FATE OF *SALMONELLA ENTERICA* AND ENTEROHEMORRHAGIC *ESCHERICHIA COLI* DURING VEGETABLE SEED GERMINATION AND IN THE PRESENCE OF BACTERIAL COMPETITORS

by

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(Under the Direction of Jinru Chen)

ABSTRACT

Vegetable seeds are potential vehicles of human pathogens and a likely source of contamination in most sprout-associated outbreaks. The objectives of this study were to observe 1) the fate of two important human pathogens, *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC), artificially inoculated on seed-producing flowers or internalized into vegetable seeds, during pilot-scale seed production or germination processes, and 2) the competition of the two human pathogens with a group of selected bacterial strains for growth in microbiological media and attachment to vegetable seeds.

Salmonella and EHEC cells were vacuum-infiltrated into seeds of alfalfa, fenugreek, tomato, and lettuce before germination at 25 ºC. Sections of sprout and seedling tissues were collected for microbiological analysis during the 9-day germination process. Results showed that the growth and distribution of the two human pathogens on sprout/seedling tissues were affected by bacterial species and strains as well as vegetable seed types.

Open flowers of alfalfa, fenugreek, tomato, and lettuce were inoculated with different strains of *Salmonella* and EHEC. Seeds from the inoculated flowers were germinated at 25 ºC

for 7 days, and sprout or seedling tissues were analyzed subsequently for *Salmonella* or EHEC. An average of 2.7% of the sprouts or seedlings developed from seeds of contaminated flowers tested positive for *Salmonella* whereas none of the samples tested positive for EHEC.

Salmonella or EHEC were set for competition with a group of selected bacterial competitors, including *Lactobacillus. rhamnosus* GG, for growth in nutrient broth and attachment to sprout seeds. The inhibitory effect of 72 h cell-free supernatants of the bacterial competitors on *Salmonella* and EHEC growth was also evaluated. The presence of competitive bacteria, especially *L. rhamnosus* GG significantly inhibited the growth of *Salmonella* and EHEC. Cell-free supernatants of *L. rhamnosus* GG spent cultures also inhibited the two pathogens. *Pseudomona*s. *fluorescens* A506 showed strong ability to reduce attachment of *Salmonella* and EHEC to seed `surfaces.

Results of this study provided a better understanding on the fate of human pathogens during seed production and germination, highlighting the importance of following Good Agricultural Practice for vegetable seed and seed sprout production.

INDEX WORDS: *Salmonella*, EHEC, attachment, biocontrol, vegetable seeds, germination, sprouts, seedlings

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DEDICATION

I dedicate this thesis to my parents for their unconditional support during the 27 years of my life and during my Ph.D. journey.

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CHAPTER 1

INTRODUCTION

Vegetable sprouts have been increasingly involved in foodborne outbreaks since 1995 (Yang et al., 2013). The U.S. Food and Drug Administration (FDA) stated that seeds are the source of contamination in most of sprouts-related outbreaks (Ding et al., 2013a). Sprouts are produced by soaking viable seeds in water and germinating themin a warm and humid environment for 3-7days, which provides ideal environment for the growth of human pathogens (Waje et al., 2009). Less than 10 colony forming units (CFU) of *Salmonella* Cubana per gram of dry alfalfa seeds can grow to more than 10^5 CFU per gram of fresh sprouts in 48 h under typical sprouting conditions (Howard & Hutcheson, 2003). *Escherichia coli* O157, various serotypes of *Salmonella enterica, Bacillus cereus, Yersinia enterocolitica, Shigella,* and *Listeria monocytogenes* have also been documented as causative agents of disease outbreaks associated with sprouts (Harmon et al., 1987; Jackson, 1998).

In response to the safety concerns about sprouts, the California Department of Health Services, FDA, and the International Sprout Growers Association initiated voluntary programs for producers and encouraged the treatment of seeds with 20,000 ppm calcium hypochlorite before sprouting (Proctor et al., 2001). The producers are also allowed to use an alternative treatment if it reduces microbial load on vegetable seeds by ≥ 3.5 log as shown by data in a refereed scientific journal. However, many studies have indicated that either the 20,000-ppm calcium hypochlorite treatment, or other treatments currently approved by regulatory agencies, cannot reliably eliminate all types of pathogens from sprout seeds (Gandhi & Matthews, 2003;

Stewart et al., 2001). The location of pathogen cells on/in seeds (surface attachment *vs*. internalization) play a role in the efficacy of the current seed disinfection strategies (Cooley et al., 2003).

Though contaminated seeds are the root cause of most sprout-related outbreaks, little is understood about the pathway(s) utilized by the pathogen cells to make contact with seeds (Dechet et al., 2014; Mahon et al., 1997). It is known that the activities of insects and wildlife, as well as the splash of contaminated irrigation water, can introduce pathogen cells to open vegetable flowers in the field (Chen et al., 2010; McArt et al., 2014). The secretion of nutrients from flower stigma can support the growth of microorganisms (McArt et al.,2014). Therefore, flowers of a vegetable plant might be an entry site for human pathogens associated with seeds. Transmission of *Salmonella* and EHEC cells to seeds or fruits following inoculation of *Arabidopsis thaliana* or tomato flowers with the pathogen cells has been reported in two pilot studies (Cooley et al., 2003; Guo et al., 2001).

The influence of resident microflora to the fate of human pathogens during seed production and processing, as well as the potential of using biological control agents to reduce the risk of postharvest seed contamination, are drawing the attention of seed safety specialists and researchers (Bacon & Hinton, 2002; C. De Roever, 1998). Microorganisms such as *Pseudomonas fluorescenc*e 2-79 and *Enterobacter asburiae* have potential in the control of human pathogens during sprouting (Liao, 2008; Ye et al., 2010).

This study was designed to understand the fate of *Salmonella* and enterohemorrhagic *Escherichia coli*, when internalized into vegetable seeds by vacuum infiltration, during seed germination (Chapter 3), to determine the ability of *Salmonella* and EHEC cells to transmit from flowers of vegetable plants, to sprouts and seedlings *via* seeds produced by the contaminated

flowers (Chapter 4), and to observe the interactions between bacterial competitors (*i.e.* biological control agents, probiotics and plant pathogens) and *Salmonella*/EHEC with regard to their growth in microbiological media and attachment to vegetable seeds (Chapter 5).

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CHAPTER 2

LITERATURE REVIEW

2.1 Seeds and seed sprouts

Sprouting has a long history in eastern countries such as China and Japan (Plaza et al., 2003). Due to the simplicity of the process, sprouting is widely used on family basis to process various types of seeds including legumes (bean, pea, soybean), grains (barley, wheat, rye), and vegetables (alfalfa, fenugreek, radish). After sprouting, the reserved nutrient (carbohydrates and lipids) content of seeds usually decreases due to the degradation of carbohydrates and lipids that provides energy and precursor molecules for embryonic growth and protein synthesis (Devi et al., 2015). The lipid content of mung bean seeds can decrease by approximately 27% and total energy by 7% over a 120 h sprouting process (Masood et al., 2014). In most sprout seeds the unsaturated fatty acids exceed the saturated ones after germination. For instance, the buckwheat sprouts were found to contain significantly higher levels of linoleic and linolenic acid, which are essential fatty acids for humans, but lower levels of oleic acid than the pre-germinated seeds (Wijngaard & Arendt, 2006). At the same time, the metabolism of actively-growing plant cells can increase the digestibility of seed nutrients and change the content of vitamin and dietary fiber in sprouts. Urbano et al. (2005) examined the proteolytic activity of green pea sprouts and the utilization of seed starch in growing rats. They found that two days of germination significantly improves the digestibility of protein and carbohydrate in green pea seed. The sprouts of alfalfa, soybean, and wheat were found to respectively contain 10, 4, and 2.6 times more B-vitamins than their

dry seeds on a dry weight basis (Plaza et al., 2003). The vitamin A content of alfalfa seeds can increase approximately 1,000 times during sprouting, from 0.42 to 531.89 retinol equivalent per 100 g dry weight (Plaza et al., 2003). The crude fiber content of mung bean seeds increased about 110% in seed sprouts after 120 h of germination (Masood et al., 2014). In addition, sprouting can enhance the production of antioxidants such as vitamin C and polyphenols in many types of seeds. Seeds of horse bean and mungo bean have been reported to respectively gain a maximum 67% and 61% of antioxidant capacity during sprouting (Shah et al., 2011). The ability of mashed mungo bean sprouts to inhibit lipid peroxidase increased by 359% after 5 and 7 days of germination.

Compared to raw seeds, sprouts usually contain less complex carbohydrates or lipids but increased amounts and varieties of protein, vitamin, crude fiber, antioxidants, and fatty acids, which makes them good candidates in a nutritious and healthy diet. Table 2-1 lists the nutrition facts of alfalfa sprouts *vs*. commonly consumed leafy green (lettuce), vegetable fruit (tomato), and seed (raw peanut). Compared to commonly consumed vegetables and seeds, alfalfa sprouts provide a higher amount of dietary fiber and omega-fatty acids per gram of carbohydrates and fat. Twenty-eight grams of alfalfa sprouts can provide 1.1 g protein, which is 3 and 5 times higher than those provided by lettuce and tomato, respectively. Comparing to peanuts, one takes about 490 kJ less energy from alfalfa sprouts to obtain the same amount of proteins (7.2 g), which makes the alfalfa sprouts a good candidate in diet menus. Alfalfa sprouts are also a good source of magnesium and phosphorous and can provide a similar amount of iron as tomato and lettuce per gram of fresh weight

Table 2-1. Nutrition facts for alfalfa sprouts, lettuce, tomato, and peanut

Nutrients [a]	Alfalfa sprouts	Lettuce	Tomato	Peanut
Calories (kJ)	26.8	20.1	20.9	666.0
From fat	6.7	2.9	2.1	481.0
Total Carbohydrates (g)	0.6	0.9	1.1	4.5
Dietary fiber	0.5	0.6	0.3	2.4
Total fat (g)	0.2	0.1	0.1	13.8
ω-3 fatty acids (mg)	49.0	31.6	0.8	0.8
ω-6 fatty acids (mg)	65.5	13.2	22.4	4355
Protein (g)	1.1	0.3	0.2	7.2
Vitamin A (IU)	43.4	2,439.0	233.0	0.0
B-vitamins (mg)	10.4	38.2	6.0	71.4
Vitamin C, D, E, K (mg)	10.8	35.4	4.4	2.3
Calcium[b]	9.0	9.2	2.8	25.8
Iron	0.3	0.3	0.1	1.3
Magnesium	7.6	3.9	3.1	47.0
Phosphorus	19.6	8.4	6.7	105.0
Potassium	22.1	69.2	66.4	197.0
Sodium	1.7	2.2	1.4	5.0
Zinc	0.3	0.1	0	0.9
Copper	0	0	0	0.3
Selenium (mcg)	0.2	0.1	0	0.2
Cholesterol	0.0	0.0	0.0	0.0
Phytosterol	ND[c]	ND	2.0	61.6

[a] The nutrients are scaled on 28 g (1.0 ounce) of each food material
[b] Minerals measured in milligram (mg) if not specified
[c] Not detected

Besides the high nutritional values, sprouts of alfalfa and fenugreek have several functional properties. The alfalfa and fenugreek seeds are rich in biogenic amines including putrescine, cadaverine, histamine, tyramine, spermidine, and spermine that can help control blood glucose and cholesterol values (Frias et al., 2007). Alfalfa sprouts are a rich source of phytoestrogen which can block estrogen receptors and reduce the risk ofcancer (Reinli & Block, 1996). In a 1994 research involving Australian postmenopausal women, a negative correlation

has been found in taking alfalfa sprout supplemented, low-estrogen diet and development of hormone-dependent cancer (Morton et al., 1994). Very interestingly, Shah and Mir reported that providing fenugreek seeds at 20% of diet dry matter for dairy cow reduced blood cholesterol concentration of the animals by 4%. The diet also improved the profile of functional fatty acids and lowered the cholesterol content of the milk produced (Shah & Mir, 2004).

The health and nutritional advantages of seed sprouts have led to the popularity of sprout-related produce in western countries where consumer demand for minimally-processed, more natural, nutritious, and healthy foods has been increasing (He & Chen, 2013). The first interest in sprouts in the United States arose during World War II when soy sprouts were taken out of the Chinese markets and placed on American tables as an excellent wartime meat substitute and a rich source of protein and vitamins B and C (Shurtleff & Aoyagi, 2011). The pioneering research on the health and nutritional values of soy sprouts was led by Dr. Clive McCay and other members in the School of Nutrition at Cornell University in the 1940s. The interest from researchers and consumers in sprout products kept fermenting until today as more health benefits of the sprout foods are unveiled and more sprout species are introduced to the diet of the country. Alfalfa sprouts are currently among the most common sprout varieties consumed in U.S. and approximately 80 million pounds of alfalfa seeds are produced each year. Approximately 15-20 million pounds of mung bean seeds are consumed annually in U.S., almost all in the form of sprouts. By 2002, a total of 300,000 tons of sprouts were produced annually in U.S. by more than 400 growers and the value of the whole industry rose over $25 million dollars (Thomas et al., 2003). In Europe, more than 129 professional production establishments are operating for sprouts production, with an industrial market value over 500 million €.

2.2 Sprout-associated foodborne illnesses

Foodborne illness is a major public concern that affects great numbers of consumers and can cause huge economic burdens to the government and tax payers. The Centers for Disease Control and Prevention (CDC) estimated that more than 48 million people in the U.S. suffer from foodborne illnesses, resulting in 128,000 hospitalizations and 3,000 deaths annually (Scallan et al., 2011). Approximately 77.7 billion U.S. dollars were spent every year on investigating foodborne illnesses caused by human pathogens (Scharff, 2012). One of the first sprout-associated outbreaks was reported in 1973, with soy, mustard, and cress sprouts grown by using a home sprouting kit. *Bacillus cereus* was found on the seeds provided by the kit and sprouting of the contaminated seeds resulted in large number of the pathogen cells on sprouts (Taormina et al., 1999).

Sprouting conditions are optimal for the growth of human pathogens thus making sprout products highly susceptible to pathogen contamination. For instance, the widely used industrial sprouting conditions for alfalfa sprouts are to grow a maximum of 100 pounds of seeds in a rotating drum and apply irrigation water of *ca*. 24 ºC every 5-10 min. The seeds are sprouted for 3-7 days in dark to expect a maximum of *ca.* 500 pounds of sprouts per drum. The high humidity brought by frequent irrigation with warm water allows rapid establishment of bacterial populations on sprouts. The population of *S.* Stanley inoculated to alfalfa seeds were reported to increase 2.5 logs in 24 h, by another 1 log in the following 18 h and only decreased slightly after 10 days of refrigerated storage (Jaquette et al., 1996). The flowing of irrigation water may further circulate pathogen cells in the drum and result in the contamination of all sprouts from a single contaminated seed. Similar sprouting conditions are used for many other types of seeds

and the sprouting times are generally long enough for the thriving of rapidly-growing pathogens (FDA, 2017).

Since seed sprouts are usually consumed raw or minimally-processed, they have been linked to multiple high-profile outbreaks of gastrointestinal infections (NACMCF, 1999). . In addition to these outbreaks, eating alfalfa sprouts contaminated with *Escherichia coli* O157 caused two serious outbreaks in Colorado and Minnesota in 2003 (Ferguson et al., 2005). An outbreak of *E. coli* O104:H4 infections in Germany and France in 2011 were linked to the consumption of contaminated fenugreek sprouts and the two outbreaks combined caused more than 3,940 illnesses and 52 deaths from uremic hemolytic syndrome (HUS) (Buchholz et al., 2011). The most recent sprout-associated outbreak of infection occurred in May 2016, and resulted in 36 illnesses and 7 hospitalizations (CDC, 2017). Among human pathogens, *Salmonella enterica* and enterohemorrhagic *E. coli* (EHEC) are most commonly linked to sprout-related outbreakS (Ding et al., 2013). Cells of *Salmonella* are rod-shaped, flagellated, facultative anaerobic, and Gram-negative. *S. enterica* is one of the two species (*S. enterica* and *S. bongori*) of the genus *Salmonella* and is responsible for the majority of *Salmonella*-associated foodborne outbreaks (Tindall et al., 2005). *S. enterica* has many human pathogenic serovars such as *S.* Typhimurium, *S.* Newport, *S.* Montevideo, and *S.* Saintpaul. *S. enterica* can infect human intestinal epithelium, causing cell death and inflammation in the gastrointestinal tract (Natvig et al., 2002). Typical syndromes of salmonellosis include diarrhea, fever, abdominal cramps, and vomiting. Although most infected individuals can naturally recover within 4-7 days after illness onset, salmonellosis could lead to more severe outcomes including death among immunity-compromised individuals that are not treated in time (CDC, 2009). According to the CDC,

Salmonella is estimated to cause one million illnesses in the U.S., with 19,000 hospitalizations and 380 deaths every year.

EHEC are a subset of pathogenic *E. coli* that infects humans and causes diarrhea or hemorrhagic colitis (Griffin & Tauxe, 1991). Hemorrhagic colitis can occasionally progress to Hemolytic-uremic syndrome (HUS), which leads to acute renal failure in children and morbidity/mortality in adults. *E. coli* O157:H7 strains are most frequently involved in EHEC-associated foodborne outbreaks. Approximately, 265,000 EHEC infections occur in the U.S. every year, and serotype O157 alone accounts for 36% of these infections (Swaminathan et al., 2001). In recent years, increasing numbers of clinical cases and outbreaks caused by other EHEC serogroups h. As stated previously, EHEC serotype O104:H4 caused a serious outbreak of hemorrhagic colitis and HUS in Germany and France in 2011 (Buchholz et al., 2011).

Many *S. enterica* and EHEC strains are resistant to various environmental stresses (dehydration, starvation, UV, etc.), which allows them to survive in non-host environments for a relatively long time. For instance, *S.* Typhimurium DT104 and DT12 survived up to 299 days in soil in a terrestrial microcosm study (Baloda et al., 2001). You et al. (2006) recovered a five-strain cocktail of *E. coli* O157:H7 in soil-manure mixtures 200 days after inoculation. Pathogen cells in soils amended with manure can potentially make contact with field crops by irrigation, rain splashing, insects, and activities of wildlife. Some *Salmonella* and *E. coli* strains can adapt themselves to specific plant hosts including those that are used to grow fresh produce. For instance, *S.* Montevideo was reported to survive and grow significantly better than nine other *S. enterica* serovars on the surface and within stem/fruit tissues of tomato plants (Shi et al., 2007). Therefore, in many ways *Salmonella* and EHEC impose a greater risk to the safety of foods and

more efforts are needed to reduce the number of outbreaks and resulting economic loss associated with fresh produce and sprouts.

2.3 Sprout seed disinfection

Epidemiological studies on sprout-associated outbreaks revealed that seeds are the most likely source of contamination. Investigations on alfalfa sprouts-related outbreaks in Finland and 17 states of the United States in 1995 revealed that the source of contamination was the same lot of alfalfa seeds contaminated with *S.* Stanley (Sivapalasingam et al., 2004). In 1997, the alfalfa sprouts involved in outbreaks of *E. coli* O157:H7 infection in Michigan and Virginia were grown from the same lot of seeds (Como-Sabetti et al., 1997). In 2003, two *E. coli* O157 outbreaks associated with alfalfa sprouts in Colorado and Minnesota were linked to a common seed source (Ferguson et al., 2005). In a 2009 sprouts-related multistate outbreak of *S.* Saintpaul infections, seeds from a single grower were identified as the source of contamination (Barton Behravesh et al., 2011).

Many studies on disinfection of seeds revealed that once the seeds are contaminated with *Salmonella* or *E. coli*, complete decontamination of the seeds can be difficult. Jaquette and coworkers (1996) investigated the efficacy of chlorine and heat treatment for disinfection of alfalfa seeds. Although washing with 100 µg/ml active chlorine solution or heating at 54 °C for 10 min significantly reduced *S.* Stanley populations on seeds, the pathogen cells were not eliminated (Beuchat & Scouten, 2002). Treating alfalfa seeds pre-inoculated with 2.68 log CFU/g of *E. coli* O157 with 20,000 ppm active chlorine did not eliminate pathogen (Taormina et al., 1999). Beuchat (1997) also treated alfalfa seeds with 1,800/2,000 ppm of active chlorine, 6% hydrogen peroxide, or 80% ethanol for 10 min and *Salmonella* cells could still be recovered from the water used to wash the treated seeds. The underlying mechanisms of *Salmonella* and *E. coli*

strains to resist and survive the disinfection process are not yet fully understood, but several have been proposed. Wang and coworkers (2009) examined the response of *S. enterica* and *E. coli* O157 cells to chlorine treatment and found that certain stress-resistant mechanisms can be triggered when the pathogen cells are exposed to sub-lethal concentrations of active chlorine. The interaction with the organic matters released by plant tissues may also reduce the active chlorine concentration during disinfection (Ölmez & Kretzschmar, 2009). In addition, it is known that the bacterial cells could become internalized into protected niches of vegetable seeds, either during seed development or by infiltration, which allows the cells to avoid contact with chlorine molecules (Buck et al., 2003a).

