SALMONELLA ENTERICA AND ENTEROHEMORRHAGIC *ESCHERICHIA COLI* ON VEGETABLE SEEDS – MECHANISM OF ATTACHMENT, FATE DURING GERMINATION, AND CONTROL

by

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(Under the Direction of Jinru Chen)

ABSTRACT

The number of outbreaks of human gastrointestinal infection associated with the consumption of fresh produce has increased in recent years. Contaminated seeds have been identified as a potential source of vegetable contamination, especially the contamination of sprouts. The objectives of this study were to assess the abilities of selected *Salmonella* and enterohemorrhagic *Escherichia coli* strains to attach to vegetable seeds with different surface characteristics, to examine the physiological behavior of the bacterial pathogens on germinating seeds artificially contaminated by different approaches, and to evaluate the efficacy of treatment with probiotic tomato ferments on the fate of bacterial pathogens inoculated on alfalfa seeds and sprouts. We found that bacterial attachment to vegetable seeds was influenced by seed integrity but not by the fungicide treatments on seed surface. The mean populations of the pathogens on sprout/seedlings tissues developed from seeds contaminated by immersion into bacterial

suspensions were significantly higher than those from seeds contaminated by contact with artificially-inoculated soil. Seed coats had the highest bacterial counts, followed by the roots and cotyledons; the stem tissues had the lowest pathogen counts. Treatment with filter-sterilized supernatants of probiotic tomato ferments significantly reduced the population of *S.* Cubana and *E. coli* F4546 on alfalfa seeds and sprouts. The study provides a better understanding on how pathogens attach to, and behave on germinating, vegetable seeds and the performance of tomato ferments for sanitizing sprout seeds and seed sprouts. This information will help reduce the economic losses associated with vegetable-related outbreaks of infections.

INDEX WORDS: *Salmonella*, enterohemorrhagic *Escherichia coli*, vegetable seeds, sprouts/seedlings, probiotic tomato ferments

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CHAPTER 1

INTRODUCTION

The number of foodborne outbreaks of infections associated with the consumption of fresh produce has risen dramatically over the last two decades (Sapers, 2014). During the period of 2004 – 2013, 19% of the foodborne outbreaks and 24% of the total number of foodborne illnesses reported to the CDC were linked to fresh produce consumption, which made fresh produce the most common vehicle for transmitting foodborne illnesses (Fischer, Bourne, & Plunkett, 2015). From 2004 to 2012, the food items most frequently implicated in fresh produce-related outbreaks in the U.S. included lettuce, tomatoes, and sprouts (Callejón et al., 2015; Franz & Van Bruggen, 2008). Similar to historic epidemiological data, several recent outbreaks of human gastrointestinal infections have been linked to the consumption of contaminated lettuce, tomatoes, as well as alfalfa and fenugreek sprouts (Beutin & Martin, 2012; CDC, 2007, 2016a, 2016b; Slayton et al., 2013). A wide spectrum of microorganisms are involved in produce-associated outbreaks (Lynch, Tauxe, & Hedberg, 2009). Among them, *Salmonella enterica* and enterohemorrhagic *Escherichia coli* were the top two bacterial pathogens responsible for fresh produce-associated outbreaks (Callejón et al., 2015).

Vegetable seeds can be passive carriers as well as effective vectors of transmitting human pathogens. Vegetable seeds may become contaminated through contact with birds and rodents during storage and transportation or by irrigation waters, animals, and animal feces during seed production (Brooks et al., 2001; Hanning, Nutt, & Ricke, 2009).

Contaminated seeds could be a potential source of vegetable contamination, especially the contamination of seed sprouts (Hanning, Nutt, & Ricke, 2009). The seeds could be destined as raw agricultural product or for sprouting; however, the decision is often made after seeds are harvested. Some seed producers may not follow good agricultural practices, making sprout seeds prone to microbial contamination (Robertson, Johannessen, Gjerde, & Loncarevic, 2002). According to the FDA (2012), most sprout-related outbreaks have been due to seeds contaminated with bacterial pathogens before the sprouting process begins. Many pathogens can survive for months under the dry conditions used for seed storage (Van der Linden et al., 2013). However, pathogen populations in/on sprout seeds are exceptionally low, making them difficult to detect by routine seed testing. Furthermore, seed sanitation treatments are not always effective in eliminating bacterial pathogens associated with sprout seeds (Beuchat, 1997; Fu, Reineke, Chirtel, & VanPelt, 2008; Sapers, 2014).

