuer et al., 2001; CDC, 2016). In a previous study of our laboratory, cells of individual *Salmonella* and EHEC strains exhibited a differential attachment behavior on the surface of alfalfa, fenugreek, lettuce, and tomato seeds (Cui, Walcott, & Chen, 2017). Bacterial attachment to vegetable seeds is the initial step of vegetable sprout/seedling contamination by pathogenic bacteria. This study was undertaken to observe the physiological behavior of seed-borne *Salmonella* and EHEC cells on various tissue sections of alfalfa and fenugreek sprouts and lettuce and tomato seedlings during the germination process.

2. Materials and methods

2.1. Vegetable seeds

Alfalfa (*Medicago sativa*), fenugreek (*Trigonella foenum-graecum*), lettuce (*Lactuca sativa,* cv. Iceberg), and tomato (*Solanum lycopersicum,* cv. Roma) seeds were obtained from a commercial source (Otis S. Twilley Seed Co. Inc., Hodges, SC) and were stored at 10 °C before being used in the study.

2.2. Bacterial strains and growth conditions

S. enterica serovar Baildon (tomato outbreak strain), Cubana (alfalfa sprout outbreak strain), Montevideo (tomato outbreak strain), and Stanley (alfalfa sprout outbreak strain), as well as *E. coli* O157:H7 F4546 (alfalfa sprout outbreak strain), K4499 (spinach outbreak strain), and H1730 (lettuce outbreak strain), and *E. coli* O104:H4 BAA-2326 (fenugreek outbreak strain) were used in the study. The bacterial strains were retrieved from frozen storage at −70 °C and grown on tryptic soy agar (TSA) at 37 °C for 16 h. The resulting cultures were purified on bismuth sulfite agar (BSA), sorbitol MacConkey (SMAC) agar, and MacConkey (MAC) agar (Becton Dickenson, Sparks, MD), respectively. Spontaneous nalidixic acid (NA; MP Biomedicals, Santa Ana, CA) resistant cells of each bacterial strain were selected and used throughout the study.

2.3. Bacterial attachment to vegetable seeds

The experimental approach used for bacterial attachment to vegetable seeds has been described previously (Cui et al., 2017). Briefly, each type of vegetable seeds (2 g) were placed in a 50 ml Falcon centrifuge tube (Fisher Scientific, Asheville, NC) and sanitized with 10 ml of 20,000 ppm sodium hypochlorite solution (pH 6.8; Becton Dickenson, Sparks, MD) at room temperature for 15 min with gentle mixing. Residual sanitizers on vegetable seeds were neutralized with 10 ml of Dey-Engley neutralizing broth (Becton Dickenson, Sparks, MD) for 10 min with gentle mixing and then rinsed twice, each with 10 ml of sterile deionized water for 1 min. An overnight culture of individual *Salmonella* and EHEC strain grown in Luria-Bertani no salt broth supplemented with NA (50 μg/ml) was serially diluted in sterile water and 20 ml of each inoculum with a cell population of *ca*. 10^4 CFU/ml was added to the centrifuge tubes with sanitized seeds. The precise numbers of bacterial cells in the bacterial suspensions were determined by plating 0.1 ml of appropriately diluted cultures on TSA containing NA. Vegetable seeds in the centrifuge tubes were agitated horizontally at 100 rpm in an orbital platform shaker (Model: 3520, Lab-line, IL, U.S.A) at 20 °C for 1 h and the inocula were then discarded. Pathogen cells on each type of contaminated seeds were enumerated to determine the initial level of bacterial contamination on vegetable seeds.

2.4. Seed germination and sprout/seedling growth

Each type of vegetable seeds with the attached cells of an individual bacterial strain (n = 50) were placed with a proper spacing (10 seeds/plate), using small, sterile curved forceps (Fisher Scientific, Asheville, NC), onto 1% (*w/v*) water agar (Becton Dickenson, Sparks, MD) in sterile squared Petri dishes with grid (10 × 10 cm; Electron Microscopy Sciences, Hatfield, PA). The Petri dishes with the vegetable seeds were placed in transparent plastic boxes (Walmart, Bentonville, AR), the bottom of which was covered with a layer of paper towel moisturized with deionized water. The boxes were placed in a 25 °C incubation room in the dark to allow the seeds to germinate for 9 days. Bacterial populations on different tissues of sprouts/seedlings developed from each type of vegetable seeds were microbiologically analyzed every other day. Two independent trials were conducted. A total of 1,600 vegetable seeds were included in the experiment involving *Salmonella* or EHEC, with 800 seeds in an individual trial.

2.5. Sample preparation and microbiological analyses

On the first day of germination, pre-germinated vegetable seeds (inoculated seeds with no differentiated tissue sections) were analyzed whereas on the third day of germination, developed sprouts/seedlings were carefully dissected using sterile forceps and scissors, and the seed coat/cotyledon (combination of seed coat and cotyledon due to difficulty in separating the two tissue sections), stem, and root tissues were collected. The roots were tissues with fibrils and the boundary between root and stem tissues was determined by their positions in relation to the surface of water agar. The portion above the surface of water agar was taken as stem tissues and that beneath the surface of agar was the root tissues. On the fifth day and forward, seed coat and cotyledon were analyzed separately. Different tissue sections of sprouting seeds sampled in the study are shown in Fig. 1.

An individual tissue section of sprouts/seedlings developed from a composite sample of 5 vegetable seeds of an individual type and inoculated with a single pathogen strain was ground, using a pestle, for 1 min in 5 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.4) in a whirl-pak bag (1 oz., Nasco, Fort Atkinson, WI). Appropriate ten-fold serial dilutions of each sample were plated onto BSA, MAC, or SMAC with NA to quantify the population of *Salmonella*, *E. coli* O104:H4, and *E. coli* O157:H7, respectively. Additionally, all samples were plated on TSA amended with NA (NA-TSA). When the numbers of cells dropped below the detection limit (<10 CFU/ml), enrichment was performed according to protocols outlined in Bacteriological Analytical Manual (FDA, 2011a, 2011b).

2.6. Statistical analysis

Fisher's least significant difference test in the general linear model was conducted, using the Statistical Analysis Software (version 9.4; SAS Institute Inc., Carey, N.C.), to determine the difference in the cell population (log CFU/ g of seedling/sprout tissues)