2.4 Seed sprouting and pathogen growth

The sprouting of seeds incorporates many steps that start with the uptake of water by the dry seed and terminates with the elongation of the embryonic axis (Bewley, 1997). Likely relying on a signal transduction cascade involving synthesis of, or sensitization to, germination-promoting gibberellins, the dormant seeds are activated and start germinating (Hilhorst, 1995). The change in phosphorylating activity of membrane Ca^{2+}-dependent protein kinases was also reported to play a role in the activation process (Trewavas, 1987).

The quick influx of water during imbibition rehydrates cells and the majority of the metabolic activity of seeds resumes. Several membrane-stabilizing phospholipids, such as *N*-acetylphosphatidylethanolamine, might be simultaneously released to repair damaged membranes and organelles caused by desiccation and rehydration of seeds (Sandoval et al., 1995). Within minutes after imbibition starts, the respiratory activity of seeds resumes followed by the recovery of various metabolites mechanisms including the synthesis of proteins and mRNA (Bewley, 1997). The energy and precursor molecules involved in the biosynthesis, cell

growth, and elongation of radicles are provided by the degradation of stored nutrients in seeds, during which some of nutrients are released as seed or root exudates. Although different levels and rates of exudation were reported for different types of seeds (Kageyama & Nelson, 2003), the exudates of sprouting seeds generally contain plentiful amount of carbohydrates, amino acids, fatty acids, and other nutrients that can be used by microorganisms (Buck et al., 2003b). It has been reported that sprouting alfalfa sprouts can indifferently support the growth of various serovars of *S. enterica* and strains of *E. coli* O157 (Charkowski et al., 2002; Howard & Hutcheson, 2003).

The growth of *Salmonella* and *E. coli* during sprouting can be rapid and only requires the presence of very few cells on seeds. Howard et al. (2003) reported that an initial inoculation level lower than 20 CFU of *Salmonella* per g of alfalfa seeds resulted in a pathogen population of more than 6 log CFU/g of alfalfa sprouts within 48 h of sprouting. The authors also found that the growth of *S. enterica* strains on germinating alfalfa seeds was independent of the pathogen serovar, isolation source, or virulence of the strain. On alfalfa sprouts, the growth of *E. coli* O157 was lower than that of *S. enterica* strains (Charkowski et al., 2002; Cui et al., 2017). After 48 h of germination, the *E. coli* O157 population was about 2 log CFU/sprout lower than that of *S.* Newport, but still increased by 100-fold and reached *ca*. 5 log CFU/sprout (Charkowski et al., 2002). Several other studies confirmed that human pathogens like *Salmonella* and EHEC can grow rapidly during sprouting of different vegetable seeds including alfalfa, fenugreek, mung bean, and radish seeds (Itoh et al., 1998; Viswanathan & Kaur, 2001).

The ability of pathogens to thrive on germinating seedlings has made disinfection of sprouts less effective. Spraying chlorine solutions on sprouting seeds or treating contaminated sprouts with chlorine-based sanitizers does not help reduce sprout contamination developed from

contaminated seeds (Fransisca et al., 2011). Warriner and coworkers (2003) reported that *E. coli* or *S.* Montevideo inoculated onto mung beans established populations both internally and externally on sprouts within 24 h of germination. Treatment of contaminated sprouts with 20,000 ppm sodium hypochlorite removed the majority of bacteria on sprout surfaces but viable pathogen cells were still recovered from apoplastic fluid and extracts of the surface-sterilized sprouts.

Once attached to seeds or having established populations on sprouts, *Salmonella* and EHEC can survive for long periods under storage conditions. When stored at 5 ºC, *Salmonella* and *E. coli* O157:H7 survived on dry alfalfa seeds for at least 52 weeks with the populations reduced by less than 1 log unit (Beuchat & Scouten, 2002). The population of *S.* Typhimurium was reported to grow *ca*. 2 log units on mung bean sprouts when stored at 15 ºC for 7 days. Lowering storage temperature to 4 ºC prevented the growth of *S.* Typhimurium on sprouts but did not significantly reduce the viable cell number of the pathogen during the 7-day storage (Tian et al., 2012). In the same study, *E. coli* O157 was unable to survive on sprouts at 15 or 4 ºC during storage. However, more strains and sprout types need to be tested before a conclusion can be drawn, as other *E. coli* O157 strains were reported to survive in apple juice for up to 24 days at 4 ºC (FDA, 2008).

Besides sprouts, contamination of crop seeds/seedlings by *Salmonella* or EHEC has also been reported to facilitate the circulation of the pathogens during crop cultivation. *E. coli* O157:H7 and *S. enterica* attached to butterhead lettuce seeds have been reported to survive for at least two years post inoculation under commercial storage conditions (Van der Linden et al., 2013). Germination of contaminated seeds yielded seedlings that tested positive for *E. coli* O157 and *Salmonella*; further cultivation of the contaminated seedlings resulted in the internalization

of *Salmonella* and *E. coli* cells to the mature lettuce plants. Using *A. thaliana* as a model system, Cooley and coworkers (2003) reported the transmission of *S. enterica* to mature *A. thaliana* plant from seeds artificially inoculated with the pathogen. Deering and coworker (2015) later reported the presence of *E. coli* O157 in mature tomato fruit grown from tomato seeds contaminated with the pathogen.

2.5 Attachment of human pathogens to seed surfaces

Among the many ways that seeds become contaminated by bacterial pathogens, pathogen attachment to seed surfaces is the most prevalent. The association of bacterial cells with their surfaces involve a number of physical, chemical, and biological processes (Garrett et al., 2008). Characklis and Marshal (1990) described the process of attachment and biofilm formation of bacterial cells on their contact surfaces, including the formation of conditioning layers, reversible and irreversible adhesion, formation of biofilm, and the following detachment of cells from the biofilm. In a typical attachment process, the initial formation of a conditioning layer by organic and inorganic matters in the bulk fluid can modify the surface charge, electrical potential, and tensions of the contact surface and improves accessibility of planktonic microbial cells to attachment sites (Marshall, 1996). Then the planktonic cells are transported from the suspension to the conditioned contact surfaces by physical forces or by bacterial surface structures such as flagella and fimbria. Factors including surface functionality, temperature, bacterial orientation, and pressure conditions may influence the firmness of attachment. The irreversible attachment of bacterial cells has been described as a result of stronger attachment of bacterial cells to contact surfaces through physical appendages (flagella, fimbriae and pili), which overcomes the repulsive forces of the electrical double layer (Pratt & Kolter, 1998). The appendages then react with chemicals in the conditioning layer by oxidation and hydration, which further facilitates the

consolidation of the bacteria onto the contact surface. The irreversibly attached bacterial cells can further form biofilms on the contact surfaces to gain stronger resistance to environmental stress, antimicrobial compounds, washing, and killing steps. The commonly used disinfectants, sodium hypochlorite (500 mg/liter), sodium hydroxide (1 M), and benzalkonium chloride (0.02%) were reported unable to eradicate the mature 168-h *Salmonella* biofilm (Corcoran et al., 2014). The structure of the biofilm reportedly protected the cells of *E. coli* O157 ATCC 43895 from 50 μg/ml active chlorine, which can kill *ca*. 9 log CFU/ml of planktonic cells of the strain within 10 min of contact (Ryu & Beuchat, 2005). Sessile cells can cease the expression of genes encoding surface appendages and express genes for the production of cell surface proteins such as Opr C and Opr E, and excretion materials such as polysaccharides. These proteins and polysaccharides fortify adhesion and cohesion (*i.e.* cell to cell contact) of bacterial cells in a biofilm and improve the stability and environmental resistance. In a previous study, as many as 57 proteins were found in biofilms of various microorganisms but not in the corresponding planktonic profile (Hall-Stoodley & Stoodley, 2002).

Multiple factors, from both the attaching pathogen cells and vegetable seed themselves may affect the attachment process (Cui et al., 2017). The characteristics of seed surfaces were reported to have a significant influence on the levels of contamination by artificially-inoculated bacterial pathogens (Rajkowski, 2009). The wrinkled seed coat and/or the presence of cracks on the seed surfaces significantly enhanced the attachment of *Salmonella* cells to alfalfa seeds. Cui and coworkers (2017) also reported the differential attachment of *S. enterica* and EHEC strains to seeds of four different types of vegetable seeds (alfalfa, fenugreek, tomato, and lettuce) and with different surface characteristics (fungicide-treated versus untreated and intact versus mechanically damaged). In addition, the hydrophobicity and the positive/negative charges

carried by bacterial surfaces have been reported to affect attachment/detachment of *Salmonella* and *E. coli* O157 cells to cantaloupe rind and meat (Ukuku & Fett, 2002).

Cui and coworkers (Unpublished) found that different strains of *Salmonella* and EHEC had significantly different levels of attachment to the vegetable seeds tested. And the same pathogen strain had different level of attachment to different type of vegetable seeds, which indicate that the characteristics of both bacteria and vegetable seeds play an essential role in the attachment of bacteria cells. *S.* Typhimurim was reported to survive in soil (through dripping of contaminated irrigation water) for at least 41 days and cause contamination to cantaloupe surfaces by direct contact with/splash of the contaminated soil (Lopez-Velasco et al., 2012). Cooley and coworkers (2003) also described the attachment and migration of *S. enterica* serovar Newport and *E. coli* O157:H7, inoculated in soil, from root to flowers of *A. thaliana* plants.

2.6 Critical genes involved in bacterial attachment

Several critical genes have been reported to greatly influence the attachment and biofilm formation of *Salmonella* and EHEC strains to contact surfaces. Using gene-knockout mutants of *S*. Typhimuium ATCC 14028, Tan et al.(2016) reported the significant role of *fliB, fliC, bcsA*, *csgA*, and *csgD* genes, characterized as encoding or regulating genes of phase 1 flagellin, phase 2 flagellin, cellulose, fimbrae and cellulose/fimbriae, in the attachment of the pathogen to plant cell walls. The synergetic effects between thin aggregative fimbrae and cellulose played a crucial role in the formation of *Salmonella* biofilm (Jain & Chen, 2007). The synthesis of thin aggregative fimbriae by *Salmonella* greatly enhanced the adhesion of *Salmonella* cells to contact surfaces. It has been reported that the deletion of *agfD,* a regulator of biosynthesis of thin aggregative fimbriae, resulted in *Salmonella* mutants lacking all forms of multicellular behavior and the ability to form biofilm (Römling et al., 1998). However, contradictory results were also

reported. Tan et al. (2016) reported a significantly higher attachment of a *agfD*-deletion *S.* Typhimurium mutant as compared to wild type cells at 37°C. The authors suggested that the result might be due to the increased expression of flagella-related genes which are also indirectly down-regulated by *csgD.* Additionally, *S. enterica* mutants lacking *yihO,* which encodes regulators for O-antigen capsule assembly and transport, displayed reduced attachment to various plant surfaces (Barak et al., 2007). In another study, transposon insertion at *rpoS,* the global stress regulator, caused the *Salmonella* mutants to attach poorly to alfalfa sprouts (Barak et al., 2005).

Production of other exopolysaccharides such as colanic acid and poly-β,1-6-N-acetyl-D-glucosamine (PGA) were reported to play a role in the attachment of *E. coli* cells to alfalfa seed and sprout surfaces (Mathews et al., 2014). Matthysse and coworkers (2008) tested the attachment of *E. coli* mutants that lack expression of *pgaC* (PGA production), *bcsB* (cellulose production) and *wcaD* (colanic acid) genes and found significantly reduced attachment of the mutants to alfalfa sprouts. In the same study, the researchers also found that the introduction of plasmid vectors carrying a cellulose synthase gene or a *bps* polysaccharide biosynthesis operon to non-attaching *Sinorhizobium meliloti* and a nonbinding mutant of *Agrobacterium tumefaciens* resulted in the attachment of the two microorganisms to tomato roots. Type I pili encoded by *pilA* gene is another essential component in the attachment of *E. coli* to contact surfaces and the formation of biofilms (Orndorff & Falkow, 1985). It was reported that type I pili of *E. coli* contains the mannose-specific adhesin, FimH, which not only facilitates pathogenesis through interactions between FimH and mannose oligosaccharides on eukaryotic cell surfaces, but also promotes the adherence of *E. coli* cells to abiotic surfaces, possibly

through a non-specific binding using a separate region of the FimH adhesin (Pratt & Kolter, 1998).

2.7 Other pathways of contamination

Infiltration is another mechanism that might be used by human pathogens to contaminate seeds (Buck et al., 2003a). The process can happen naturally during production and harvest of seeds, when water pressure overcomes the internal gas pressure and hydrophobic nature of seed surfaces, allowing bacterial cells to be infiltrated through cracks, crevices, and intercellular spaces of vegetable seeds. Such pressure differences are generally considered to be induced by the change of environmental temperatures and water activities of the seed surface.

Although no data are currently available about the true incidence of infiltration under natural conditions, the chance of pathogen infiltration into vegetable seeds is considered to be low. Thus, seeds contaminated in this manner are difficult to detect through routine sampling and testing. Internalized pathogen cells are also more difficult to kill due to the inability of the sanitizers to make contact with pathogen cells (Charkowski et al., 2001).

Besides infiltration, pathogen cells may gain entry to vegetable seeds through plant flowers, which may result in contamination of internal tissues of vegetable seeds. This pathway is used by various seed-borne phytopathogens. For instance, the transmission of *Xanthomonas campestris* pv. *campestris* (Wolf et al., 2013), *Acidovorax citrulli* (Fessehaie & Walcott, 2007), and *Pseudomonas syringae* (Dutta et al., 2014) to seeds are reported to rely on penetration of the pistil of flowers. Whether human pathogens can utilize the same pathway to contaminate crop seeds has not been extensively studied, given the fact that human pathogens have many chances to contact crops plants and their flowers through the activity of insects, livestock, wildlife, rain splash, and contaminated irrigation water (Cooley et al., 2003; Lim et al., 2014).

Several human pathogenic *Enterobacteriaceae*, including *Salmonella* and *E. coli* O157:H7, are known to be carried by insect hosts (Nadarasah & Stavrinides, 2011). Wasala and coworkers (2013) previously reported that *E. coli* O157:H7 cells acquired by flies from contaminated cattle manure and deposited in regurgitation spots of spinach leaves can survive and multiply. The feeding damage caused by phytophagous insects, such as *Frankliniella occidentalis*, was found to sustain higher *S. enterica* populations on agricultural crops at the damaged spots (Soto-Arias et al., 2013). Insect feces can also spread pathogens on plants. Feeding infested cress shoots to grape snails resulted in the recovery of *S.* Typhimurium and *E. coli* O157:H7 from the dry snail excrement up to 10^5 CFU/ml (Semenov et al., 2010).

Since plants are considered as hostile environments for human pathogens, it is not fully understood if the pathogenic cells survive in contaminated seeds and finally lead to sprout/seedling contamination (Brandl, 2006). Cooley and coworkers (2003) reported the presence of *S. enterica* strains in the seeds of *A. thaliana* grown from flowers inoculated with the same pathogen. Guo and coworkers (2001) also reported the presence of *S. enterica* cells in the pulp of tomato fruits grown from flowers contaminated with the pathogen. It is worth noting that seed infiltration might result in the internalization of pathogen cells into seeds, thus making detection and control of the pathogens before and during seed germination processes.

Enteric pathogens internalized in plant roots of agricultural crops were able to translocate to leaves and fruits (Franz et al., 2007). In a hydroponic system pre-contaminated with *S.* Typhimurium or *E. coli* O157:H7 or in the potting mixes irrigated with water contaminated with the same pathogens, viable *Salmonella* or *E. coli* cells were recovered from surface-sterilized lettuce leaves (Nthenge et al., 2007). Lepidot and Yaron (2009) also reported that *S.* Typhimurium added to irrigation water could penetrate the roots of parsley plants and translocate

to the leaves or stems. Although no data are currently available about the contamination of seeds by human pathogen through root penetration, the ability of the pathogens to survive, move within plant tissues, and localize in fruits indicate the possibility of human pathogen cells to make contact with, and contaminate to vegetable seeds.

2.8 Interactions between plant and human pathogens

The interactions between plant and animal pathogens have drawn a lot of attention as the activity of plant pathogens and the developed plant diseases can modify the phyllosphere and enhance the growth of resident enteric pathogens (Lim et al., 2014). Co-inoculation of *Pectobacterium carotovorum* subsp. *carotovorum* with *S. enterica* or *E. coli* O157:H7 onto fresh potato, carrot, and pepper disks resulted in 10-fold higher *Salmonella* or *E. coli* O157:H7 levels relative to those inoculated with the human pathogens alone. The same disks co-inoculated with *Pseudomonas viridiflava* and *S.* Typhimurium contained approximately three times the *Salmonella* populations as disks inoculated with *Salmonella* alone (Wells & Butterfield, 1997). Fungus-induced soft rot of potato, carrots, and pepper from commercial sources were also found to enhance the growth of *Salmonella* (Lim et al., 2014). The presence of *X. campestris* pv. *vesicatoria* on tomato plants did not affect the incidence of *S. enterica* in the tomato phyllosphere but enhanced the growth of *S. enterica* in the absence of disease symptoms (Barak & Liang, 2008). Pollard et al. (2014) reported an increased number of *S. enterica* cells recovered from Eastern shore Virginia tomatoes co-inoculated with *Ralstonia solanacearum* and concluded that *R. solanacearum* can impart the survival and transportation of *S. enterica* cells through the internal tissues of tomato plants.

Resident plant bacteria, in addition to plant pathogens, might also enhance the survival of human pathogens. Presence of *P. syringae* and *Erwinia herbicola* on lettuce leaves inoculated

with *S. enteric*a resulted in 10 times more *Salmonella* cells 7 days post inoculation, compared to those from leaves that were inoculated with *S. enterica* alone (Poza-Carrion et al., 2013). Potnis et al. (2014) reported that the suppression of pathogen-associated molecular pattern-triggered immunity by a virulent *X. perforans* strain creates a favorable environment for the growth of *S. enterica* in the tomato phyllosphere. Following successful colonization, the human pathogens can conversely suppress the growth of plant bacteria and dominate the growth niches. For instance, S. *enterica* reduced *P. carotovorum* populations and soft rot progression by lowering pH (Kwan et al., 2013). Therefore, the local microbiota of plants might play an important role in the survival of enteric pathogens in the phyllosphere, which is generally-regarded as a hostile environment for enteric pathogens. However, whether similar interactions between the two pathogen types occur on seeds is not known, as dry/germinating seeds represent a different environments than mature plants with respect to nutrient availability (Bewley, 1997).

2.9 Novel seed treatments and biological control agents

In addition to chlorine and thermotherapy, other treatments have been developed for disinfection of sprout seeds. High pressure is a promising seed treatment in combination with 20,000 ppm sodium hypochlorite. Seed treated at 500 to 600 MPa for 2 min at room temperature showed a reduction in *E. coli* O157:H7 load of 3.50 log CFU/g (Neetoo et al., 2009). The high-pressure seed treatment was even more effective when combined with presoaking in chlorine solution or higher temperature (Neetoo & Chen, 2010). Treatment at a lower pressure (300 MPa) for a longer time (15 min) achieved similar disinfection effects, although a delay in germination was observed (Wuytack et al., 2003). The high-pressure treatment can generally achieve a microbial reduction of 5.09 log CFU/g, which is the most effective seed disinfection strategy thus far. However, more tests are needed to examine the efficacy of the method for more seed

types and under different processing conditions before it can be used commercially (Ding et al., 2013a).

Irradiation dosage lower than 8 kGy has been approved by the FDA for decontamination of seeds (FDA, 2008a). On average, 3.18 log units of population reduction was consistently achieved on different types of seeds and pathogens when irradiation dosage was controlled lower than 2 kGv (Rajkowski & Thayer, 2001; Schoeller et al., 2002). Even at low dosage, the undesirable impact of irradiation on the appearance, yield and nutritional value of sprouts can reduce its commercial applicability of sprout (Bari et al., 2004).

Competitive exclusion of pathogen cells by using plant microbial flora has been tested on fresh produce and sprouts. Several strains of bacteria, bacteriocins, and bacteriophages have been tested to inhibit the growth of *Salmonella* or *E. coli* O157:H7 during sprout production (Matos & Garland, 2005; Nandiwada et al., 2004; Ye et al., 2010). Two strains of lactic acid bacteria effectively controlled (> 6 log CFU/g inhibition) the growth of *Salmonella* or *E. coli* O157:H7 in culture media (Wilderdyke et al., 2004). Liao (2008) reported that co-inoculation of *P. fluorescens* 2-79 onto sprouting alfalfa seeds reduced the growth of *Salmonella* by 1-2 log units during sprouting. Higher reductions (*ca*. 5 log units) in *S. enterica* growth both in broth and on sprouting alfalfa seeds were observed by Fett and coworkers (2006), who reported that the *Salmonella* growth was controlled throughout the 6-day sprouting process. Ye et al. (2010) also reported a maximum 6.4 log growth reduction in *S. enterica* population using a of *Enterobacter asburiae* JX1 and *Salmonella* phage cocktail on sprouting mung bean and alfalfa seeds. In general, results from the limited studies about the biological control of human pathogens on seeds/sprouts indicated the treatment could potentially achieve a similar effect as 20,000 ppm calcium hypochlorite in the reduction of microbial populations. However, due to the

complexity of the inhibition mechanisms, uncertainty about its efficacy on an industrial scale, and the concern of potential adverse health effects (as some of the biological control agents used, such as *B. subtilis*, are opportunistic human pathogens), whether this strategy can be applicable in sprout production remains to be determined.