In the current body of literature, limited studies have addressed the attachment ability of bacterial pathogens to vegetable seeds (Fransisca & Feng, 2012; Van der Linden et al., 2013), particularly lettuce, tomato, and fenugreek seeds. Most of the earlier studies concerning bacterial pathogens and vegetable seeds have focused on the efficacy of chemical treatments in reducing seed-borne bacterial pathogen populations (Buchholz & Matthews, 2010). Physical mechanisms of pathogen attachment to vegetable seeds have not been adequately addressed. Attachment to vegetable seeds is the first step of colonization by bacterial cells. The fate of pathogen cells during seed germination could have a significant impact on microbial safety of fresh produce, especially seed sprouts.

Currently, the National Advisory Committee on Microbiological Criteria for Foods recommends the use of 20,000 ppm calcium hypochlorite to sanitize seeds before sprouting (NACMCF, 1999), but this treatment does not guarantee the removal of all types of human pathogens (Montville & Schaffner, 2004). In addition, chlorine-based sanitation treatments may lead to potential formation of carcinogenic by-products (Van Haute, Sampers, Jacxsens, & Uyttendaele, 2015). Therefore, the need for alternative treatments for sprout seeds and seed sprouts has drawn the attention of seed safety specialists and researchers.

This project was undertaken

1) To elucidate the physical mechanisms that selected human pathogens, *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) use to attach to vegetable seeds with different surface characteristics (Chapter 3);
2) To examine the physiological behavior of the bacterial pathogens on germinating seeds contaminated by two different inoculation methods (immersion into bacterial suspensions *vs.* contact with artificially-inoculated soil; Chapter 4); and
3) To evaluate the efficacy of probiotic tomato ferments on inactivation of the bacterial pathogens on alfalfa seeds and sprouts (Chapter 5).

The information and knowledge generated from this research will lead to a better understanding of how pathogens attach to, and colonize on germinating, vegetable seeds, and help reduce the economic losses associated with vegetable-related outbreaks of infections.

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CHAPTER 2

LITERATURE REVIEW

2.1. Foodborne outbreaks associated with fresh produce, including sprouts

2.1.1. Consumption of fresh produce, including sprouts

Consumption of fresh produce is associated with a healthy lifestyle (Callejón et al., 2015). The World Health Organization encourages a daily intake of at least 400 g of fresh produce (excluding potatoes and other starchy roots) for reducing the risk of chronic diseases and ensuring an adequate daily intake of dietary fiber (WHO, 2003). According to United States Department of Agriculture Economic Research Service, annual U.S. per capita consumption of fresh produce increased 12.8% from 1976 to 2009 (Cook, 2011).

Sprouts originate from the *Leguminosae* family. There are currently different varieties of sprouts existing in the market, including alfalfa, mung bean, radish, and fenugreek sprouts (Robertson, Johannessen, Gjerde, & Loncarevic, 2002). Sprouts are a healthy food, rich in proteins, carbohydrates, minerals, vitamins, and anti-cholesterol constituents (Gabriel et al., 2007). Consumption of sprouts may help protect against certain chronic diseases and cancers due to the high content of phytochemicals known as glucosinolates which function as antioxidants and aid in cancer prevention (Bellostas, Kachlicki, Sørensen, & Sørensen, 2007). In the United States, about 10% of the

populations consumes sprouts regularly, leading to a $250 million market. According to the International Sprout Growers Association, approximately 300,000 tons of sprouts were produced annually by about 475 U.S. sprouts growers (Sikin, Zoellner, & Rizvi, 2013). Among them, alfalfa sprouts are the most common sprout varieties consumed. Approximately 80 million pounds of alfalfa seeds are produced each year, with a small fraction used by the sprout industry (Mueller, 2008).

2.1.2. Foodborne outbreaks associated with fresh produce

As the increase of fresh produce consumption, a corresponding increase in the number of foodborne outbreaks associated with fresh produce was noticed since most fresh produce receives minimal preparation and is often consumed raw (Lynch, Tauxe, & Hedberg, 2009; Olaimat & Holley, 2012). In the United States during the period of 2004-2013, 19% of the foodborne outbreaks and 24% of the total number of foodborne illnesses reported to CDC were associated with fresh produce consumption, which made fresh produce the most common vehicle for transmitting foodborne illnesses (Fischer, Bourne, & Plunkett, 2015). Among reported fresh produce-associated outbreaks in the United States from 2004-2012, 13% (n = 377) implicated more than one State (Callejón et al., 2015) and the food item most frequently implicated was salad (124, [32.9%], followed by leafy greens (98 [25.9%]), tomatoes (24 [6.4%]), and sprouts (20 [5.3%]) (Callejón et al., 2015; Franz & Van Bruggen, 2008). The types of sprouts implicated included alfalfa, fenugreek, and mung bean. Lettuce was the leafy greens that were most commonly associated with outbreaks of infection.