Using combinations of treatments can be an option when the efficacy of individual treatment is unsatisfactory. Lang and coworkers (2000) reported that using lactic acid/acetic acid in combination with 2,000 ppm sodium hypochlorite resulted greater reductions in *E. coli* O157:H7 population on alfalfa seeds than individual chemicals. Treating radish seeds with electrolyzed oxidizing water/chlorine dioxide, coupled with a drying treatment significantly reduced the pathogen load pre-inoculated onto the seeds (Kim et al., 2010). Fransisica and coworkers (2012) reported the use of a malic acid/thiamine dilauryl sulfate mixture as an alternative to the traditional 20,000 ppm calcium hypochlorite treatment for alfalfa seeds. Other studies also reported different degrees of success for different combinations of biological, physical, and chemical treatments to control human pathogens on sprouts and fresh produce (Bang et al., 2011; Pierre & Ryser, 2006; Zhao et al., 2010). Identification of an optimal combination might be challenging due to the complexity introduced by the application of multiple treatments and the susceptibility of microorganisms to these treatments on in vivo. When the combinations contain one or more biological control agents, it is important to understand the antagonistic mechanisms used by each agent and make the best use of potential synergistic effects.

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CHAPTER 3

FATE OF *SALMONELLA ENTERICA* AND ENTEROHEMORRHAGIC *ESCHERICHIA COLI* CELLS, ARTIFICIALLY INTERNALIZED INTO VEGETABLE SEEDS DURING GERMINATION

Liu, D., Cui, Y., Walcott, R., & Chen, J. (2017). *Applied and Environmental Microbiology*, AEM-01888. Reprinted here with permission of the publisher

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^{o}C for 9 days and different sprout/seedling tissues were microbiologically analyzed every other day. Internalization of *Salmonella* and EHEC cells into vegetable seeds was confirmed by the absence of pathogens in seed-rinsing water and presence of pathogens in seed homogenates after post-internalization seed surface decontamination. Results show that 317 (62%) and 343 (67%) out of the 512 collected sprout/seedling tissue samples were positive for *Salmonella* and EHEC, respectively. Average *Salmonella* populations were significantly higher (p<0.05) than the EHEC populations. Significantly higher *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 pre-germinated seeds. Three *Salmonella*, and two EHEC, strains had significantly higher cell populations on sprout/seedling tissues than other strains used in the study. *Salmonella* and EHEC populations from fenugreek and alfalfa tissues were significantly higher than those from tomato and lettuce tissues. The study observed 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.

Keywords: Alfalfa, EHEC, fenugreek, lettuce, *Salmonella*, seedlings, sprouts, tomato, vegetable seeds

3.1 Introduction

Consumption of vegetable seed sprouts has become popular worldwide in recent decades due to the shift of consumers' preference to healthier and nutritious foods. However, seed germination process has made sprouts highly susceptible to microbial contamination and thus, concerns have been raised about the safety of seeds used for sprout production (NACMCF, 1999). 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 (C. De Roever, 1998). In 1995, an international outbreak in Finland and 17 states in the United States was traced to alfalfa seeds contaminated with *S.* Stanley (Mahon et al., 1997). In 1997, simultaneous outbreaks of *E. coli* O157:H7 infection in Michigan and Virginia were linked to consumption of alfalfa sprouts grown from the same lot of seeds (Como-Sabetti et al., 1997). In 2003, two *E. coli* O157 outbreaks associated with alfalfa sprouts in Colorado and Minnesota were linked to a common seed source (Ferguson et al., 2005). In a 2009 sprouts-related multistate outbreak of *S.* Saintpaul infections, seeds from a single grower were identified as the source of contamination (Hanning et al., 2009).

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 (Winthrop et al., 2003). 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 space of vegetable seeds (Ding et al., 2013a). Pathogenic bacteria can naturally infiltrate cracks, crevices, and intercellular space of seeds when a negative pressure is created across the seed coat by changes in environmental temperature and hydraulic status of seed surfaces (Buck et al., 2003b). 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 surface of seeds and seedlings (Charkowski et al., 2002; Liao, 2008; Warriner et al., 2003). 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 germinating seeds (Kigel, 1995). 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.

3.2 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 3-1 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 to 3.20 log CFU/g at the end of 9-day germination process and the EHEC population increased from 0.88 to 2.30 log CFU/g. 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 *Salmonella*, significant increase in EHEC population was not observed until 7 to 9 days into the germination process. Cotyledon and seed coat tissues had the highest populations of *Salmonella* cells. On average, more *Salmonella* cells were recovered from the root tissues than the stem tissues and pre-germinated seeds. The *Salmonella* population from seed coat/cotyledon tissues was higher than that from pre-germinated seeds but was not significantly different from the cell populations from the stem and root tissues. On average, the highest EHEC population was found on the cotyledon tissues, but this population was not significantly different from that on other sprout/seedling tissues except pre-germinated seeds ($p < 0.05$). Among the four *Salmonella* strains included in the study, *S.* Montevideo had the lowest cell population and the populations of the other three *Salmonella* strains were not significantly different. The average population of *E. coli* F4546 was significantly lower 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 higher ($p < 0.05$) 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 Table 3-2 and 3-3, respectively. Higher numbers of *Salmonella* and EHEC cells were recovered from fenugreek and alfalfa, than tomato and lettuce, tissues, except *E. coli* F4546 (Table 3-2 and 3-3).

The mean populations of *S.* Montevideo on fenugreek and alfalfa tissues were significantly lower ($p < 0.05$) than the cell populations of the other three *Salmonella* strains (Table 3-2). On tomato seedling tissues, the average populations of *S.* Stanley and *S.* Baildon were significantly higher than the population of *S.* Montevideo, and these three populations were

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

The average cell populations of all 4 *Salmonella* or EHEC strains from different tissue sections of each type of sprout/seedling over the 9-day germination period are shown in Table 3-4 and 5, respectively. Similar to the result shown in Table 3-2 and 3-3, the average *Salmonella* and EHEC populations on individual tissue sections of alfalfa and fenugreek sprouts were significantly higher ($p < 0.05$) than those of tomato and lettuce seedlings (Table 3-4 and 3-5).

Cotyledons and seed coats of all 4 types of sprouts/seedlings had higher *Salmonella* counts (Table 3-4). Root tissues of tomato and lettuce seedlings, seed coat/cotyledon tissues of alfalfa sprout also had higher *Salmonella* counts than other tissues of corresponding sprouts/seedlings (Table 3-4). Lower *Salmonella* counts were associated with stem tissues and pre-germinated seeds. However, the growth trend of EHEC on tissues developed from each type of seeds was not as clear as that of *Salmonella* (Table 3-5).

Daily changes in *Salmonella* and EHEC population on alfalfa, fenugreek, tomato and lettuce sprout/seedling samples during the germination process are shown in Figure 3-1 and 3-2,

respectively. In general, cell populations of *Salmonella* and EHEC on all samples increased as germination time increased.

3.3 Discussion

Infiltration of human and plant pathogens into vegetable seeds can occur naturally at various stages during seed production (Buck et al., 2003b). When water pressure overcomes the internal gas pressure and hydrophobic nature of seed surfaces, bacterial cells can infiltrate through cracks, crevices, and intercellular spaces of vegetable seeds (Beuchat, 2002). Vacuum infiltration, first described by Boosalis (Boosalis, 1950), has been used to mimic this natural process and inoculate microorganisms into plant and seed tissues. Under vacuum, the air trapped in the intercellular space of seed tissue is removed to yield a lower internal gas pressure. A subsequent sudden release of the vacuum creates a positive pressure difference towards the internal seed tissues, which allows bacterial cells in a suspension to be drawn into seeds (Dutta et al., 2014). The efficacy of seed inoculation by vacuum infiltration was previously discussed by Prathuangwong and coworkers (Prathuangwong & Khandej, 1998) who found that a 20-min vacuuming at 20 lb./inch^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-microorganism interactions (Bashan et al., 1982; A. Charkowski et al., 2002; Dutta et al., 2012; Dutta et al., 2014; Howard & Hutcheson, 2003). 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 surface 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. Treatment of vacuum-infiltrated vegetable

seeds with sodium hypochlorite successfully inactivated *Salmonella* or EHEC cells on seed surface as no pathogen cells were detected from the seed-rinsing water. However, a low number (0.26-0.95 log CFU) of pathogen cells were 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 was demonstrated in the present study (Table 3-1). The precise mechanism for the observed phenomenon is not known. However, Cooley *et al.* (Cooley et al., 2003) reported that *Salmonella* and *E. coli* could migrate along plant surfaces by either diffusion or active movement. Kroupitski *et al*. (Kroupitski et al., 2009) found that cells of some *Salmonella* strains sensed and moved towards specific chemoattractant, such as sucrose, on plant surfaces. Since cotyledon and root tissues and seed coat are closely related to seed/seedling exudation, abundant nutrients and water exuded from these tissues can support the growth of microorganisms (Wolswinkel & Ammerlaan, 1985). Klerks *et al.* (Klerks et al., 2007) observed the active movement of *S.* Dublin towards root exudates of lettuce seedlings in soil and glass capillary tubes. Effective chemoattractants of *E. coil,* such as d-glucose, fructose, and maltose (Adler, 1973), are frequently present in vegetable seed and root exudates (Kamilova et al., 2005). As *Salmonella* populations in seed coats and roots, and *Salmonella* and *E. coli* populations in cotyledon were higher than other tissue sections according to the result of the present study (Table 3-1), 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 alfalfa and fenugreek sprouts (Table 3-1). 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 chemical and physical surface properties which may have influenced the efficacy of vacuum infiltration.

The chemical composition of vegetable seeds and seed exudates might also affect the growth of bacterial cells on sprouts/seedlings during germination (El-Gali, 2015; Emmert et al., 1998; Green et al., 2007). Generally, seeds and seed exudates that contain more essential nutrients and fewer growth inhibitors better support the growth of microorganisms (Barak & Schroeder, 2012). Previous studies have shown that alfalfa and fenugreek seeds contain arabinose (Kylen & McCready, 1975; Naidu et al., 2011) which is absent in tomato (Abdel-Rahman, 1982) and lettuce seeds (Xu et al., 2012). 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 (Abbott et al., 1994; Hendriksen, 2003). 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 (López-Garrido et al., 2015). In addition to arabinose, the abundancy of threonine differs in the four types of vegetable seeds used in the study (Hume et al., 1997). The amounts of threonine in unit weight of alfalfa and fenugreek seeds are higher than tomato and lettuce seeds (Abdel-Rahman, 1982; Kylen & McCready, 1975; Naidu et al., 2011; Xu et al., 2012). 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 *vs*. lettuce and tomato seedlings in the present study.

Tu (1972) compared the seed and early root exudates of 19 crop species and reported significantly varied abundancy in amino acids, amines, amides, and reducing sugars. The researchers recovered only trace amount 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 (Kamilova et al., 2006; Lugtenberg et al., 1999). Neumann *et al.* (Neumann et al., 2014) reported the presence of benzoic and lauric acid, 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 or not 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.* (2015) recovered *E. coli* O157:H7 cells in tomato fruits grown from seeds artificially inoculated with the pathogen. Gu *et al.* (2013) 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.* (2003) observed a temporal reduction of contamination frequency of plants grown from contaminated *A. thaliana* seeds during 30 days of cultivation.

Howard and Hutcheson (2003) previously reported that the ability of *S. enterica* to grow 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 hours of germination. The present study, however, revealed that the average population of *S.* Montevideo recovered from different sprout/seedling tissues was significantly lower than the other *Salmonella* strains used in the study (Table 3-1 and 3-2). This

indicates that the *S.* Montevideo strain might be poorly adapted in germinating seeds. Interestingly, *S.* Montevideo was reported in early studies as a better adapter on tomato plants than nine other *S. enterica* serotypes, such as *S*. Newport, *S*. Dublin, and *S*. Typhimurium (Shi et al., 2009; Zheng et al., 2013). In contrast to previous studies that used mature tomato plants (Kus et al., 2002), germinating tomato seeds were used in the present study. Furthermore, only four *S. enterica* strains were tested in the current study compared to approximately 100 documented pathogenic *Salmonella* serotypes that are frequently associated with human infections (Olsen et al., 2001). More strains/serotypes should be tested before a conclusion can be drawn with respect to 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 lower than that of *Salmonella*. Similar results were reported by Charkowski *et al.* (2002), who compared the growth of five *S. enterica* strains and six *E. coli* O157: H7 strains on germinating alfalfa seeds for two days. The population of *S. enterica* was 0.5-2.0 log CFU/sprout higher than that of *E. coli* O157:H7. The authors ascribed the lower growth rate of *E. coli* on alfalfa sprouts poor adaptation to alfalfa seed exudates and/or inability of the pathogen to firmly attach to sprout surfaces. Roy *et al.* (2013) 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* internalized into vegetable seeds caused the contamination of different tissues of sprouts/seedlings, and pathogen growth on germinating seeds is bacterial species- and vegetable seed type-dependent. Average *Salmonella* populations recovered from different tissue sections of sprouts/seedlings were higher than the *E.*

coli populations. Alfalfa and fenugreek sprout tissues had, on average, higher pathogen populations than lettuce and tomato seedling tissues. Whether or not 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.

3.4 Material and Methods

3.4.1 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 (Taormina et al., 1999). Nalidixic acid (NA) resistant mutants of each bacterial strain were selected on tryptic soy agar (Becton, Dickinson and Company [BD], 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 16-18 h at 37 °C. Resulting bacterial cultures were diluted in sterile water to an approximate cell concentration of 10^4 CFU/ml. The exact cell population in the inocula was determined using standard plate count assay on NATSA.

3.4.2 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 (n = 2 g/seed type) were 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 Shaker, 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 vigorously vortexed for 20 s. Vacuum infiltration was performed according to a procedure previously described by Darrasse *et al.* (Darrasse et al., 2010) with modifications. Specifically, the seeds were placed in 20 ml of each *Salmonella* or *E. coli* inoculum at room temperature for 30 min before being exposed to a vacuum of 25-inch Hg for 10 min. The vacuum was then broken to create a negative pressure. The draw and release of vacuum was repeated three times (Curtis, 2004) 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 duplicate, as described above, to determine the initial bacterial loads.

3.4.3 Germination of vegetable seeds

For seed germination, 1.0% (w/v) water agar was prepared in sterile 100 x 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.

3.4.4 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 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 duplicate and incubated at 37°C for 24 h before colonies were enumerated. Enrichment of samples with negative plating results was performed according to FDA's Bacteriological Analytical Manual (Andrews et al., 2007; Feng et al., 2011)

3.4.5 Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and Fisher's 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 smaller than 0.05 were considered as significant.

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Table 3-1. Overall mean populations of *Salmonella enterica* and EHEC recovered at different sampling points during germination and from different types of sprouts/seedlings and different tissue sections of each type of sprout/seedling

Main effects (*Salmonella*)	Mean population [a] (log CFU/gram)		Main effects (EHEC)	Mean population (log CFU/gram)	
	BSA [b]	NATSA [c]		NASMAC [d]	NATSA
Group			***Group***		
Trial 1 (n=256)	2.79a	3.00 a	Trial 1 (n=256)	1.60 a	1.93 a
Trial 2 (n=256)	2.78a	2.95 a	Trial 2 (n=256)	1.54 a	1.89 a
***Sampling time during germination* (day)**			***Sampling time during germination* (day)**		
9 (n=128)	3.20 a	3.57 a	9 (n=128)	2.30 a	2.91 a
7 (n=128)	3.19 a	3.40 a	7 (n=128)	1.76 b	2.27 b
5 (n=128)	3.13 a	3.30 a	5 (n=128)	1.43 b	1.54 c
3 (n=96)	1.63 b	1.67 b	3 (n=96)	1.11 bc	1.15 cd
1 (n=32)	1.45 b	1.48 b	1 (n=32)	0.88 c	1.03 d
Bacterial strains used			***Bacterial strains used***		
S. Stanley (n=128)	3.09 a	3.52 a	*E. coli* K4492 (n=128)	1.70 a	1.91 a
S. Baildon (n=128)	3.30 a	3.37 a	*E. coli* H1730 (n=128)	1.72 a	1.93 a
S. Cubana (n=128)	3.16 a	3.24 a	*E. coli* BAA-2326 (n=128)	1.49 ab	1.90 a
S. Montevideo (n=128)	1.55 b	1.75 b	*E. coli* F4546 (n=128)	1.37 b	1.91 a
Tissue sections of sprouts/seedlings			***Tissue sections of sprouts/seedlings***		
Cotyledon (n=96)	3.63 a	3.82 a	Cotyledon (n=96)	1.83 a	2.19 a
Seed Coat (n=96)	3.52 a	3.80 a	Seed Coat/Cotyledon (n=32)	1.66 a	2.18 ab
Root (n=128)	2.69 b	2.96 b	Seed Coat (n=96)	1.67 a	1.95 ab
Seed Coat/Cotyledon(n=32)	2.11 bc	2.13 c	Root (n=128)	1.53 ab	1.85 ab
Stem (n=128)	2.17 c	2.30 c	Stem (n=128)	1.44 ab	1.68 b

Pre-germinated seeds (n=32)	1.45 d	1.48 d	Pre-germinated seeds (n=32)	1.11 b	1.15 c
Vegetable seed type			***Vegetable seed type***		
Fenugreek (n=128)	4.08 a	4.36 a	Alfalfa (n=128)	3.31 a	3.70a
Alfalfa (n=128)	4.21 a	4.30 a	Fenugreek (n=128)	1.93 b	2.27b
Tomato (n=128)	1.47 b	1.68 b	Lettuce (n=128)	0.67 c	1.54c
Lettuce (n=128)	1.35 b	1.55 b	Tomato (n=128)	0.38 c	1.15d

[a] Mean populations of *S. enterica* within a column that are not followed by the same letter are significantly different ($P<0.05$).

[b] Bismuth sulfite agar.

[c] Tryptic soy agar supplemented with nalidixic acid.

[d]. Sorbitol MacConkey agar supplemented with nalidixic acid.

Table 3-2. Mean populations of individual *Salmonella* strains recovered from different types of sprouts/seedlings over the 9-day germination period

Seed type	Mean population [a] (log CFU/gram)			
	S. Stanley	*S.* Baildon	*S.* Cubana	*S.* Montevideo
Fenugreek	4.44 Aa	4.02Aa	4.12Aa	1.59Ba
Alfalfa	3.88 Aa	4.14 Aa	4.10Aa	2.01Ba
Tomato	1.49 Ab	1.32 Ab	1.18ABb	0.88Bb
Lettuce	0.90 BCb	1.99Ab	1.27Bb	0.67Cb

[a] Mean values within a column that are not followed by the same lowercase letter 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 3-3. Mean populations of individual EHEC strains recovered from different types of sprouts/seedlings over the 9-day germination period

Seed type	Mean population [a] (log CFU/gram)			
	E. coli K4492	*E. coli* H1730	*E. coli* BAA-2326	*E. coli* F4546
Alfalfa	2.52Ba	2.83Ba	1.59Cb	3.72Aa
Fenugreek	1.64Bb	1.74ABb	2.46Aa	0.21Cb
Lettuce	0.59ABc	0.82Ac	0.07Bc	0.51ABb
Tomato	0.42Ac	0.00Bc	0.58Ac	0.06Bb

[a] Mean values within a column that are not followed by the same lowercase letter 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 3-4. 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 [a] (log CFU/gram)					
	Cotyledon	Seed coat	Root	Seed coat/ cotyledon	Stem	Pre-germinated seeds
Fenugreek	4.38Aa	4.24Aa	3.23Ba	2.52BCb	2.61Ba	1.59Cb
Alfalfa	4.21Aa	4.18Aa	2.99Ba	4.73Aa	2.68Ba	3.14Ba
Tomato	1.59Ab	1.21Ab	1.56Ab	1.14ABbc	0.86Bb	0.00Bc
Lettuce	1.75aAb	1.44Ab	1.45Ab	0.00Bc	0.85Bb	0.00Bc

[a] Mean values within a column that are not followed by the same lowercase letter are significantly different ($P<0.05$). Mean values within a row that are not followed by the same capitalized letters are significantly

Table 3-5. Mean populations of all 4 EHEC strains recovered from different types of sprouts/seedlings over the 9-day germination period

Seed type	Mean population [a] (log CFU/gram)					
	Cotyledon	Seed coat/ cotyledon	Seed coat	Root	Stem	Pre-germinated seed
Alfalfa	3.18ABa	3.14ABa	3.03Aa	2.56ABa	2.01Ba	3.36Aa
Fenugreek	1.87Ab	2.29Aa	1.58Ab	1.55Ab	1.38ABb	0.30Bb
Lettuce	0.51ABc	0.00Bb	0.50ABc	0.68Ac	0.49ABc	0.00Bc
Tomato	0.34ABc	0.15ABb	0.06ABc	0.46Ac	0.27ABc	0.00Bc

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

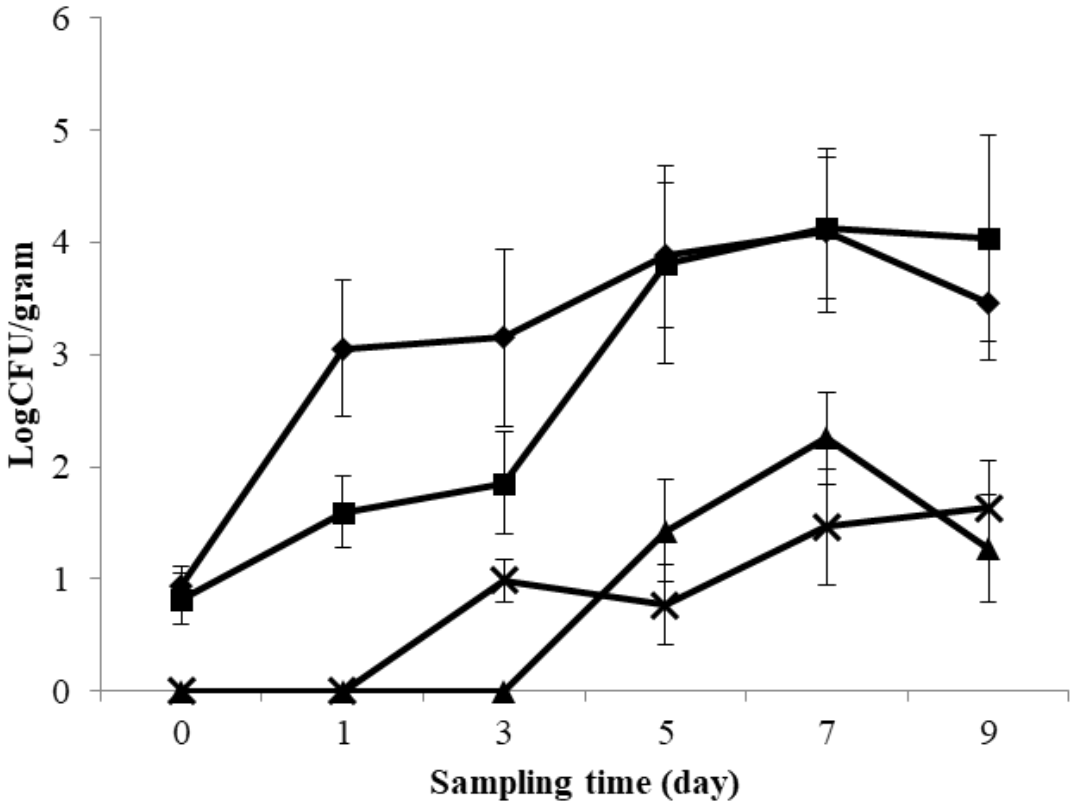

Fig. 3-1. Growth of *S. enterica* on sprouting seeds of alfalfa (♦), fenugreek (■), tomato (●) and lettuce (▲). Values are averages populations of *S.* Montevideo, *S.* Stanley, *S.* Cubana, and *S.* Baildon recovered from various tissues of different types of germinating seeds.