A wide spectrum of microorganisms is involved in produce-associated outbreaks (Lynch, Tauxe, & Hedberg, 2009). Annually, more than 9 million foodborne illnesses are estimated to be caused by major pathogens in the U.S. (Scallan et al., 2011). Among all microorganisms, *Salmonella enterica* and *Escherichia coli* were the top two bacterial pathogens responsible for fresh produce related outbreaks in the U.S. (Callejón et al., 2015). *S. enterica* and *E. coli* are gram-negative, rod-shaped, and facultative anaerobic bacteria in the gastrointestinal tracts of mammals and belong to *Enterobacteriaceae* family.

Salmonella causes salmonellosis and patients with salmonellosis often develop symptoms like diarrhea, fever, and abdominal cramps 12 to 72 hours after exposure. These symptoms usually last 4 to 7 days (CDC, 2010). Infants, elderly, and immunocompromised people are more likely to develop severe complications.

All pathogenic *E. coli* present a significant health risk, and those belonging to enterohemorrhagic *Escherichia coli* (EHEC) group are of most concern (Beutin & Martin, 2012). EHEC can cause hemorrhagic colitis in humans, which occasionally progresses to hemolytic uremic syndrome, an important cause of acute renal failure in children and morbidity and mortality in adults (Page & Liles, 2013). The best known EHEC strain, is *E. coli* O157:H7, however, EHEC also includes strains in other serogroups such as O111, O145, O113, O103, O91, O26 and O104 (Berger et al., 2010; Grant et al., 2011).

Among outbreaks associated with fresh produce in the U.S. from 2004-2012, several food-pathogen combinations were reported, *Salmonella* was the microorganism involved in the majority of sprouts and tomato-associated outbreaks in the United States

from 2004-2012 while EHEC outbreaks were associated with the consumption of leafy greens, especially lettuce (Callejón et al., 2015).

2.1.3. Foodborne outbreaks associated with sprouts

Outbreaks associated with sprout consumption have occurred throughout history, and the first recorded case was in the 1970s. The most notable early outbreak that was linked to bean sprouts contaminated with *Salmonella* occurred in Sweden and England in 1988 (O'mahony et al., 1990). A critical point was reached in 1996 with an outbreak involving radish sprouts contaminated with *E. coli* O157:H7 in Japan, with at least 6,000 cases confirmed (Itoh et al., 1998).

In the past 10 years, there have been 32 reported outbreaks associated with sprouts consumption in the United States (CDC, 2017). However, the actual number of the foodborne outbreaks might be higher since some outbreaks are likely underreported (Sapers, 2014). Alfalfa sprouts were implicated in most of the outbreaks that occurred from 2007 to 2016, followed by a small number of outbreaks attributed to bean sprouts and clover sprouts (Table 1). The bacterial pathogens attributed to these outbreaks are *S. enterica, E. coli* O157:H7, and *E. coli* O104:H4. The latter was the etiological agent in one of the worst foodborne outbreaks associated with sprouts, which affected 3,842 people, with 855 developing hemolytic uremic syndrome and 53 deaths in Germany in 2011 (Beutin & Martin, 2012). Evolved from enteroaggregative *E. coli*, *E. coli* O104:H4 was previously thought to be of limited pathogenic potential in humans. However, it was found to have acquired new virulence factors (Beutin & Martin, 2012).

2.2. Vegetable seeds, especially sprout seeds as potential source of contamination

2.2.1. Potential sources of fresh produce contamination

Fresh produce can become contaminated at any point in the production chain: during growth, harvesting, processing, transporting, and final preparation. Pre-harvest contamination of fresh produce could occur directly or indirectly *via* soil, feces, dust, insects, wild or domestic animals, and human handling (Olaimat & Holley, 2012). Elements involved in introducing human pathogens into the fields and contributing to the contamination cycle in agricultural areas include bioaerosols, insects, untreated manure, animal feces, and contaminated irrigation water (Brandl, 2006); the most important of which is the application of improperly-composted manure as a fertilizer (Franz & Van Bruggen, 2008). Foodborne pathogens such as *E. coli* O157:H7 were able to survive in animal manure for about 50 d at 22 °C (Semenov, Van Bruggen, Van Overbeek, Termorshuizen, & Semenov, 2007). Pathogens could be transferred to fresh produce by application of inadequately composted or raw animal manures or sewage (Santamaria & Toranzos, 2003). In an *E. coli* O157:H7 outbreak of infection associated with bagged spinach, epidemiological studies were traced back to a ranch where isolates from feral swine and cattle feces matched the outbreak strains based on the pattern of pulsed-field gel electrophoresis and multilocus variable number tandem repeat analysis (Jay et al., 2007).