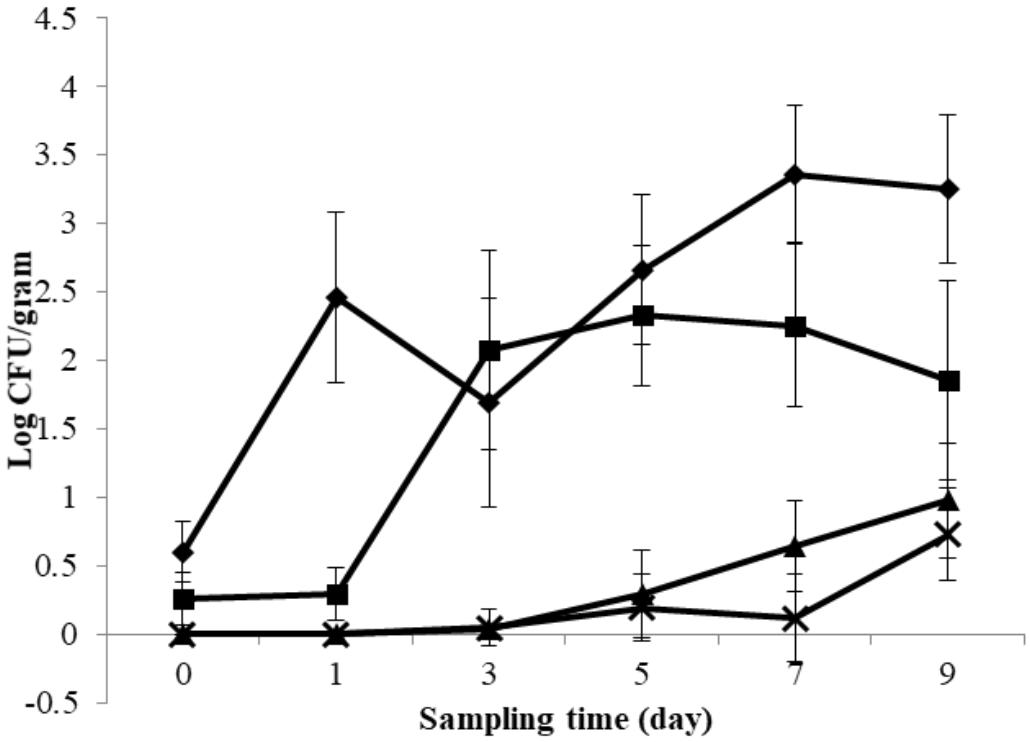

Fig. 3-2. Growth of EHEC on sprouting seeds of alfalfa (♦), fenugreek (■), tomato (●) and lettuce (▲). Values are average populations of *E. coli* F4546, *E. coli* K4492, *E. coli* H1730, and *E. coli* ATCC BAA-2326 recovered from various tissues of different types of germinating seeds.

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CHAPTER 4

TRANSMISSION OF HUMAN ENTERIC PATHOGENS FROM ARTIFICIALLY-INOCULATED FLOWERS TO VEGETABLE SPROUTS/SEEDLINGS DEVELOPED FROM CONTAMINATED SEEDS

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Abstract

Seeds contaminated with bacterial pathogens were found to be the primary cause of sprout-associated outbreaks of human gastrointestinal infections. This study was undertaken to determine if cells of selected *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) strains, artificially inoculated onto the flowers of vegetable plants, will result in contamination of sprouts/seedlings that develop from seeds produced by the inoculated flowers. Pistils of alfalfa, fenugreek, lettuce, and tomato flowers were inoculated with cells of selected *S. enterica* or EHEC strains. A total of 906, 715, 1,237, and 1,276 mature seeds, produced by lettuce, tomato, alfalfa or fenugreek flowers inoculated with *Salmonella* were collected as 48, 94, 109, and 116 composite samples (367 in total), respectively. Correspondingly, 934, 640, 1,827 and 1,027 seeds, produced by the four respective types of flowers after inoculation with *E. coli* were divided into 42, 81, 162, and 107 composite samples (392 in total), respectively. Seeds in each composite sample were surface-decontaminated with NaOCl solution and germinated at 25 °C in the dark for 5 days. Subsequently, pathogen populations on the sprouts/seedlings developed from each composite seed sample were determined by the plate count assay. The overall *Salmonella* detection percentage from vegetable sprouts/seedlings developed from the 367 composite seed samples was 2.7%, while none of the sprouts/seedlings grown from the 392 composite seed samples with *E. coli* flowerinoculation tested positive for the pathogen. One of the 94 tomato seedling samples contained 4 *Salmonella* CFU/seedling and five samples tested positive by enrichment (6.4%). Two out of 109 (1.8%) alfalfa and 116 (1.7%) fenugreek composite samples tested positive for *Salmonella* with enrichment. However, none of the lettuce seedlings tested positive for *Salmonella* or *E. coli* even after enrichment. This study suggests that under controlled environmental conditions, human pathogens inoculated onto flowers of

vegetable plants can result in the contamination of sprouts/seedlings *via* seeds produced by the inoculated flowers. However, the frequency of sprout/seedling contamination was low and could be affected by characteristics of the pathogens and plant species tested.

Keywords: EHEC, *Salmonella*, seedlings, sprouts, vegetable seeds

4.1 Introduction

Fresh fruits and vegetables have been a cornerstone of the American diet. The consumption of fruits and vegetables has continuously risen since the 1990s (Patterson et al., 1990). Correspondingly, outbreaks of human gastrointestinal infections associated with the consumption of fruits and minimally-processed vegetables have occurred more frequently, and have become significant public health and financial concerns (Callejón et al., 2015). According to the 17th annual report of Center for Science in the Public Interest, contaminated fresh produce was the most significant cause of foodborne outbreaks (Fischer et al., 2016). *Salmonella enterica* and enterohemorrhagic *E. coli* (EHEC) caused approximately 2.73 million illnesses, 21,000 hospitalizations, and 400 deaths each year in the United States and were increasingly involved in outbreaks associated with sprouts (Taormina et al., 1999), tomato (Guo et al., 2001; Weissinger et al., 2000), lettuce (Ethelberg et al., 2010), green pepper (Barton Behravesh et al., 2011; Mukherjee et al., 2004), and other fresh produce (Jay et al., 2007).

Tomato and lettuce are among the most commonly consumed fresh produce in the United States (Olaimat & Holley, 2012). In 2014, the combined consumption of fresh tomato and lettuce was more than 540 million kilograms. Eating contaminated fresh tomato and lettuce cumulatively was responsible for more than 158 foodborne illness outbreaks from 1988 to 2015 (Valadez et al., 2012). In comparison, more than 9.1 million kilograms of mung bean and alfalfa sprouts were consumed annually in the United States. From 1998 to 2010, 33 foodborne outbreaks of human gastrointestinal infections were linked to the consumption of vegetable sprouts (Dechet et al., 2014).

Epidemiological investigations indicate that seeds are the primary source of contamination in most sprout-associated outbreaks of human gastrointestinal infections (Puohiniemi et al.,

1997). Sanitizing artificially-inoculated sprout seeds with CaOCl or NaOCl reduced the pathogen population by 3.08 log CFU/g; but the treatments failed to eliminate bacterial pathogens, even at low contamination levels (Rivera & Velez, 2005). This might be due to the inability of the sanitizers to reach bacterial cells that become localized in the internal tissues of seeds (Buck et al., 2003b).

Transmission of plant pathogenic bacteria, such as *Xanthomonas campestris* pv. *campestris* (Van der Wolf et al., 2013), *Acidovorax citrulli* (Lessl et al., 2007), and *Pseudomonas syringae* (Dutta et al., 2014) from flowers to seeds has been previously described. However, little is known about the pathway of ingress of human pathogens into the internal tissues of vegetable seeds. It is not clear whether human pathogens, when present on plant flowers, could contaminate vegetable seeds. It is also not known if seeds contaminated in such a manner could lead to sprout/seedling contamination by human pathogens. Hence, the objective of this study was to determine if cells of selected *S. enterica* and EHEC strains could be transmitted to vegetable sprouts/seedlings from seeds produced by inoculation of alfalfa, fenugreek, tomato, and lettuce flowers.

4.2 Material and Methods

4.2.1 Bacterial strains

Three *S. enterica* (*S.* Baildon, *S.* Montevideo, and *S.* Stanley), two *E. coli* O157:H7 (K4492 and F4546) and one *E. coli* O104:H4 (BAA-2326) strains were used in the study because of their previous association with fresh produce-associated outbreaks of illnesses (Table 4-1). Nalidixic acid (NA)-resistant mutants of each strain were selected on tryptic soy agar (TSA) (Becton, Dickinson and Company [BD], Sparks, MD) supplemented with 50 μg/ml of NA (NATSA). *S.* Baildon, *S.* Montevideo, and *S.* Stanley were used to inoculate the flowers of

lettuce (*Lactuca sativa 'Iceberg'*), tomato (*Solanum lycopersicum* 'Roma'), alfalfa (*Medicago sativa*), and fenugreek (*Trigonella foenum-graecum*), respectively. *E. coli* K4492 was used to inoculate tomato and lettuce flowers and *E. coli* F4546 and *E. coli* BAA-2326 were used to inoculate the flowers of alfalfa and fenugreek, respectively. One day prior to inoculation, the *S. enterica* and EHEC strains were grown separately overnight in tryptic soy broth supplemented with 50 μg/ml of NA at 37 °C. Bacterial cell suspensions were prepared by ten-fold serially diluting the overnight cultures with phosphate buffered saline (PBS, pH 7.4) until an approximate cell concentration of 10^7 CFU/ml was reached. Appropriate dilutions of each cell suspension were plated on bismuth sulfite agar (BSA, BD) and sorbitol MacConkey agar (SMAC, BD) supplemented with 50 μg/ml of NA (NA-SMAC) to determine the exact concentrations of cells in each suspension.

4.2.2 Plant cultivation

Seeds used in the study were purchased from Twilley Seed Company (Hodges, SC) and stored at 10 °C until used. Lettuce seeds were grown in three 11.4 L plastic pots (Hydroponics Organic 3 Gal. Nursery Pots, Home Depot, GA) containing commercial potting mix (Fafard 3B mix, Sungro, Agawam, MA). Polypropylene catch pans were placed under each pot to retain water lost from the soil during irrigation. Pots were spaced approximately 2.0 m apart in an environmental chamber (PGW 36, Controlled Environments, Pembina, ND) under constant conditions of 24 ºC, mean relative humidity (RH) of 76%, 12-h of combined fluorescent and incandescent light daily, and automatic irrigation. In each pot, one healthy lettuce plant was retained until flowering. Similar pots, potting mix, and growth chamber parameters were used to grow tomato, alfalfa, and fenugreek plants. Fourteen pots each containing 2-3 healthy tomato seedlings were evenly assigned to two chambers and grown to flowering. Cultivation of alfalfa

and fenugreek was performed as for lettuce, except that different numbers of plants and pots were used, *i.e*. 5 to 6 alfalfa plants/pot in 12 pots, and 2 to 3 fenugreek plants/pot in 10 pots.

4.2.3 Flower inoculation

For lettuce, pathogen inoculation was performed from the beginning of anthesis until 50 flowers on each plant were inoculated. For flower inoculation, sterilized cotton-head applicators (Q-Tips® cotton swabs, Unilever, London) were dipped briefly in each bacterial suspension and then used to directly apply the bacterial suspension onto the pistils of each flower. Flowers on a single lettuce plant were inoculated with the same bacterial strain, *S*. Baildon or *E. coli* K4492 to avoid cross-contamination. A similar method was used to inoculate tomato, alfalfa, and fenugreek flowers. At least 10 tomato flowers on each plant were inoculated with *S*. Montevideo or *E. coli* K4492. For alfalfa, 40 to 80 flower clusters on each of 5 to 6 plants grown in the same pot were inoculated with *S*. Stanley or *E. coli* F4546. Ten to twelve flowers on each of 2 to 3 fenugreek plants were inoculated with *S*. Stanley or *E. coli* BAA-2366. For each plant type, PBS was used to inoculate flowers on a separate pot of plants growing in the same chamber to serve as negative controls.

4.2.4 Seed harvest

At 20 days after inoculation, lettuce seeds were manually harvested. Seeds from a single lettuce capitulum were placed in a ~ 60 ml sample bag (WHIRL-PAK™, eNasco, WI) until 50 bags, each containing 10~25 seeds, were obtained from each plant. Seeds in the same sample bag were used as a composite sample for seedling production and microbiological analysis. All harvested seeds were immediately transported to the laboratory and analyzed within 24 h of harvest. In a biosafety cabinet (Class II type A/B 3, Nuaire, Plymouth, MN), the seed pappus was manually removed using sterilized latex gloves (Microflex, Reno, NV).

Tomato fruits were allowed to mature for 40 to 45 d after flower inoculation. At maturity, tomato fruits were collected in plastic bags (Ziploc®, S.C. Johnson & Son, Inc., Racine, WI) and transported to the laboratory within 1 h of harvest. Each fruit was disinfected with 70% ethanol before the seeds were extracted from individual fruits with sterilized knives and tweezers. Seeds extracted from the fruits were air-dried for 2 h inside a biosafety cabinet before being collected as composite samples. Each composite sample consisted of 2 to 17 seeds from a single fruit.

Alfalfa pods were left to mature for 42 to 48 d after flower inoculation. Three to eleven dry pods produced from a single flower cluster were collected in individual ~ 60 ml sample bags (eNasco) and transported to the laboratory within 1 h of harvest. The pods were surface-disinfected by immersing in 70% ethanol for 90 s and then air-dried for 2 h in a biosafety cabinet. The seeds were aseptically extracted from the pods and all seeds obtained from a single pod cluster were regarded as a composite sample. Dry fenugreek pods were harvested at 48 to 55 d after flower inoculation. Disinfection of pod surfaces and extraction of fenugreek seeds were performed as described in the alfalfa experiment. Seeds extracted from each pod were used as a composite sample in sprout/seedling production and microbiological analysis.

4.2.5 Sprout/seedling production

All composite seed samples were surface-disinfected according to a previous protocol with modifications (Cui et al., 2017). Specifically, each sample was soaked in 5 ml of 20,000 ppm NaOCl (pH 6.8) at room temperature for 10 min with agitation at 120 rpm on a platform shaker (Orbit Shaker, Lab-Line Instruments, Inc.). Residual NaOCl on vegetable seeds was neutralized with 10 ml Dey-Engley neutralization broth (Becton, Dickinson and Company). Disinfected seeds were rinsed twice with 10 ml sterilized distilled water. The seed samples were then placed on multiple 1% (w/v) water agar plates. Each agar plate contained seeds from a

single composite sample, with an average of 15, 12, 13, and 8 seeds of lettuce, tomato, alfalfa, and fenugreek per plate, respectively. Seeds were germinated at 25 °C in the dark and sprouts/seedlings samples were harvested 5 d after planting.

4.2.6 Microbiological analysis

Sprouts/seedlings (n = 8-15) developed from a composite seed sample were homogenized in 1 ml 0.1 M PBS and diluted homogenates were plated on BSA or NASMAC plates in duplicate to quantify presumptive *Salmonella* and *E. coli* colonies. When no countable colonies were obtained, enrichment of sprout/seedling homogenates was performed as recommended by the FDA (Andrews et al., 2007; Feng et al., 2011). The enriched seed homogenates were plated on BSA and NASMAC plates and the inoculated plates were incubated at 37 °C for 24-48 h.

4.2.7 ERIC-PCR fingerprints of isolated Salmonella *strains*

Presumptive *Salmonella* isolates recovered from seedling samples were first confirmed by a slide agglutination assay, as follows. Specifically, single colonies selected from BSA plates were mixed with a drop of Difco™ *Salmonella* O Antiserum Poly A – I & Vi (BD) on clean glass slides and the colonies that tested positive in the slide agglutination assay were selected for further analysis. Enterobacterial repetitive intergenic consensus (ERIC)-PCR was performed to compare DNA banding patterns of isolates recovered from sprouts/seedlings to those of the inoculated strains (Burr et al., 1998). The nucleotide sequence of the primer was (5'- AAG TAA GTG ACT GGG GTG AGC G-3', based on a highly conserved, 126-bp enterobacterial repetitive intergenic consensus sequence restricted to transcribed regions of the bacterial genome (Guo et al., 2001). Crude total cellular DNA was prepared as previously described with modifications (Guo et al., 2001). Specifically, 1 ml of overnight culture was centrifuged at 10,000 ×*g* for 10

min and supernatant was decanted. Pellets were washed twice, each time with 1 ml sterilized distilled water, re-suspended in 100 µl of sterilized distilled water, boiled for 10 min, and centrifuged as described above. The aqueous supernatant was stored at 4 °C until used as DNA template for PCR assay. The 25-µl PCR mixture contained PCR buffer, deoxynucleoside triphosphates (1 mM each), primer (1 µM), *Taq* polymerase (1 U; ThermoFisher Scientific Inc., MA), and 5 µl of DNA template. PCR assays were performed in a DNA thermal cycler 480 apparatus (Perkin Elmer, Norwalk, CT) using a cycle at 94°C for 5 min, followed by 40 cycles of 92°C for 45 s, 25°C for 1 min, and 68°C for 10 min, plus a final extension at 72°C for 20 min. The PCR amplicons were analyzed using gel electrophoresis on 1% agarose (GIBCO BRL, Rockville, MD) gels in Tris-borate-EDTA buffer (0.089 M Tris-borate, 0.002 M EDTA, pH 8.0). After electrophoresis, gels were stained with 1% ethidium bromide and visualized using a Gel Doc System 2000 (Bio-Rad Laboratories, Hercules, CA).

4.3 Results

4.3.1 Recovery of S. enterica *and EHEC from sprouts/seedlings*

Neither *S. enterica* nor EHEC was recovered from the sprouts/seedlings that developed from seeds produced by the negative control flowers (inoculated with 0.1 M PBS) of lettuce, tomato, alfalfa, and fenugreek. A total of 48, 42, and 20 composite lettuce samples, comprised of seedlings that developed from 906, 934, and 409 seeds produced from lettuce flowers inoculated with *S*. Baildon, *E. coli* K4492, and PBS, respectively, were microbiologically analyzed. No *Salmonella* or *E. coli* cells were recovered from harvested lettuce seedlings by direct plating and enrichment (Tables 4-2).

Approximately 41, 48, and 44% of inoculated tomato flowers abscised, after inoculation with *S*. Montevideo, *E. coli* K4492, and PBS, respectively. In total, 715, 640, and 249 seeds

were extracted from the 94, 81, and 25 fruits harvested from tomato flowers inoculated with *Salmonella*, *E. coli*, and PBS, respectively. One tomato seedling sample grown from a composite of 16 seeds yielded an average of 4 *Salmonella* CFU per seedling by direct plating. In addition, five tomato seedling samples tested positive for *Salmonella* after enrichment (Table 4-1). The overall incidence of *S.* Montevideo in tomato seedlings was 6.4% (Table 4-1). No *E. coli* cells were recovered from tomato seedlings by direct plating and enrichment.

For alfalfa, 109, 162, and 42 composite seed samples were collected from plants inoculated with *S.* Stanley, *E. coli* F4546, and PBS, respectively. Each composite sample from *Salmonella* or *E. coli*-inoculated plants contained 2 to 12 or 3 to 11 pods yielding 4 to 22 and 6 to 25 seeds, respectively. The average alfalfa seed yield per pod for *Salmonella*-inoculated, *E. coli*-inoculated, or PBS-inoculated flowers was 1.8, 2.4, and 2.2, respectively. Two composite alfalfa sprout samples tested positive for *Salmonella* after enrichment and the overall incidence of *Salmonella* on alfalfa sprouts was 1.8%. No *E. coli* cells were recovered from alfalfa sprouts, even after enrichment (Table 4-2).

A total of 116, 107, and 40 composite seed samples were harvested from 10 pots of fenugreek plants inoculated with *S.* Stanley, *E. coli* BAA-2326, or PBS, and the average fenugreek seed yield was 11, 9.6, and 10.4 seeds per pod, respectively. *Salmonella* was recovered from 2 out of 116 harvested sprout samples (2.6%) while *E. coli* was not detected on sprouts.

4.3.2 ERIC-PCR fingerprints of recovered Salmonella

All presumptive *Salmonella* isolates recovered from seedling samples tested positive in the slide agglutinin assay using Difco™ *Salmonella* O Antiserum Poly A – I & Vi (BD) and had typical growth on lysine iron agar and triple iron slants. ERIC-PCR fingerprint patterns of the

Salmonella isolates recovered from tomato, alfalfa, and fenugreek samples matched those of *Salmonella* strains used for flower inoculation (Fig. 4-1).

4.4 Discussion

Salmonella cells inoculated onto alfalfa, fenugreek and tomato flowers survived seed development and germination, and resulted in the contamination of sprouts/seedlings. Guo and coworkers (2001) previously observed the transmission of *Salmonella* to tomato fruits from inoculated flowers although the potential contamination of tomato seeds by *Salmonella* was not examined. The researchers brushed open tomato flowers with 9 log CFU/ml of *Salmonella* and found that the pathogen was detected in the fruit pulp of 2 out of the 8 (25%) harvested tomato fruits. Several other studies also demonstrated the ability of *Salmonella* to contaminate tomato fruits *via* artificial inoculation of open flowers (Iturriaga et al., 2007; Rathinasabapathi, 2004; Shi et al., 2007). In addition to tomato fruit pulp, flower inoculation with *Salmonella* also resulted in contamination of other seed-contacting plant tissues such as the chaff of *Arabidopsis thaliana* (Cooley et al., 2003; Guo et al., 2001; Shi et al., 2009). Thus, pathogen cells inoculated onto open flowers could be transmitted to seeds through direct contact between seeds and pathogen cells that have relocated to seed-contacting tissues during seed development. A similar transmission pattern was previously described by Dutta and coworkers (2016) who observed the transmission of *A. citrulli* from flowers to the seeds of watermelon. They also observed that *A. citrulli* cells co-localized with growing pollen germ tubes and subsequently translocated to ovules and seeds (Dutta et al., 2012). It is not currently known, however, if *Salmonella* cells inoculated onto open flowers could enter the vegetable seeds through a similar pathway.

Seed surface treatment with NaOCl did not completely disinfect sprouts and seedlings in the present study (Table 4-1). A similar observation was made by Cooley *et al.* (2003) who

found that *A. thaliana* seeds grown from *S. enterica*- or *E. coli* O157:H7-infested flowers could not be sanitized by extensive washing and NaOCl treatment. This may be due to the internalization of pathogen cells into the protected niches of seeds such as cracks and crevices beneath the seed surface. It is possible that the process of pathogen infiltration, induced by changes in environmental temperature and hydrostatic conditions of seed surface, could aid the translocation of pathogen cells across the seed coat (Buck et al., 2003b). In a previous work, cells of *A. citrulli* were found to selectively localize at the perisperm-endosperm layer (97%) and embryo tissues (94%) relative to the testa (< 8%) of seeds after the pathogen was inoculated on watermelon flowers (Dutta et al., 2012). However, it is not yet known if the floral invasion pathway can provide human pathogen cells similar access to internal seed tissues.

The absence of *E. coli* O157 cells in seeds might be due to poor adaptation of the pathogen to plant tissues. *Salmonella*, in general, displayed better survivability than *E. coli* O157 in non-host terrestrial habitats. This survival advantage could be attributed to the higher genomic complexity of *Salmonella* compared to *E. coli* (Van Elsas et al., 2011). Furthermore, *S. enterica* displayed a relatively high tolerance to desiccation and persisted on plant surfaces despite wide fluctuations in water activity (Brandl, 2006). The inactivation rate of an *E. coli* O157:H7 strain on the surface of lettuce leaves was significantly higher than that of a *S. enterica* strain at both high (68.6%-99.6%) and low humidity (28.6%-67.7%) (Stine et al., 2005). Thus, after flower inoculation, *Salmonella* cells were more likely to survive in the flower niche before relocating to protected niches of vegetable seeds. Thilmony and coworkers (2006) found that lettuce plants inoculated with *S. enterica* exhibited more obvious phenotypic changes than those inoculated with *E. coli*. Lettuce plants infested by *E. coli* was displayed a significantly higher level of expression of plant defense-related marker genes, such as *PR1,* than those infested by

Salmonella (Roy et al., 2013). Subversion of plant immunity by *Salmonella* was reported by Schikora and coworkers (2011), who also concluded that *Salmonella,* rather than *E. coli* O157:H7, should be considered a pathogen of plants.

In the current study, the prevalence of *S. enterica* was higher (6.4%) on tomato than on alfalfa (1.7%) and fenugreek sprouts/seedlings (1.8%; Table 4-2). During seed development, the fruit pulp might have protected the pathogen cells from desiccation and other unfavorable environmental conditions (Heaton & Jones, 2008). Although tomato pulp has a pH of 4.0, this level of acidity was not likely to affect the survival of *Salmonella* cells (Weissinger et al., 2000). Additionally, some *Salmonella* serotypes, such as *S.* Montevideo, have a greater fitness in tomato plants than other *Salmonella* serovars, such as *S.* Dublin and *S.* Typhimurium (Guo et al., 2001). Zheng *et al.* (2013) studied the serovar-specific niche colonization on tomato plants and suggested that *S.* Montevideo has a superior fitness on the leaves and blossoms. To our knowledge, such fitness has not previously been reported for *S.* Stanley on alfalfa and fenugreek plants. Another factor that could influence the survival of enteric pathogens is the water activity of vegetable seeds. During development, the water activity of alfalfa and fenugreek seeds gradually reduce to approximately 0.62 at harvest maturity (Kreitlow & Hart, 1974; Neetoo et al., 2009). The moisture content of lettuce seeds can drop to > 40% during the 20-day maturation at day/night temperature of 20 °C/10 °C (Contreras et al., 2009). However, the water activity of tomato fruit remains *ca*. 0.97 throughout the maturation process (Fontana et al., 2008). It has been reported that at water activity lower than 0.7, *Salmonella* populations gradually decline and become undetectable by direct plating after 14 d of incubation in broth culture at 25 °C (Chen, 2015). This may partly explain why only *Salmonella* cells were recovered from tomato seedlings by direct plating.

The overall mean detection rate of *S. enterica* and EHEC in sprouts/seedlings in the current study was *ca*.1.2%, suggesting that transmission of pathogen cells from flowers to seeds and then sprouts/seedlings was a rare event. Eight out of nine (88.9%) *Salmonella*-positive samples obtained in the present study were from enriched sprout/seedling homogenates (Table 4-2), indicating that the *Salmonella* populations were low. It is possible that some of the pathogen cells might have been inactivated when subjected to various environmental stresses and plant immune responses during seed development. Compared to active infection of seeds by plant pathogenic fungi, the transmission of *Salmonella* and *E. coli* to internal tissues of seeds is passive and thereby subject to more environmental variation. Hence, it could be significantly affected by plant immunity in the pistil tissue, where strong immune responses are triggered by incompatible pollen and foreign microorganisms (Mariana, 2017).

The environmental conditions in the growth chamber are less variable and extreme compared to field conditions, as dramatic fluctuations in temperature and relative humidity can happen throughout the day (Hatfield & Prueger, 2015; Samra et al., 2003). Such fluctuations in temperature can expedite or retard the inactivation of pathogen cells (Winfield & Groisman, 2003). The survival rates of *S*. Typhimurium and *E. coli* O157:H7 under fluctuating temperatures were lower than under constant temperature (Semenov et al., 2007). The bacterial population reduction was more significant when the amplitude of the temperature oscillations was enhanced (7 °C *vs*. 4 °C). In addition, the native plant bacterial community, whose composition varies among plant type and geographical location of the crop field, may play a role in the transmission of enteric pathogens to seeds (Brandl et al., 2013; Godoy-Vitorino et al., 2012). Pollard *et al.* (2014) suggested that the presence of *Ralstonia solanacearum* played a role in *S. enterica* survival and transportation through the internal tissues of tomato plants. However,

the activation of effector-triggered immunity by an avirulent *X. perforans* strain could reduce *S. enterica* populations in tomato plants (Potnis et al., 2014). Thus, although the transmission of enteric bacteria through the floral pathway was observed in the present study under growth chamber conditions, the frequency of occurrence in crop production fields could be different. The dynamic environmental conditions and diverse microflora of different crop species grown in different geographic regions might either promote or suppress the transmission of enteric pathogens to seeds.

The contamination of seeds by enteric bacteria through flower pistil invasion also depends on the ability of the pathogen cells to survive on flower tissues. Splash of contaminated rain and irrigation water could deposit enteric bacteria onto plant surfaces such as leaves and flowers (Allende & Monaghan, 2015). Even if the pathogen cells were not directly introduced onto flowers, they might migrate across plant surfaces and eventually reach the reproductive organs. Active migration of *S. enterica* and GFP-labeled *E. coli* O157:H7 cells from roots to sepals and floral buds of *A. thaliana* was previously reported (Cooley et al., 2003). In addition, cells of enteric bacteria could be transmitted to flowers and other plant surfaces by pollinators (De Roever, 1999) and plant-feeding insects (Soto-Arias et al., 2014). Therefore, studies are needed to address the influence of external factors, such as weather, native microbiota, and wild life/insect activities, on the transmission of enteric pathogens to seeds through the floral pathway.

4.5 Conclusion

Under controlled environmental conditions, we observed a low-frequency of transmission of *Salmonella* cells from inoculated flowers to alfalfa and fenugreek sprouts and tomato seedlings *via* contaminated seeds. Our observation revealed the ability of *Salmonella*, when present on open flowers of vegetable plants, not only to survive and transmit to seeds, but also to establish

populations on vegetable seedlings. Whether this transmission pattern represents a serious risk of seed/seedling contamination requires further investigation, as pathogen migration influenced by environmental factors and plant microbiota is poorly understood. Nevertheless, the ability of enteric bacteria to persist in a non-host environment, migrate along plant surface, and be transmitted to plants through various routes (irrigation water, insects, livestock, wildlife, etc.) emphasize the importance of following Good Agricultural Practices and other agricultural guidelines to ensure the safety of fresh produce and vegetable seeds.

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Table 4-1. Bacterial strains used in this study

Bacterial strains	Serotypes	Description
Salmonella enterica	Stanley	Sprout-related outbreak, Finland, Canada and the US, 1997
	Baildon	Fresh vegetables-related outbreak, tomato and lettuce, 1999
	Montevideo	Tomato-related outbreak, 1993
Enterohemorrhagic *Escherichia coli*	F4546	Sprout-related outbreak, 1997
	ATCC BAA-2326	Outbreak associated with fenugreek, Germany, 2011
	K4492	Spinach-related outbreak, 2006

Table 4-2. Proportion of *Salmonella*-positive sprouts/seedlings produced from seeds harvested from artificially-inoculated flowers.

Plant	Seed maturation time [a] (days)	Direct plating [b]			Enrichment [c]	
		No. of samples		Positive (%)	No. of positives	Presence (%)
		Examined	Positive			
Lettuce	22-27	48	0	0	0	0
Tomato	41-46	94	1	1.1	5	6.4
Alfalfa	42-48	109	0	0	2	1.8
Fenugreek	55-60	116	0	0	2	2.6

[a] Time between flower inoculation and seed harvest

[b] *Salmonella* recovered from vegetable seedlings by plate count assay

[c] *Salmonella* recovered from enriched vegetable seedling homogenates

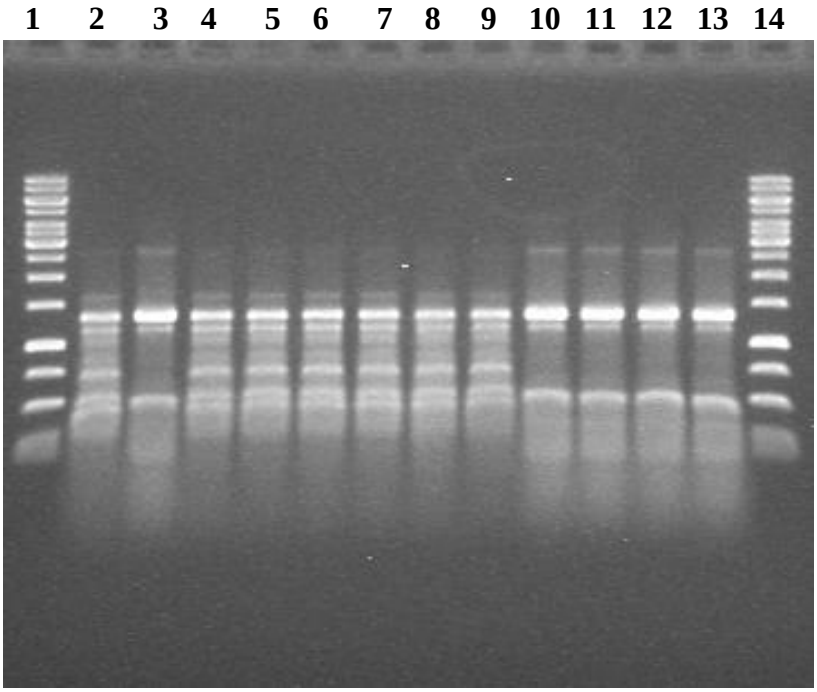

Figure 4-1. ERIC-PCR DNA fingerprints of *Salmonella* colonies recovered from alfalfa, fenugreek, and tomato sprouts/seedlings grown from seeds produced by artificially-inoculated flowers. Lane 1 and 14, 1 kb DNA ladder; lanes 2 and 3, DNA profiles of *S.* Montevideo and *S.* Stanley, respectively; lanes 4 through 9, DNA profiles of the six isolates recovered from tomato seedling samples; lanes 10 and 11, DNA profiles of the two isolates recovered from alfalfa sprout samples; lanes 12 and 13, DNA profiles of the two isolates from fenugreek sprout samples.

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CHAPTER 5

INFLUENCE OF BACTERIAL COMPETITORS ON *SALMONELLA ENTERICA* AND ENTEROHEMORRHAGIC *ESCHERICHIA COLI* GROWTH IN MICROBIOLOGICAL MEDIA AND ATTACHMENT TO VEGETABLE SEEDS

Liu, D., to be submitted to *Food Microbiology*

Abstract

Interests in using biological agents for control of human pathogens on vegetable seeds and seed sprouts is rising. This study was undertaken to evaluate whether a selected plant pathogen and probiotic bacterium (*Lactobacillus rhamnosus* GG), as well as bacterial strains previously used as biocontrol agents could compete with human pathogens, such as *Salmonella* and EHEC, in microbiological media and affect attachment to vegetable seeds; and to determine whether the metabolites in cell-free supernatants of competitive bacterial spent cultures could inhibit the growth of *Salmonella* and EHEC. Results of overall revealed that the presence of competitive bacteria, especially *L. rhamnosus* GG significantly inhibited the growth of *Salmonella* and EHEC. Cell-free supernatants of *L. rhamnosus* GG significantly reduced the pathogen populations. Although not as effective as *L. rhamnosus* GG in inhibiting *Salmonella* and EHEC growth, the biocontrol agents were more effective in competing for attachment to sprout seeds. The present study revealed the inhibition of human bacterial pathogens by competitive bacteria or their metabolites and the competitive attachment to sprout seeds among all bacteria involved. The results will help strategize interventions for the production of safe vegetable seeds and seed sprouts.

Keywords: *Salmonella*, EHEC, vegetable seeds, biological control, bacterial attachment, growth inhibition

5.1 Introduction

Consumption of raw and lightly cooked sprouts has been linked to multiple high-profile outbreaks of human gastrointestinal infections (Como-Sabetti et al., 1997; Mahon et al., 1997; Winthrop et al., 2003). The most likely source of pathogens in sprout-associated outbreaks is contaminated seeds (Taormina et al., 1999). Pathogens such as *Salmonella* and *E. coli* O157:H7, when present on seeds, can grow rapidly from a low contamination level of *ca*. 0.1 log CFU/g to as high as 10^6 log units under sprouting conditions (Howard & Hutcheson, 2003). Although seeds are not generally regarded as a contamination source of fresh produce other than sprouts, a recent study by Deering et al. (2015) reported the presence of *E. coli* O157:H7 in mature tomato fruits grown from seeds contaminated with the pathogen. Therefore, the sanitation of seeds needs to be addressed to reduce the growing incidence of foodborne outbreaks associated with fresh produce.

Hypochlorite-based sanitizers have been recommended by the U.S. Food and Drug Administration to inactivate *Salmonella* and enterohemorrhagic *E. coli* (EHEC) on vegetable seeds (Andrews et al., 2007; Feng et al., 2011). In general, a 3-log population reduction can be reached by applying the sanitizer on sprout seeds for 10 min (Fett, 2002). However, the pathogen cannot be reliably eliminated due to bacterial strain-specific resistance to chlorine and/or the internalization of pathogen cells into protected niches of vegetable seeds (Cooley et al., 2003; Davidson & Harrison, 2002).

The potential of using antagonistic microorganisms as control agents on harvested vegetable seeds has been discussed (Kamilova et al., 2006; Kamilova et al., 2005). Inoculation of *Acidovorax avenae* subsp. *avenae* on watermelon seeds before planting reduced the transmission of bacterial fruit blotch of cucurbits caused by *Acidovorax avenae* subsp. *citrulli* by

96.5% (Fessehaie & Walcott, 2005). Treating alfalfa seeds with *Pseudomonas* strain 2-79 has reduced the population of *Salmonella* by 1-2 log units during sprouting (Liao, 2008). An advantage of using biocontrol agents over chemical/physical disinfection for pathogen control is that once the biocontrol agents establish a population on vegetable seeds/plants, a sustainable protection can be maintained throughout the sprouting/cultivation process (Ye et al., 2010).

The mechanisms of biocontrol include competition, for available nutrients, between plant/human pathogens and the microorganisms used as biocontrol agents and inhibitory effects of antagonistic metabolites, produced by biocontrol agents (Compant et al., 2005). For instance, the preemptive colonization of orange flowers by *Pseudomonas fluorescens* A506 can prevent future *Erwinia amylovora* infections (Cabrefiga et al., 2007). Active production of lactic acid and bacteriocin by *Lactobacillus* spp. has led to its application as *Salmonella*-control agents on meat products (Bredholt et al., 2001). Beyond that, metabolites such as fatty acids and biosurfactants produced by *Bacillus* spp. have been reported to reduce adhesion and biofilm formation of human pathogen cells on food contact surfaces (Nitschke et al., 2009).

The objectives of the study were to observe whether a selected plant pathogen and probiotic bacterium, as well as strains of bacteria previously used as biocontrol agents against plant diseases could compete with foodborne human pathogens such as *Salmonella* and EHEC for growth in microbiological media and attachment to vegetable seeds, and to determine whether the metabolites in cell-free supernatants of spent cultures of bacteria could significantly inhibit *in vitro* growth of *Salmonella* and EHEC.

5.2 Material and methods

5.2.1 Bacterial strains and vegetable seeds

Four *S. enterica*, three *E. coli* O157:H7, and one *E. coli* O104:H4 strains were used in the study due to their previous association with sprout- or fresh produce-associated outbreaks of infections (Table 5-1). Nalidixic acid (NA) resistant mutants of each bacterial strain were selected on tryptic soy agar (TSA) supplemented with 50 μg/ml of NA (NATSA). Three bacterial strains previously used as biological control agents including *P. fluorescens* A506, *B. mojavensis* RRC 101, and *B. subtilis* ATCC 6051 were obtained from the laboratory collection of Dr. Ronald Walcott (Table 5-1). A well-characterized probiotic strain, *Lactobacillus rhamnosus* GG was obtained from a commercial source (*Culturelle*®, i-Helath Inc., Cromwell, CT). A plant pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000), was also included to observe its interactions with the human pathogenic bacterial strains used in the study. All bacterial cultures were maintained at -80 °C until use. Microbiological media used in the study were purchased from Becton, Dickinson and Company (Sparks, MD) unless specified.