Contamination of irrigation water was another important source of fresh produce contamination in the field (Franz & Van Bruggen, 2008). Mitra et al. (2009) reported

that *E. coli* O157:H7 could uptake and colonize spinach leaves after contaminated irrigation water was dropped on the plant. It has been suggested that the quality of irrigation water and type of irrigation system influenced the microbial safety of fresh produce (Keith Warriner, Huber, Namvar, Fan, & Dunfield, 2009). Flood and overhead sprinkler irrigation posed a greater risk than surface irrigation since contaminated water can be directly deposited onto the edible parts of fresh produce. Solomon, Potenski, and Matthews (2002) reported that 90% of lettuce plants that had been sprinkler-irrigated with water inoculated with 7 log CFU/ml of *E. coli* O157:H7 tested positive for the pathogen, while only 19% were found to be *E. coli* O157:H7 positive when inoculated surface irrigation was used.

Post-harvest processes, including distribution, storage, and processing, are also possible mechanisms of contamination for fresh produce (Wachtel & Charkowski, 2002). The conditions of fresh produce distribution can facilitate or prevent microbial contamination depending on the environmental temperatures. Rosset, Cornu, Noël, Morelli, and Poumeyrol (2004) showed that foodborne illness events developed when an abusive temperature was used for food distribution. Processing of fresh produce, which may include human and mechanical contact, cutting or slicing, and immersion in water, not only have the potential to contaminate produce with pathogens, but also can enhance the proliferation of pathogens (Berger et al., 2010). The cutting and shredding of fresh produce is often the last processing step and usually there is no application of thermal intervention before consumption (Zilelidou, Tsourou, Poimenidou, Loukou, & Skandamis, 2015). Foodborne pathogens could be transferred between contaminated fresh produce and food contact surfaces (Buchholz, Davidson, Marks, Todd, & Ryser,

2012a, 2012b). In addition, cut surfaces of lettuce leaves are a specific target for pathogenic bacteria such as *Salmonella* which show a specific preference towards them (Yulia Kroupitski, Pinto, Brandl, Belausov, & Sela, 2009), and cutting melons may carry pathogens from the rind into the edible part of the fruit where bacteria may multiply under abusive storage condition (Ukuku & Sapers, 2007). The use of inadequately decontaminated water in hydrocoolers, which are used to store and process large quantities of fresh produce, can lead to contamination of an entire lot (Gagliardi, Millner, Lester, & Ingram, 2003).

2.2.2. Vegetable seeds as potential contaminated source

Vegetable seeds can be a potential source and efficient vector of human and plant pathogens. Unsanitized vegetable seeds could lead to the contamination of fresh produce, (Hanning, Ricke, & Nutt, 2009). Most harvested seeds are destined to be raw agricultural product and only a small proportion is used for sprouting. The decision for use is often made after the harvest. Thus, some seed growers may not follow good agricultural practices (Robertson, Johannessen, Gjerde, & Loncarevic, 2002). Seeds usually harbor native microbiota (2 - 6 log CFU/g) and fecal coliforms (2-3 log CFU/g) from the environment (Feng, 1997). Pathogen populations in the seeds are low and unevenly distributed, making them difficult to detect by routine seed testing; however, these pathogens can survive for months in stored seeds. Van der Linden et al. (2013) reported that *S. enterica* and *E. coli* O157:H7 could survive on butterhead lettuce seeds for more than two years under commercial storage conditions.