Four types of vegetable seeds, alfalfa (*Medicago sativa*), fenugreek (*Trigonella foenum-graecum*), lettuce (*Lactuca sativa* 'Iceberg'), and tomato (*Solanum lycopersicum* 'Roma') were included in the study. Their seeds or the fresh produce developed from them had a previous link to outbreaks of human gastrointestinal infections. The seeds were purchased from Twilley Seed Company (Hodges, SC) and stored at 10 °C until use.

5.2.2 Competitive growth between Salmonella *or EHEC and selected plant pathogen and biocontrol agents*

A previously described (De Keersmaecker et al., 2006), 1:1 (*v/v*) mixture of De Man, Rogosa and Sharpe (MRS) and tryptic soy broth (TSB) (M/T broth) was used in this portion of

the study to minimize the influence of microbiological media on the growth of *L. rhamnosus* GG *vs*. other bacterial strains. Fresh M/T broth was prepared by aseptically mixing an equal volume of pre-autoclaved MRS and TSB broth before use. Individual cultures of *L. rhamnosus* GG, *Salmonella*, and EHEC strains were grown at 37 °C and those of the biocontrol agents/plant pathogen were grown 25 °C, all in 10 ml M/T broth for 18 h. In single wells of sterillized 24-well tissue culture plates (Corning Incorporated, Durham, NC), 1.0 ml of a *Salmonella* or EHEC overnight culture pre-diluted to *ca*. 3.0 log CFU/ml with M/T broth was respectively mixed with 1.0 ml of an overnight culture of *P. fluorescens* A506, *B. mojavensis* RRC 101, *B. subtilis* ATCC 6051, *L. rhamnosus* GG, and their cocktails, as well as Pst DC3000, all pre-diluted to *ca*. 5.0 log CFU/ml with M/T broth. One of the cocktails (Cocktail 1) had an equal concentration *B. subtilis* ATCC6051, *B. mojavensis* RRC101, and *P. fluorescens* A506 cells; whereas the other cocktail (Cocktail 2) contained an equal concentration of the same three bacterial strains plus *L. rhamnosus* GG. The mixed cultures in the tissue culture plates were incubated at 25 °C with agitation at 100 rpm on a platform shaker (Orbit Shaker, Lab-Line Instruments, INC.). A separate set of tissue culture plates containing the mixture of *L. rhamnosus* GG and each *Salmonella/E. coli* strain were prepared in the same manner except that the incubation temperature was set to 37 °C since *L. rhamnosus* GG, unlike other competitive bacterial strains used in the study, has optimal growth at this temperature. The control sample was prepared by mixing 1 ml overnight culture of each *S. enterica* and EHEC strain pre-diluted to *ca*. 3 log CFU/ml in M/T broth with 1 ml of M/T broth in tissue culture plates. Broth culture samples were collected after 6, 12, 24, 48, and 72 h of incubation at the set temperatures and the collected samples were diluted in PBS (pH 7.4) and plated in duplicate on XLT4 or sorbitol MacConkey agar supplemented with 50 μg/ml of NA (NASMAC). Presumptive colonies of *S. enterica*, *E.*

coli O157:H7, and *E. coli* O104:H4 were counted after 24-48 h of incubation at 37 °C. The experiment was repeated three times.

5.2.3 Competitive attachment between Salmonella *or EHEC and selected plant pathogen and biocontrol agents*

Competitive attachment between *Salmonella* or EHEC cells and those of selected plant pathogen and biocontrol agents to vegetable seeds was studied using a previously published protocol with modifications (Cui et al., 2017). Two grams of each type of vegetable seeds described above were placed in 50-ml centrifuge tubes (Fisher Scientific, Asheville, NC) and sanitized with 10 ml of 20,000-ppm sodium hypochlorite solution (pH 6.8; BD) at room temperature for 10 min with gentle mixing. The sanitizer solution was then decanted, and remaining chlorine was removed by soaking the seeds in 10 ml of Dey-Engley neutralizing broth for 10 min and rinsing twice, each with 10 ml of sterilized deionized water.

Two pathogen cocktails were prepared, each with an equal number of the four *Salmonella* or EHEC overnight cultures grown in M/T broth at 37 °C. Overnight cultures of each competitive bacterial strain and a mixture containing an equal number of each bacterial competitor were prepared in the same broth at 25 °C. The pathogen cocktails and overnight cultures of competitive strains/mixtures were individually diluted to a cell density of 10^4 CFU/ml in PBS. Ten milliliters of a diluted *Salmonella* or EHEC cocktail was then mixed with equal volume of each competitive strain/mixture in Falcon centrifuge tubes containing sanitized vegetable seeds. Ten milliliters of each diluted *Salmonella* or EHEC cocktail and 10 ml of PBS was added to a separate set of seeds as controls. The inoculation levels were determined by plating 0.1 ml of appropriately-diluted cell suspensions on TSA or NATSA. Vegetable seeds in the centrifuge tubes were agitated horizontally at 100 rpm in an orbital platform shaker (model

3520; Lab-Line, IL, USA) at 20°C for 5 h. The inocula were then decanted and seeds were rinsed twice, each with 10 ml sterillized water for 1 min with gentle mixing. Seeds were then soaked overnight at 4 °C in 5.0 ml of PBS to release attached bacterial cells. On the next day, seed samples were vortexed at max speed (56 ×g) (Fisher Scientific, Asheville, NC) for 50 s before 0.1 ml of soaking solution was spread on XLT4 or NASMAC plates in duplicate. The plates were incubated at 37 °C for 24-48 h for the enumeration of *Salmonella* and *E. coli* colonies. The ratio of the number of attached cells to the number of inoculated cells (attachment ratio) was reported. The experiment was repeated three times.

5.2.4 Effect of metabolites in cell-free supernatants of the spend cultures of biocontrol agents and plant pathogen on Salmonella *and EHEC*

For the preparation of cell-free supernatants, *L. rhamnosus* GG was grown in 10 ml of MRS broth at 37 °C for 72 h. Each biological control agent was grown individually in 10 ml of TSB at 25 °C for 72 h. Cultures of *S. enterica* and EHEC strains were prepared in 10 ml TSB at 37 °C overnight. Obtained bacterial cultures were centrifuged at 6,000 x *g* for 10 min and resulting supernatants were carefully removed. Suspended bacterial cells were removed by filtering the supernatants through 0.45-µm sterillized syringe filter (Fischer Scientific). One-milliliter aliquot of each filtered supernatant was mixed with 1 ml of overnight culture of each *Salmonella* and *E. coli* strain pre-diluted to *ca*. 10^5 CFU/ml with PBS in 24-well tissue culture plates. The starting cell concentration was determined by plating 0.1 ml of diluted CFS/pathogen mixture on XLT4 or NASMAC plates and incubated at 37 °C for 24-48 h. The tissue culture plates were incubated at 25°C and samples were collected after 2, 4, 8, 12, 24, and 48 h during incubation. Collected samples (0.1 ml) were plated in duplicate on the same media stated above

and presumptive colonies of *Salmonella* and EHEC were counted after incubation at 37 °C for 24-48 h. The experiment was performed in triplicate.

5.2.5 Statistical analysis

The mean populations of different *Salmonella* and EHEC strains exposed to the presence of different competitive agents or their cell-free metabolites and at different sampling times were compared by Fisher's Least Significance Difference test using the SAS software (Version 4). A similar statistical analysis was used to compare the attachment of *Salmonella* and EHEC cells in co-cultures to alfalfa, fenugreek, lettuce, and tomato seeds. For all comparisons, *P* values less than 0.05 was considered as significant.

5.3 Results

5.3.1 Competitive growth between Salmonella*/EHEC and bacterial competitors*

The mean populations of all four *Salmonella* or EHEC strains in co-cultures with individual competitive bacterial strains/cocktails are shown in Table 5-2. The mean populations of *Salmonella* and EHEC in the co-cultures were significantly lower than those in the respective controls. *Salmonella* populations in co-culture with *L. rhamnosus* GG were significantly lower than those in other co-cultures, at 37 °C and 25 °C. The mean population of *Salmonella* in co-culture with *L. rhamnosus* GG at 37°C was 5.36 log units lower than in the control and 3.53-log lower than the *Salmonella* population in the co-culture grown at 25°C. Adding *L. rhamnosus* GG to cocktail 1 did not affect *Salmonella* growth but a different result was observed with EHEC over the 72-h co-incubation period. Mean *Salmonella* and EHEC populations in the co-culture with cocktail 2 were significantly higher than with *L. rhamnosus* GG at 25 °C. The populations of EHEC were significantly lower when they were co-cultured with cocktail 2 compared to individual bacterial strains. Mean *Salmonella* populations were similar in the co-cultures with

the two cocktails and *P. fluorescens* A506, and the three populations were significantly lower than those in the co-cultures with *B. subtilis* ATCC 6051, *B. mojavensis* RRC 101 and Pst DC3000. It is worth noting that although statistically significant, *Salmonella* and EHEC population differences in co-cultures with competitive bacterial strains other than *L. rhamnosus* GG were all below 1 log unit. Among the three tested biocontrol agents, *Salmonella* and EHEC populations in co-culture with *P. fluorescens* A506 were the lowest. EHEC or *Salmonella* populations cultured with *B. mojavensis* RRC 101 were similar to that with *B. subtilis* ATCC 6051. The presence of, Pst DC3000 in co-cultures also retarded the growth of *Salmonella* and EHEC during the 72 h co-incubation period.

When co-cultured with their growth competitors, the mean populations of *S*. Cubana and *S*. Stanley were significantly lower than those of *S*. Montevideo and *S*. Baildon (Table 5-2). *E. coli* F4546 and *E. coli* BAA 2326 populations from the co-cultures were significantly different but these populations were significantly lower than the mean populations of *E. coli* H1730 and K4492. Significant increase in the mean populations of *Salmonella* and EHEC was seen at most sampling points except for *Salmonella* population at the 24 h sampling point, which was not significantly different from that at the 48 h sampling point.

No significant difference was observed among the populations of the four individual *Salmonella* strains co-cultured with different bacterial competitors (Table 5-3) except with *L. rhamnosus* GG. The mean populations of the four individual *Salmonella* strains in other cultures were not significantly different from each other nor the controls. Furthermore, all four *Salmonella* strains had similar mean cell populations in the co-cultures with the similar bacterial antagonists. Different observations were made with EHEC, and co-culture with *L. rhamnosus*

GG only significantly reduced the population of H1730 and K4492. The mean populations of *E. coli* BAA2326 were the lowest in all cultures (Table 5-4).

The mean populations of *Salmonella* in the control and co-cultures with each bacterium increased exponentially within the first 24 h of incubation before entering the stationary growth phase. The population difference between the controls and co-cultures with individual bacterial strains ranged from 0.9 to 1.36 log units at the 24 h sampling point. No significant improvement in *Salmonella* inhibition was observed at the 48 h sampling point. At the 72 h sampling point however, the mean *Salmonella* population in the co-culture with *L. rhamnosus* GG was 4.57 log units lower than the control. *Salmonella* population difference among other co-cultures ranged from 0.55 to 1.42 log units at this sampling point.

Compared to the control, the mean population of all four tested EHEC strains was 0.7-2.98 log units lower when co-incubated with the bacterial competitors throughout the 72-h incubation period (Fig. 5-1b). At the 72-h sampling point, the EHEC population was 1.42 log CFU/ml lower in the co-cultures with cocktail 1, 1.37 log CFU/ml lower in co-culture with cocktail 2, and 2.86 log CFU/ml lower in co-culture with *L. rhamnosus* GG than in the control culture. Similar to what was observed with *Salmonella*, a significant decrease in EHEC population was observed between 48 and 72 h in co-culture with *L. rhamnosus* GG, but the level of population decrease was less profound than that of *Salmonella*.

The ability of *L. rhamnosus* GG to inhibit*Salmonella* and EHEC growth was much stronger at 37ºC than 25ºC. After 24 h of co-incubation with *L. rhamnosus* GG, the mean populations of all four tested *Salmonella* or EHEC strains were lower at 37 ºC than at 25 ºC (Fig. 5-2). *Salmonella* cells were not detected from the co-culture with *L. rhamnosus* GG at 37 ºC after the 48 h sampling point (detection limit > 10 CFU/ml). The mean populations of EHEC recovered

from the co-cultures with *L. rhamnosus* GG were higher than those of *Salmonella* incubated at the same temperatures.

5.3.2 Competitive attachment to vegetable seeds by Salmonella*/EHEC and bacterial competitors*

The mean attachment ratios of *Salmonella* or EHEC for the two types of vegetable seeds were significantly lower when the competitive bacterial strains were present (Table 5-5). When no competitor bacteria were used, the mean attachment ratios of *Salmonella* and EHEC cells were 10.5% and 3.9%, respectively. In the co-cultures with *P. fluorescens* A506, *Salmonella* (7.0 %) and EHEC (2.4%) strains had the lowest attachment ratios. The attachment ratios of *Salmonella* in the co-cultures with *B. mojavensis* RRC 101 (7.9%), *B. subtilis* ATCC 6051 (8.1%), and Pst DC 3000 (8.2%) were statistically similar but were significantly lower than attachment ratios in co-cultures with *L. rhamnosus* GG (9.4%) and the cocktail (9.0%). Except for the controls, the highest attachment ratios of EHEC were seen in the co-cultures with *L. rhamnosus* GG (3.4%) and Pst DC3000 (3.5%), followed by the cocktail (3.1%), *B. mojavensis* RRC 101 (2.9%), and *B. subtilis* ATCC 6051(2.8%). The attachment ratios of *Salmonella* and EHEC cells were the highest on fenugreek seeds (12.5% and 6.7%), followed by alfalfa (11.8% and 2.0 %) and lettuce (8.9% and 1.7%). No attachment was detectable from tomato seeds.

5.3.3 Inhibition of Salmonella *and EHEC by cell-free supernatants of bacterial competitors*

Changes in the mean population of all four *Salmonella* and EHEC strains grown in PBS amended with the CFS of 72-h spent cultures of the three biocontrol agents and *L. rhamnosus* GG are shown in Fig. 5-3. The addition of *L. rhamnosus* GG CFS to the diluted *Salmonella* and EHEC cultures resulted in significant reductions in the populations of *Salmonella* and EHEC. The mean populations of the four *Salmonella* strains reduced approximately 1 log unit after 2 h of incubation. *Salmonella* cells became undetectable (< 10 CFU/ml) at the 12 h sampling point

and forward, and a 5 log unit reduction was achieved after the 24 h incubation period. The mean population of the four EHEC strains decreased 2.4 log CFU/ml from the 4 h to the 8 h sampling points and a total of 4 log CFU/ml reduction was observed after 24 h incubation. In comparison, the growth of *Salmonella* and EHEC in other co-cultures was not inhibited and the pathogen populations increased exponentially before entering the stationary phase after 24 h. The populations of *Salmonella* and EHEC recovered from these co-cultures did not differ, with the maximum population difference of 0.69 log units for *Salmonella* and 0.42 log units for EHEC.

The responses of the four individual *Salmonella* or EHEC strains to the CFS of *L. rhamnosus* GG over the 48-h incubation period are shown in Fig. 5-4. Populations of the four *Salmonella* strains decreased rapidly after 4 h of incubation and populations of *S.* Baildon and *S.* Cubana fell below the detection limit of the plate count assay after 8 h of incubation (Fig. 5-4a). *S.* Montevideo and *S.* Stanley cells were not detectable after 12 h of incubation. Populations of all 4 EHEC strains also decreased rapidly after initial 4 h of incubation and cells of *E. coli* BAA 2326 became undetectable after 12 h of incubation in M/T broth amended with CFS (Fig. 5-4b). Cells of *E. coli* F4546 and H1730 were detectable until 24 h of incubation. The population of K4492 increased to 3.39 and 3.57 log CFU/ml at the 24 and 48 h sampling points after dropping from the 5.2 log CFU/ml inoculation level to 2.74 log CFU/ml during the initial 12 h of incubation.

5.4 Discussion

The mean populations of *Salmonella* and EHEC in co-cultures with each of the competitive bacterial strains used in the study over the 72-h co-incubation were significantly lowered than those in the controls (Table 5-2). Co-cultures with *L. rhamnosus* GG alone had the lowest *Salmonella* and EHEC populations at both 25 and 37 °C compared to co-cultures with

other bacterial competitors. Production of various organic acids and antimicrobial peptides including bacteriocin by *L. rhamnosus* GG might be the underlying mechanisms for the observed phenomenon. Organic acid produced by *L. rhamnosus* GG can lower the pH of microbiological media and thereby inhibit the growth of bacterial pathogens (Fayol-Messaoudi et al., 2005). Bacteriocins are proteinaceous or peptidic toxins produced by bacterial cells, which can kill susceptible pathogens by changing the permeability of bacterial membranes or interfering with the biological function of essential bacterial enzymes (Pithva et al., 2011). Antimicrobial peptides produced by *L. rhamnosus* GG belong to a poorly-characterized class of complex bacteriocins with a broad spectrum of antagonistic activity against Gram-positive and Gram-negative bacteria (Pithva et al., 2011). Previous studies have shown that the bacteriocin produced by *L. rhamnosus* GG can also inhibit the growth of some plant pathogens such as *Pseudomonas aeruginosa* and several major food spoilage microorganisms (Alexandre et al., 2014; Tharmaraj & Shah, 2009). These findings suggest that the probiotic has potential for application as a biological control agent for improving the microbial safety of sprout seeds.

When *L. rhamnosus* GG was co-cultured with *Salmonella* or EHEC at 25 °C, the incubation time required for observable pathogen inhibition was longer than at 37 °C. Furthermore, *Salmonella* and EHEC population differences were much smaller between different sampling points at 25 °C (Fig. 5-2). The relatively lower antagonistic ability of *L. rhamnosus* GG at 25 °C might be the results of slower growth rate and accumulation of bactericidal metabolites since this incubation temperature was less than optimal (Fayol-Messaoudi et al., 2005). The growth rates of *L. rhamnosus* GG in MRS broth (pH 6.5) at 37 °C and 25 °C were reported as 0.95 and 0.36 OD_{600}/h, respectively (Deepika et al., 2012). At 25 °C, the maximum *L. rhamnosus* GG population was 0.5-1.5 OD_{600} readings lower, and the time required for *L.*

rhamnosus GG to reach stationary phase was about 12 h longer, than at 37 °C. When grown in Ultra-high temperature processed milk for 48 h, the amount of organic acids and acetaldehyde produced by *L. rhamnosus* GG at 30 °C was found significantly lower than at 37 °C (Østlie et al., 2005). Fayol-Messaoudi and coworkers (2005) observed that when incubated at 32 °C in MRS medium, the killing activity of CFSs of the 24 h-culture of *L. rhamnosus* GG was significantly lower although the lactic acid concentration in CFSs did not differ significantly from that at 37 °C. The authors believed that the production of bactericidal metabolites other than organic acid is also affected by incubation temperature used to cultivate the probiotic bacterium.

In general, lower populations of *Salmonella* than EHEC were observed in the co-cultures with *L. rhamnosus* GG (Table 5-2) or in broth supplemented with its CFS (Fig. 5-3). Similar observations were made by Arias O and co-workers (2013) who reported that the inhibition of *L. rhamnosus* and its CFS to *S.* Typhimurium was significantly stronger than for *E. coli* O157:H7. *E. coli* O157 strains are known to be more acid resistant than *Salmonella* at pH from 2.5-3.8 (Breidt et al., 2013). Higher tolerance of *E. coli* O157 to acetic acid and lactic acid were also reported (Berry & Cutter, 2000; Brackett et al., 1994). Since the production of organic acid and the resulting low environmental pH are one of the important antagonistic mechanisms used by *L. rhamnosus* GG (Vanderhoof et al., 1999), the ability of *E. coli* O157 in better adapting to acidic environment might have contributed to the lower inhibition effects of *L. rhamnosus* GG and its CFS observed in the present study. *E. coli* O104:H4 strain BAA 2326 used in the present study had a similar response to co-culture with *L. rhamnosus* GG and in broth supplemented with its CFS compared to the two O157 strains, F4546 and H1730. The higher cell counts of *E. coli* K4492 in co-cultures with *L. rhamnosus* GG CFS indicates that the resistance of EHEC to the metabolites of *L. rhamnosus* GG is likely strain-dependent (Figure 5-3b).

P. fluorescens A506 is known to compete with plant pathogen *Erwinia amylovora via* preemptive utilization of growth-limiting nutrients at growth niches such as nectars of pear blossoms (Stockwell et al., 2010). In the present study, a 100-fold higher start inoculum of *P. fluorescens* A506 over the tested *Salmonella* and EHEC strains resulted in a significant, but less than 1 log unit difference in *Salmonella* and EHEC population during the 72 h incubation period (Table 5-4). The observed population reduction is likely due to competition for nutrients as the CFS of *P. fluorescens* A506 did not cause observable inhibition of *Salmonella* and EHEC (Fig. 5-3). Similar observations were made by other authors. The presence of a *P. fluorescens* strain in the mixed culture with *E. coli* O157:H7 ATCC 43895 reduced the pathogen population overtime at 10, 15, and 25 ºC (Samelis & Sofos, 2002). The incubation temperature of 25 ºC might also give *P. fluorescens* A506 competition advantages over the two human pathogens, as the optimal growth temperature of *P. fluorescens* A506 is 25 ºC while that of *Salmonella* and EHEC is 37 ºC.