Contaminated sprout seeds have been recognized as the primary and most common source of sprout-associated illnesses. Starting with the *E. coli* O157:H7 outbreak linked to radish sprouts in Japan that caused more than 10,000 cases of infection in 1996, contaminated seeds led to the contamination of sprouts and ultimately human illness (Nathan, 1997). According to the U.S. Food and Drug Administration (NACMCF, 1999; Zilelidou, Tsourou, Poimenidou, Loukou, & Skandamis, 2015), most sprout outbreaks have been caused by seeds contaminated with bacterial pathogens before the sprouting process begins. Seed sanitation treatments have been shown to be ineffective in eliminating bacterial pathogens (Beuchat & Scouten, 2002; Scouten & Beuchat, 2002; Sikin, Zoellner, & Rizvi, 2013), especially those that are located in surface cavities (Beuchat, 1997) or internal vegetable seed tissues (Fu, Reineke, Chirtel, & VanPelt, 2008). Investigations on alfalfa sprouts-related outbreak in Michigan and Virginia in 1997 revealed that the common source of contamination was the same lot of alfalfa seeds contaminated with *E. coli* O157:H7 (Breuer et al., 2001). The *E. coli* O157:H7 outbreaks associated with alfalfa sprouts in Colorado and Minnesota in 2003 were also traced back to a common seed lot (Ferguson et al., 2005). A single lot of seeds from Egypt was identified as the most likely source of the *E. coli* O104:H4 outbreak of infection associated with fenugreek sprouts in Germany in 2011 (Muniesa, Hammerl, Hertwig, Appel, & Brüssow, 2012). More recently, collaborative investigation of 2015 alfalfa sprouts-related multistate outbreak of *S.* Muenchen and *S.* Kentucky indicated that alfalfa sprouts produced by multiple sprouting facilities from one lot of contaminated seeds were the likely source of this outbreak (CDC, 2016).

2.3. Seed production system and possible contamination routes

2.3.1. Seed production system

Historically, seeds for subsequent season's crop were collected as a by-product of production. Although some seeds may still be produced in this way, commercial seed production has become a specialized industry (TeKrony, 2006). Large amounts of vegetable seeds are produced in areas characterized by low humidity and limited rainfall during seed harvest to provide good seed yields (Watkins, 1992). Vegetable seeds are harvested and produced under different conditions depending on seed type. Some seeds are produced mechanically by passing through a harvester while others may involve multiple steps (Anonymous, 2010). For example, harvesting alfalfa seedpods is primarily a combination of an initial cutting and drying with passage through the harvester (Sapers, 2014). Tomato seeds are first separated from fruit pulp by juice extracting equipment and the resulting extract is fermented for 2 to 3 days. Following extraction, seeds are washed and dried under the sun or in dehydrators (Anonymous, 2010). However, postharvest processing of seeds is similar regardless of seed type. This involves an initial cleaning step by passing seeds through a series of sieves. The seeds are then sorted based on weight *via* a gravity table to remove damaged seeds and foreign objects such as stone and soil. The seeds are tested for germination percentage (normally > 90%) before packaging and distribution.

2.3.2. Possible routes of seed contamination

How seeds are contaminated by microorganisms has not been studied extensively, although the causes are likely to be the same as for fresh produce contamination (Sapers, 2014). There are three different mechanisms by which pathogens become associated with seeds: systemic seed infection, seed infestation, and concomitant contamination (Agarwal & Sinclair, 1996).

Systemic seed infection

Seed infection refers to the establishment of a pathogen within the seed, which results in contamination of internal tissues. Pathogen cells may gain entry to vegetable seeds through vascular system in flower, fruit, or seed stalk, or penetration of the ovary wall and natural openings (Agarwal & Sinclair, 1996). This mechanism is usually used by seedborne phytopathogens. For example, systemic invasion of seeds through fruit vasculature was observed with *Clavibacter michiganensis* subsp. *michiganensis* and *Pseudomonas syringae* in tomato (Dutta, Gitaitis, Smith, & Langston Jr, 2014; Tancos, Chalupowicz, Barash, Manulis-Sasson, & Smart, 2013). It is also possible for human pathogens such as *Salmonella* and EHEC to infect seeds in a similar manner, since they have the opportunity to contact with plants and flowers through the activity of insects, domestic animals, rainfall, and irrigation water (Franz & Van Bruggen, 2008). However, the incidence of systemic seed infection might be low since *Salmonella* and EHEC may encounter harsh physicochemical conditions in field, such as temperature and osmotic conditions and are not as well adapted as plant pathogens to utilize the full spectrum of

nutrients present in plants (Brandl, 2006). For example, unlike many plant-associated bacteria, *S. enterica* is typically unable to assimilate sucrose (Lin et al., 1996), one of the main sugars present in leaves (Lindow & Brandl, 2003) and root exudates (Brandl, 2006). In addition, while on plants, enteric pathogens have to compete with plant pathogens and other microflora, which make it harder to persist on plant tissues. For example, *Arabidopsis thaliana* plants grown from 26.7% of the contaminated seeds in a gnotobiotic system tested positive for *E. coli* O157:H7 and those from 12.5% of the seeds tested positive for *S.* Newport. In contrast, these two pathogens were not detected in the presence of a competitor, *Enterobacter asburiae* which was isolated from soil-grown *A. thaliana* (Cooley, Miller, & Mandrell, 2003).