Slight, but significant differences in *Salmonella* and EHEC population were observed when they were co-cultured with *B. mojavensis* RRC101 and *B. subtilis* ATCC6051 (Table 5-2). The antagonistic activities of the two biocontrol agents were reported to rely on the release of specific fungicidal cyclic lipopeptides and/or bio-surfactants (Bacon & Hinton, 2002; Snook et al., 2009). Whether the bio-surfactants effectively inhibited the growth of *Salmonella* and EHEC remains contradictory. The surfactin extracted from a 7-day culture of *B. licheniformis* M104 inhibited the growth of *S*. Typhimurium ATCC 14028 and two *E. coli* strains ATCC 11775 and 11246 in a disc diffusion assay (Gomaa, 2013). However, Mireles and coworkers found that surfactins of *B. subtilis* inhibited biofilm formation, but not the growth of *S. etnerica* and *E. coli* cells (Mireles et al., 2001). .

Using multiple biocontrol agents simultaneously could be one of the solutions to the unsatisfactory inhibition to target pathogen (s) by individual biocontrol agents. Knowing the inhibitory mechanisms of each biocontrol agent is crucial in finding the most efficient components of a biological control cocktail, ideally different modes of action can be applied simultaneously to avoid adaption of the target pathogen (Vijayakumar & Muriana, 2017). It is also important to understand if synergistic/antagonistic effects exist among all candidates used in the same cocktail. Ye and coworkers (2010) previously reported the success in using a combination of *E. asburiae* JX1 and a cocktail of *Salmonella* lytic phages to achieve a 3-log reduction in *Salmonella* population on sprouting mung bean and alfalfa seed. Other studies also described the potential of using cocktails of antagonistic strains to control of various plant and human pathogens (Bonaterra et al., 2012; Nisbet, 2002; Vijayakumar & Muriana, 2017). In the current study, slightly lower (0.16-0.46 log CFU/ml) pathogen populations were observed when the two cocktails were used. It is not yet clear if the slight larger pathogen population differences caused by the presence of the two cocktails were the results of synergistic interactions among the biocontrol agents or simply the effect of a greater overlap of nutrition requirements among the bacteria and *Salmonella*/EHEC strains. Further research is needed to fully understand and improve the inhibitory effects of the cocktails on human pathogens in the co-culture systems.

Although the presence of *L. rhamnosus* GG alone in a co-culture had the lowest *Salmonella* and EHEC populations, adding *L. rhamnosus* GG to cocktail 1 did not significantly change the ability of the biocontrol cocktail to inhibit *Salmonella* (Table 5-2). The reduction in EHEC population caused by the presence of the two cocktails differed only by 0.11 log units. The pH of 72-h mixed cultures containing cocktail 2 and *Salmonella* or EHEC ranged from 6.8

to 7.2, which was higher than the pH of 3.7-4.3 from the co-cultures of *L. rhamnosus* GG with *Salmonella*/EHEC (data not shown). This indicates a poor *L. rhamnosus* GG growth in the mixed culture containing cocktail 2, subsequently a low level of accumulation of anti-*Salmonella* and –EHEC metabolites in the cocktail. Since the biocontrol agents used in cocktail 2 had an optimal growth temperature of 25 °C, it is likely that *L. rhamnosus* GG was outgrown by the biocontrol agents in the cocktail at this incubation temperature. The higher pH in cocktail 2 could have also affected the function of non-organic acid antimicrobial molecules produced by *Lactobacillus* GG. It has been reported that the antagonistic compounds produced by *L. rhamnosus* GG suppressed the growth of *S.* Typhimurium at pH 4.5 but not at pH 6.5 (Fayol-Messaoudi et al., 2005). Synergistic effects between lactic acid and non-lactic acid antimicrobial molecules released by *Lactobacillus* strains have been described by Alakomi and coworkers (2000) who reported that the lactic acid molecules can modify the permeability of the outer membrane of gram-negative pathogens, allowing antimicrobial molecules to more easily across cell membranes. As stated previously, a good biocontrol cocktail should not contain bacterial strains that are antagonistic to each another.

While less effective than *L. rhamnosus* GG in inhibiting the growth of *Salmonella* and EHEC, the presence of the three biocontrol agents significantly reduced the attachment of *Salmonella* and EHEC to tested vegetable seeds (Table 5-5). The lower level of *Salmonella* and EHEC attachment caused by these bacterial strains might be due to competition among bacterial strains including those of human pathogens for limited niches of attachment on vegetable seeds. Ideal attachment sites for microorganisms usually include the wrinkles, cracks, and crevices on the surface of vegetable seeds (Charkowski et al., 2001). The production of surfactin-like molecules by *Lactobacillus* GG (Sangeetha, 2016) and the two *Bacillus* strains (Snook et al.,

2009) might have interfered with the attachment by *Salmonella* and *E. coli* cells to seed surfaces (Gomaa, 2013). Most significant reductions in *Salmonella* and EHEC attachment to vegetable seeds were observed when *P. fluorescens* A506 was present. The reason for the observed phenomenon is unclear, but it may due to the fact that *P. fluorescens* is a better colonizer of seeds, root, and plant tissues in the presence of other competitive plant microorganisms (Dekkers et al., 1998).

The interactions between plant and human pathogens have been discussed widely as plant pathogens might play a role in the survival of human pathogens various plants (Aruscavage et al., 2008; Cooley et al., 2006). Certain activities of plant pathogens (breach of plant cell walls, necrotic release of nutrients from plant cells, suppression of plant immunity, *etc*.) might boost the survival of human pathogens that are in close proximity. Esseilli and coworkers (2015) reported that the necrotic lesions created by *Xanthomonas campestris* pv. *vitians* or cucumber mosaic virus strain *Fny* enhanced the postharvest survival of human pathogens on leafy green vegetables. Water-soaking of tomato leaves from the infection by *X. euvesicatoria* and *X. gardneri* supported the persistence and/or growth of *S. enterica* (Potnis et al., 2014). It is evident that most of the known interactions between plant and human pathogens were observed in mature plant hosts. Although *P. syringae* and its multiple pathovars (syringae, tomato) have been reported to enhance the survival and growth of *S.* Typhimurium and pathogenic *E. coli* on leaves of different plants (Poza-Carrion et al., 2013), Pst DC3000 only slightly inhibited the growth of the two pathogens. Whether and how the two types of pathogens interact with each other outside mature plants, such as on vegetable seeds and seedlings, demand further, and more systematic investigation.

5.5 Conclusion

Biological control of human pathogens on vegetable seeds at both pre- and post-harvest stages has drawn the attention of many researchers, as it can lead to the production of safe vegetable seeds and fresh produce. Significantly lower populations of *Salmonella* and EHEC were observed when they were in cocultured with *L. rhamnosus* GG and in broth supplemented with its CFS. Since *L. rhamnosus* GG is a probiotic organism and its beneficial effect on human gut health has been proven by numerous clinic trials, it could improve the microbial safety of vegetable seeds, especially at postharvest stages. Although not as competitive as *L. rhamnosus* GG in inhibiting the growth of *Salmonella* and EHEC, the three biocontrol agents were more effectively in competing with *Salmonella* and EHEC for attachment to vegetable seeds. Thus, in addition to control of plant pathogens, these agents could also be used to control human pathogens during vegetable seed production. The different responses of the *S. enterica* and EHEC strains to each competitive bacterium and cocktails highlights the importance of choosing appropriate bacteria for targeted pathogens. Future studies should focus on the identification of effective bacterial strains with negligible adverse influences on seed/fresh produce quality as well as better understanding of the synergistic mechanisms of participating bacterial strains to maximize their antagonistic effects.

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Table 5-1. Human pathogen, biocontrol agents, probiotic and plant pathogen strains used in this study

Bacterium	Serovar/Strain	Description
Salmonella enterica	Stanley	Sprout-related outbreak, Finland, Canada, and the US, 1997
	Baildon	Multistate outbreak, the US, raw domestic tomato and lettuce, 1999
	Montevideo	Tomato-related outbreak, the US, 1993
	Cubana	Sprout-related outbreak, the US, 2012
Enterohemorrhagic *Escherichia coli*	F4546	Sprout-related outbreak, the US, 1997
	BAA-2326	Outbreak associated with fenugreek, Germany, 2011
	K4492	Spinach-related outbreak, the US, 2006
	H1730	Lettuce outbreak, the US, 2003
Pseudomonas fluorescens	A506	Commercial available biocontrol agent for *Erwinia amylovora* (fire blight) on fruits
Bacillus mojavensis	RRC 101	Commercial available biocontrol agent for *Fusarium verticillioides* in maize and other crops; surfactin- producing
Bacillus subtilis	ATCC 6051	surfactin-producing *B. subtilis* strain
Lactobacillus rhamnosus	GG	Commercial available probiotics
Pseudomonas syringae pv. *tomato*	DC 3000	Frequently seen seedborne pathogen on tomato plant

Table 5-2. Overall mean *S. enterica* and EHEC populations in co-cultures with different competitive bacterial strains and at different sampling points

Main effect	Mean population (log CFU/ml)		Main effect	Mean population (log CFU/ml)
	S. enterica [a]	*E. coli* [b]		
Competitive strains			***S. enterica* strains**	
Control	7.84A	8.12A	*S.* Montevideo	6.75A
P. syringae pv. *tomato* DC 3000	7.40B	7.82B	*S.* Baildon	6.64A
B. subtilis ATCC 6051	7.25BC	7.62C	*S.* Stanley	6.11B
B. mojavensis RRC 101	7.21C	7.51C	*S.* Cubana	6.07B
P. fluorescens A506	7.02D	7.36D	***E. coli* strains**	
Cocktail 1[d]	6.93D	7.31D	*E. coli* H1730	7.85A
Cocktail 2[c]	6.86D	7.15E	*E. coli* K4492	7.73A
L. rhamnosus GG (25 °C)	6.01E	6.79F	*E. coli* F4546	7.12B
L. rhamnosus GG (37 °C)	2.48F	6.63G	*E. coli* BAA 2326	6.35C
Sampling points (h)				
24	7.49A	8.66B		
48	7.45A	8.97A		
72	6.92B	8.43C		
12	6.24C	6.32D		
6	3.84D	4.05E		

[a] Mean populations of *S. enterica* within a category that are not followed by the same letter are significantly different ($P<0.05$).

[b] Mean populations of *E. coli* within a category that are not followed by the same letter are significantly different ($P<0.05$).

[c] Mixed culture of *P. fluorescens* A506, *B. mojavensis* RRC 101 and *B. subtilis* ATCC 6051

[d] Mixed culture of *P. fluorescens* A506, *B. mojavensis* RRC 101, *B. subtilis* ATCC 6051 and *L. rhamnosus* GG

Table 5-3. Mean populations of individual *Salmonella* strains in co-cultures with different competitive bacterial strains and a plant pathogen

Salmonella strains	Mean population[a] (log CFU/ml)							
	P. fluorescens A506	Cocktail 2	Cocktail 1	*L. Rhamnosus* GG	Pst DC3000	*B. mojavensis* RRC101	Control	*B. subtilis* ATCC6051
Montevideo	7.58aAB	7.44aAB	7.34aAB	6.46aB	7.80aA	7.64aAB	8.00aA	7.63aAB
Baildon	7.20aA	7.23aA	7.40aA	5.89aB	7.82aA	7.37aA	7.95aA	7.57aA
Stanley	6.75aAB	6.27aAB	6.54aAB	5.74aB	7.20aA	7.00aAB	7.49aA	6.98aAB
Cubana	6.53aAB	6.53aAB	6.45aAB	5.88aB	6.78aAB	6.82aAB	7.88aA	6.81aAB

[a] Mean values within a column that are not followed by the same lowercase letter 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 5-4. Mean populations of individual EHEC strains in co-cultures with different competitive bacterial strains and a plant pathogen

EHEC strains	Mean population[a] (log CFU/ml)							
	*P. fluorescens*A506	Cocktail 2	Cocktail 1	*L. rhamnosus* GG	Pst DC3000	*B. mojavensis* RRC101	Control	*B. subtilis* ATCC6051
H1730	8.17aAB	7.50abAB	7.68abAB	7.36aB	8.55aAB	8.21aAB	8.61abA	8.32aAB
K4492	8.14aAB	7.58aAB	7.71aAB	7.07aB	8.43aA	8.28aA	8.72aA	8.34aA
F4546	6.85abA	7.30abA	7.29abA	6.67aA	7.66abA	7.31abA	7.87abA	7.22abA
BAA2326	6.29bA	6.20bA	6.56bA	6.03aA	6.63bA	6.66bA	7.26bA	6.57bA

[a] Mean values within a column that are not followed by the same lowercase letter are significantly different ($P<0.05$).

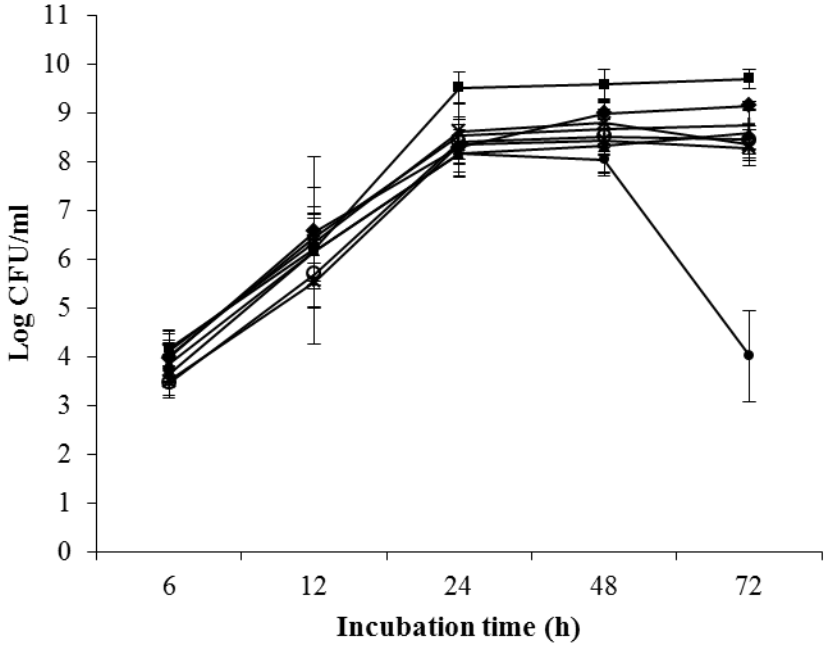
Mean values within a row that are not followed by the same uppercase letter are significantly different ($P<0.05$).

Table 5-5. Mean attachment ratios of the four-strain *S. enterica* or EHEC mixture in the presence of individual competitive bacterial strains and their cocktail

	Attachment ratio	
	Salmonella [a]	EHEC
Competitive strain		
Control	0.105A	0.039A
L. rhamnosus GG	0.094B	0.034B
Cocktail	0.090C	0.031C
B. mojavensis RRC 101	0.079D	0.029C
B. subtilis ATCC 6051	0.081D	0.028C
P. syringae pv. *tomato* DC 3000	0.082D	0.035B
P. fluorescens A506	0.070E	0.024D
Seed type		
Fenugreek	0.125A	0.067A
Alfalfa	0.118B	0.02B
Lettuce	0.089C	0.017C

[a] Mean attachment ratios within a column that are not followed by the same letter are significantly different ($P<0.05$).

(a)


Log CFU/ml
0
1
2
3
4
5
6
7
8
9
10
11
6
12
24
48
72
Incubation time (h)

(b)

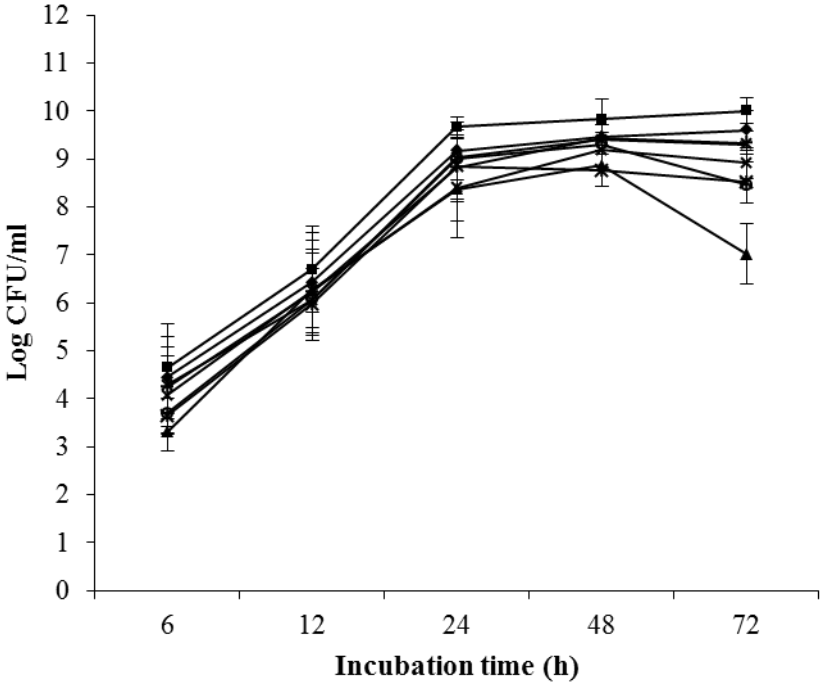

Log CFU/ml
0
1
2
3
4
5
6
7
8
9
10
11
12
6
12
24
48
72
Incubation time (h)

Figure 5-1. Mean populations of four strains of *S. enterica* (a) and *E. coli* (b) in the controls ( ) and the mixed cultures with *P. fluorescens* A506 ( ), *B. subtilis* ATCC 6051 ( ), *B. mojavensis* RRC 101( ), cocktail 1 ( ), cocktail 2( ), *L. rhamnosus* GG ( ), and *P. syringae* pv. *tomato* DC3000 ( ) after 6, 12, 24, 48, and 72 h of co-incubation.

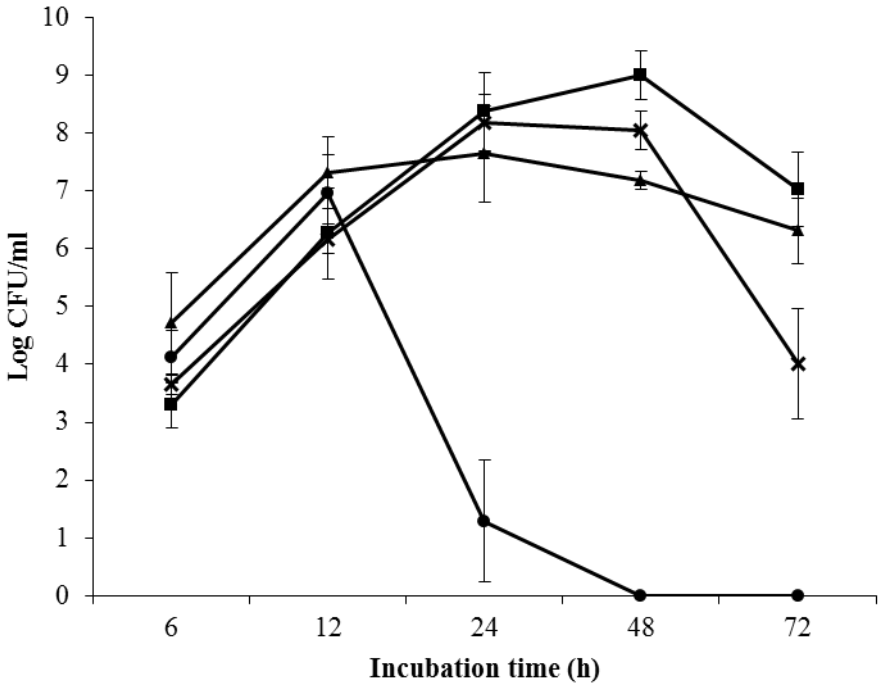


Figure 5-2. Mean population s of all four *S. enterica* and *E. coli* when co-cultured with *L. rhamnosus* GG at 37 °C (*S. enterica* —●—; *E. coli* —▲—) and 25 °C (*S. enterica* —✕—; *E. coli* —■—) after 6, 12, 24, 48, and 72 h of incubation.

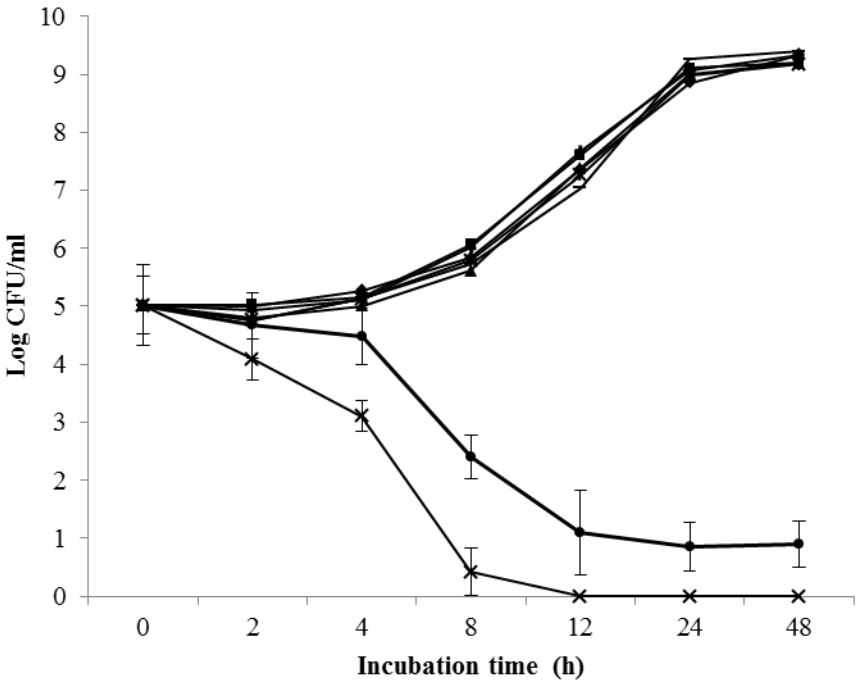
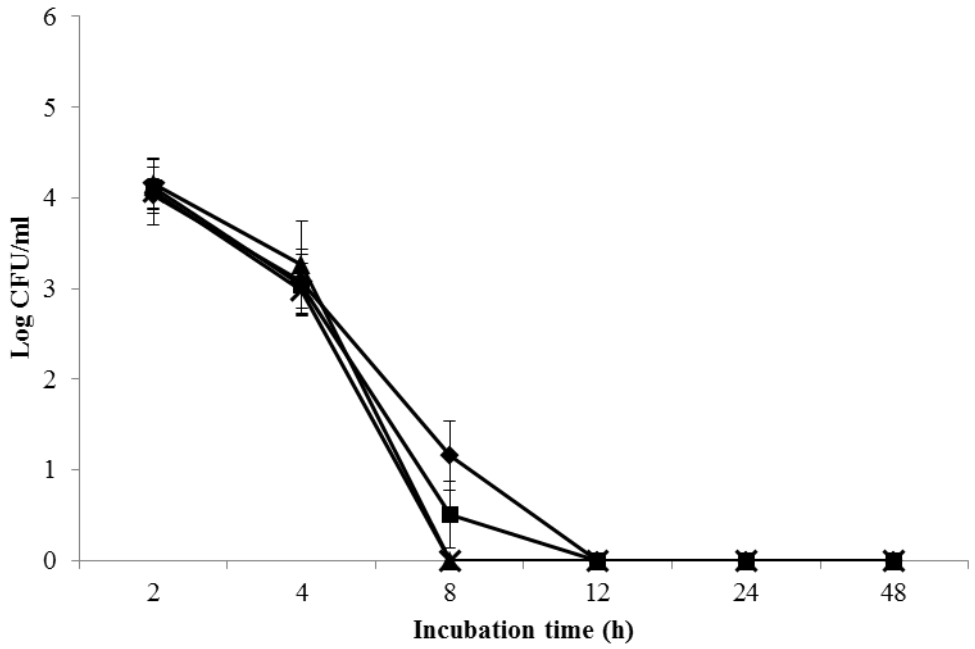


Figure 5-3. Mean populations of all four *S. enetrica* and EHEC cultured in MRS/TSB broth amended with the cell free supernatants of *P. fluorescens* A506 (*S. enterica* ; EHEC), *B. subtilis* ATCC 6051 (*S. enterica* ; EHEC), *B. mojavensis* RRC 101 (*S. enterica* ; EHEC), and *L. rhamnosus* GG (*S. enterica* ; EHEC) at the 2, 4, 8, 12, 24, and 48 h sampling points

(a)


Log CFU/ml
0
1
2
3
4
5
6
2
4
8
12
24
48
Incubation time (h)

(b)

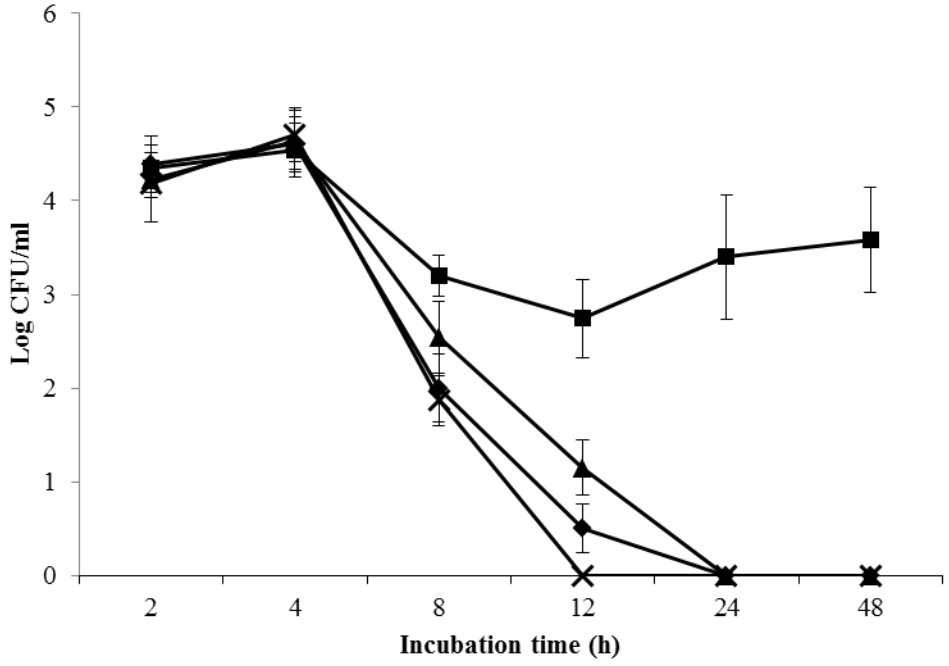

Log CFU/ml
0
1
2
3
4
5
6
2
4
8
12
24
48
Incubation time (h)

Figure 5-4. Mean populations of individual *S. enterica* (a) and *E. coli* (b) strains grown in MRS/TSB (1:1) broth amended with cell free supernatant of *L. rhamnosus* GG for 2, 4, 8, 12, 24, and 48 h. The strains included were *S*. Montevideo and *E. coli* F4546 (—◆—), *S*. Stanley and *E. coli* K4492 (—■—), *S*. Cubana and *E. coli* H1730 (—▲—), and *S*. Baildon and *E. coli* BAA-2326 (—✕—)

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CHAPTER 6

SUMMARY

This research observed the fate of *S. enterica* and EHEC during pilot-scale vegetable seed production and germination, and in the presence of potential antagonistic bacteria.

Salmonella and EHEC cells, when internalized into seeds of alfalfa, fenugreek, tomato and lettuce, can contaminate tissues of sprouts and seedlings during germination. The pathogen growth on germinating seeds was dependent on bacterial species and strains as well as type of vegetable seeds involved. In general, *Salmonella* grew to higher populations than EHEC on sprouting vegetable seeds, likely due to a stronger adaption of *Salmonella* to plant hosts. On average, alfalfa/fenugreek sprout tissues supported *Salmonella* and EHEC growth to higher populations than lettuce and tomato seedling tissues. Whether the presence of pathogen cells on vegetable seedlings, represent a threat to fresh produce safety will depend on the fate of the pathogens at later stages of plant development. However, sprouted alfalfa and fenugreek seeds are often consumed raw or minimally processed and have a greater potential for pathogen transmission.

Under controlled greenhouse conditions, we observed a low-level transmission of *Salmonella* cells from inoculated flowers to alfalfa/fenugreek sprouts and tomato seedlings *via* contaminated seeds, at a low-frequency. When inoculated onto pistils of vegetable flowers, *Salmonella* cells not only survived and transmitted to seeds, but established populations on vegetable seedlings. Further investigations are needed to fully understand whether this transmission pattern imposes a risk to the microbial safety of seeds/seedlings, as the survival and

migration of pathogen cells are not well understood. Several studies have demonstrated the ability of human pathogens to survive on plant hosts and be transmitted to open flowers (*i.e.* via irrigation water, insects, livestock, wildlife, etc.). Therefore, it is important for seed producers to follow Good Agricultural Practices and other agricultural guidelines to ensure the safety of their products.

Among the tested pathogen antagonists, *L. rhamnosus* GG demonstrated a significantly stronger ability to inhibit the growth of *Salmonella* and EHEC, either when co-incubated directly with the two pathogens in microbiological media, or when the media containing *Salmonella* or EHEC cells was amended with 72-h cell free supernatants of bacterial competitors. Considering the beneficial effects that *L. rhamnosus* GG (as a probiotic organism) might have on human gut health, it could be a candidate biocontrol agent for improving the microbial safety of vegetable seeds. While not as competitive as *L. rhamnosus* GG in inhibiting pathogen growth, other bacterial competitors were more effective in reducing the attachment of *Salmonella* and EHEC cells to alfalfa, fenugreek, and lettuce seeds. These agents could also be used to control human and plant pathogens during vegetable seeds production. The importance of choosing appropriate biocontrol agents for targeted pathogens was indicated by the different responses of tested *Salmonella* serovars/EHEC strains to bacterial competitors.

APPENDIX A. IDENTIFICATION OF GENES CRITICAL FOR ATTACHMENT AND BIOFILM FORMATION IN *ESCHERICHIA COLI* O157

A.1 Introduction

Vegetable seeds are potential vehicles of foodborne bacterial pathogens and likely source of contamination for most sprout-related outbreaks (Buck et al., 2003b). Enterohemorrhagic *Escherichia coli* (EHEC) are among the major bacterial pathogens that have been found on vegetable seeds and seed sprouts (Feng et al., 2011). From the year of 1997 to 2008, at least 14 sprouts-associated foodborne outbreaks in the U.S. were linked to EHEC O157 (Ding et al., 2013b). Non-O157 strains, including *E. coli* O104:H4, have also been documented as causative agents in sprout-related outbreaks. In 2011, more than 4,000 persons were involved in the *E. coli* O104: H4 outbreak of infections associated with the consumption of fenugreek spouts (King et al., 2012).

It was believed that most sprout-related outbreaks have been caused by seeds contaminated before the sprouting process initiates, but how pathogen cells were introduced to seeds remained unclear (Andrews et al., 2007). It has been reported that EHEC cells can attach to the surface of vegetable seeds and survive for as long as two years under dry storage conditions; and germinating these seeds can result in rapid establishment of pathogen populations on sprouts and seedlings (Tian et al., 2012). EHEC cells irreversibly attached to seed surface through their cell surface appendages can overcome the repulsive forces of the electrical double layer formed between the *E. coli* cells and their contact surfaces (Pratt & Kolter,

1998). These attached cells can grow, produce excessive amount of extracellular substances, and form biofilms to gain stronger resistance to physical and chemical treatments.

Although studies have described the underlying mechanisms of bacterial attachment and biofilm formation, not all have looked into the genetic factors that play an important role during the process. Matthysse and coworkers (2008) tested the attachment of *E. coli* mutants that lack expression of *pga*C (PGA production), *bcs*B (cellulose production) and *wca*D (colanic acid) and found a significantly reduced level of attachment of the mutant cells to alfalfa sprouts. The *pilA* gene, which encodes type I pili, was also reported to play an essential role in the attachment and biofilm formation by *E. coli* (Orndorff & Falkow, 1985). Whether the expression of these genes also affects the attachment of EHEC to the surface & vegetable seeds and whether other genes are involved in the attachment processis not well understood. The objective of this study is to identify genes that are critical for the attachment and biofilm formation of selected *E. coli* O157 strains on their contact surface.

A.2 Material and methods

A.2.1 Donor and recipient strains

Two *E. coli* O157 strains, *E. coli* F4546 and *E. coli* 5-11 were used in the study as recipients. Nalidixic acid-resistant mutants of the two strains were selected on tryptic soy agar (TSA) supplemented with 50 μg/ml of nalidixic acid. Sensitivity of the selected nalidixic acid-resistant mutants to ampicillin was confirmed on TSA plates supplemented with 100 μg/ml ampicillin. All cultural media and test reagents were purchased from Becton, Dickinson and Company (Sparks, MD). Stock cultures of the selected nalidixic acid-resistant mutants were stored at -80 °C until use.

E. coli SM 10 *λpir* with a pUT vector which contains a *mini-Tn5 luxAB-Tc* (Biomedal, S.L. Avda. Americo Vespucio) was used as the donor strain. The pUT vector also carries an ampicillin resistant gene *bla* thus the strain were maintained on Luria-Bertani (LB) agar plates supplemented with 100 μg/ml of ampicillin and 12.5 μg/ml of tetracycline.

A.2.2 Transposon mutagenesis

The donor and recipients (F4546 and 5-11) were grown at 37 °C overnight in tryptic soy broth (TSB) supplemented with 100 μg/ml of ampicillin and 12.5 μg/ml of tetracycline or 50 μg/ml nalidixic acid, respectively. The cells of donor and recipient strains were washed twice by centrifugation at 8,000 *g* for 2 min, followed by decantation of supernatants and re-suspension of cell pellets in 1.0 ml of TSB. Cells of the donor and each recipient were mixed at 1:5 ratio and the mixtures were spot-inoculated on TSA plates. The inoculated plates were incubated at 37 °C for 24 h. The cells on TSA plates were then collected and suspended in phosphate buffered saline (pH 7.4). Suspended cells were inoculated on TSA agar plates containing tetracycline (TSA-TC), and then on TSA plates containing ampicillin (TSA-AMP). The transconjugants that grew on TSA-TC but were inhibited on TSA-AMP were selected to comprise two mutant pools for *E. coli* F4546 and 5-11, respectively.

A.2.3 Selection of attachment mutants

The enrichment of attachment mutants was performed using a previous protocol described by Barak *et al.* (Barak et al., 2005) with modifications. Specifically, 2.0 ml overnight cultures of the mutant pools grown at 37 °C in LB no-salt (LBNS) broth supplemented with 50 μg/ml of nalidixic acid and 12.5 μg/ml of tetracycline (LBNS-NT) were diluted in PBS to a concentration of *ca*. 10^6 CFU/ml and allocated to single wells of 24-well tissue culture plates containing 2.0 ml of LBNS-NT. The mutant pools were incubated for 3 h at 25 °C with constant

agitation at 40 rpm on an orbit shaker (Orbit Shaker, Lab-Line Instruments, INC.). The cell suspension incubated in each well was then transferred to different wells with the same broth described above and incubated for another 3 h. The transfer of cell suspensions was performed at least three times a day and the resulted cell suspensions were incubated over overnight in LBNS-NT at 37 °C. The selection procedure was continued for 10 consecutive days, repeating attachment and regrowth of unattached cells. After 10 days, the cell suspensions were streaked on TSA plates supplemented with 50 μg/ml of nalidixic acid and 12.5 μg/ml of tetracycline and single mutant colonies were picked for further analysis.

A.2.4 Selection of mutants with altered ability in forming biofilms

The biofilm formation experiment was performed according to a previous protocol with modifications (Park & Chen, 2015). Specifically, 2.0 ml diluted overnight cultures of selected mutants, grown in LBNS at 37 °C for 24 h, were inoculated to individual wells of 24-well tissue culture plates (Corning Incorporated, Durham, NC). After incubation for 7 days at 28 °C, the broth culture was withdrawn and loosely attached bacterial cells were removed by washing the plate twice with sterile water. The plates were then dried at 60 °C for 2 h and the biofilms were fixed by passing the plates several times over the flame of a burner (Fisher Scientific, Asheville, NC). The fixed biofilm mass was then stained by adding 2 ml of 1% crystal violet to each well and let stay for 15 min under room temperature. Then the stain was washed away with running deionized water and the tissue culture plates were dried for 2 h at 60 °C. The crystal violet in the biofilms was extracted using 2 ml per well of ethanol-acetone solution (80:20) and the concentrations of extracted crystal violet were determined based on the absorbance of the extraction solutions at the wavelength of 550 nm.

A.2.5 PCR amplification and sequencing

The PCR was conducted using a transposon-specific primer directed toward the transposon-chromosomal junction, and a primer with the recognizing sequence of frequent-cutting restriction enzymes toward the particular insertion site to be detected. The primers used to amplify the transposon and its downstream sequence are shown in Table A1. Crude total cellular DNA was extracted from the selected mutants using a previous protocol with modifications (Guo et al., 2001). Specifically, 1 ml of overnight culture was centrifuged at 10,000 ×*g* for 10 min and supernatant was decanted. Pellets were washed twice, each time with 1 ml sterilized distilled water, re-suspended in 100 µl of sterilized distilled water, boiled for 10 min, and centrifuged as described above. The aqueous supernatant was stored at 4 °C until use. The 25-µl PCR mixture contained PCR buffer, deoxynucleoside triphosphates (1 mM each), primer (1 µM), *Taq* polymerase (0.5 U; ThermoFisher scientific Inc., MA), and 5 µl of DNA template. PCR assays were performed in a DNA thermal cycler 480 (Perkin Elmer, Norwalk, CT) using a cycle at 94°C for 5 min, followed by 35 cycles of 92°C for 1 min, 52-68°C for 1 min (depending on the annealing temperature of each primer), and 72°C for 2 min. The PCR amplicons were analyzed using gel electrophoresis on 1% agarose (GIBCO BRL, Rockville, MD) gels in Tris-borate-EDTA buffer (0.089 M Tris-borate, 0.002 M EDTA, pH 8.0). After electrophoresis, gels were stained with 1% ethidium bromide and visualized using a Gel Doc System 2000 (Bio-Rad Laboratories, Hercules, CA). Specific amplifications marked by strong single band on gel image were sent to Eurofins Genomics for sequencing and the sequences obtained were compared with the published sequence of *E. coli* O157 strains by a BLAST search.

A.3 Current Results

More than 2,400 colonies were selected from the mutant pool. The amounts of biofilm accumulation by some of the mutants were shown in Table A2. Chromosomal DNA was extracted from 35 transconjugants that were sensitive to ampicillin and demonstrated a significant difference in the ability to form biofilms. Although specific PCR products were obtained (Figure A1-A3), the sequencing data were not clean enough to determine the insertion site of the transposon.

Table A1. Oligonucleotide Primers used in the study

Primers	Sequence (5' to 3')	Size
fP1	CCAGGCAGGTAGATGACGACc	20
fP10f	TCGCTAACGGATTCACCACT	20
rP1	GAATTC	6
rP2	CTGCAG	6

fP: forward primer

rP: reverse primer

[a]: Uppercase letter represent sequence within the *Tc* gene

[b]: lowercase letter represent sequence within *sm/spc*[r]

TableA2. Absorbance of the extracted crystal violet at 550 nm of biofilm masses formed by each tested mutants or controls

Bacterial strain	A_{550}
Controls	
5-11	0.822
F4546	1.046
5-11 mutants	
2	0.256
67	0.387
66	0.455
F4546 mutants	
F39	0.140

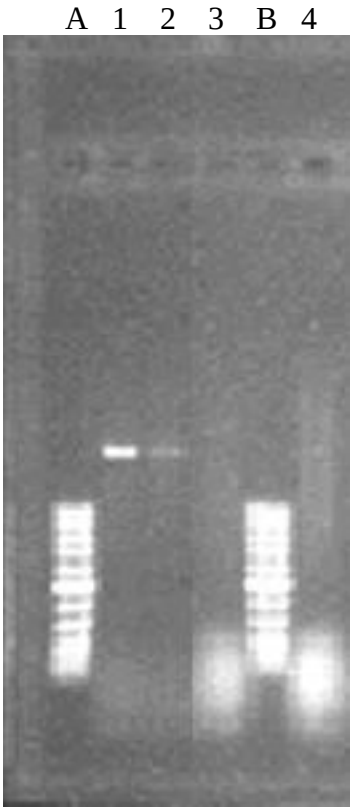

Figure A1. Amplifications of transposon-chromosomal DNA junction using the primer set fP10f - rP1. Lane1-2: M2, M67 (mutants of *E. coli* 5-11); Lane 3 and 4: F4546 and 5-11 (negative controls). Lane A and B: 1 kb DNA ladder.

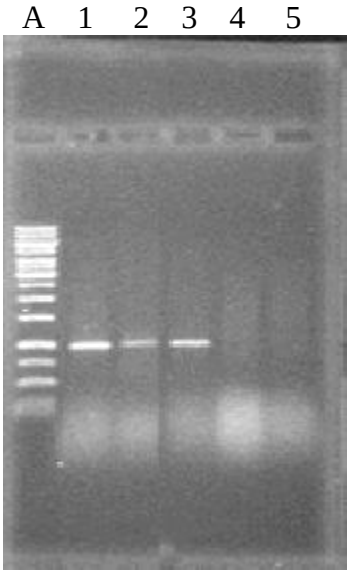

Figure A2. Amplifications of transposon-chromosomal DNA junction using the primer set fP1-rP1. Lane A: 1 kb DNA ladder; Lane 1-5: M2, M67, F39, F4546, 5-11.

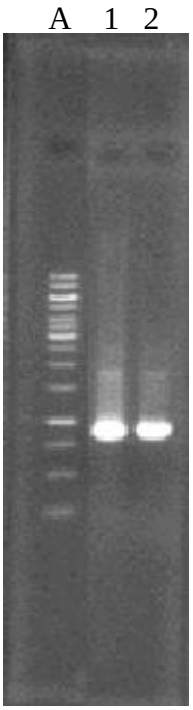

Figure A3. Amplifications of transposon-chromosomal DNA junction using the primer set fP1-rP2. Lane A: 1 kb DNA ladder; Lane 1-2, M67 and F39.

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