Seed infestation (Surface attachment)

Seed infestation refers to pathogen attachment to the seed surface. Pathogens may adhere to the seed surface at any time during harvest, extraction, threshing, or processing. Bacterial attachment to the surfaces can be either active or passive depending on bacterial motility or transportation of the cells by gravity, diffusion, or fluid dynamic forces (Kumar & Anand, 1998). It begins with a weak, reversible phase, *via* van der Waals forces, electrostatic forces, and hydrophobic interactions (Vandermei, Devries, & Busscher, 1993; Vanloosdrecht, Lyklema, Norde, Schraa, & Zehnder, 1987). During this initial attachment, bacterial cells are in Brownian motion and could be easily removed by fluid shear forces, *e.g.* rinsing (Marshall, Stout, & Mitchell, 1971). Once appendages on the cell surface are anchored and extracellular polymers are produced, the cells irreversibly attach to the substratum. At this stage, contact occurs due to the bonding

between bacterial appendages (*e.g.* pili, fimbriae, flagella, and adhesion proteins) and the substratum by various short-range forces, including dipole-dipole interaction, hydrogen, ionic and covalent bonding (Hori & Matsumoto, 2010; Kumar & Anand, 1998). Irreversibly attached cells can only be removed by a stronger physical force, such as scraping, vortexing, or chemical cleaner (Palmer, Flint, & Brooks, 2007). Irreversibly attached bacterial cells can form biofilms on contact surfaces, and biofilm-embedded bacterial cells are slow growing and have better tolerance to nutrient deficiency, differences in gene expression, and enhanced resistance to environmental stresses, such as antimicrobial treatment (Simões, Simoes, & Vieira, 2010).

Currently, studies relevant to *S. enterica* and EHEC attachment to fresh produce mainly focus on various plant tissues, including alfalfa sprouts (Barak, Gorski, Naraghi-Arani, & Charkowski, 2005; Barak, Whitehand, & Charkowski, 2002), green pepper (Han, Sherman, Linton, Nielsen, & Nelson, 2000; Liao & Cooke, 2001), spinach leaves (Berger et al., 2009; D. Macarisin, Patel, & Sharma, 2014; Salazar et al., 2013), lettuce leaves (Berger et al., 2009; Fink et al., 2012; Y. Kroupitski, Pinto, Belausov, & Sela, 2011; Patel & Sharma, 2010), cantaloupes (Ukuku & Sapers, 2001), and tomato (Iturriaga, Escartin, Beuchat, & Martinez-Peniche, 2003; Shaw et al., 2011). Only a few studies have assessed the attachment ability of bacterial pathogens on vegetable seeds. In addition, most of the previous studies concerning bacterial pathogens and vegetable seeds used a high bacterial inoculation level (7-9 log CFU/ml) and focused on the efficacy of chemical treatments in reducing seedborne bacterial pathogen populations (Buchholz & Matthews, 2010). The physical mechanisms of pathogen attachment to vegetable seeds have not been adequately addressed.

Several intrinsic and extrinsic factors influence the attachment of a bacterial cell to its contact surface, including surface conditioning, mass transport, surface charge, hydrophobicity, surface roughness, and surface micro-topography (Hori & Matsumoto, 2010). Seed coat characteristics have a significant impact on the levels of contamination by artificially inoculated bacterial pathogens (Rajkowski, 2009). It was found that wrinkled or rough alfalfa seeds were likely to harbor more bacteria and were also more resistant to sanitizers compared to smooth seeds (Charkowski, Sarreal, & Mandrell, 2001). Cells of *L. monocytogenes* and *E. coli* O157:H7 were more likely to attach to cut edges of cabbage and lettuce (Ells & Hansen, 2006; Dumitru Macarisin, Patel, Bauchan, Giron, & Sharma, 2012; Takeuchi, Matute, Hassan, & Frank, 2000).

Concomitant contamination

Concomitant contamination of seeds refers to the mixture of inoculum with seeds in the form of plant pathogen propagules, infected plant debris, such as infested straw or pieces of chaff, or contaminated soil (Agarwal & Sinclair, 1996). Concomitant contamination by infested soil particles has been observed with some plant pathogens such as *Verticillium alboatrum* in alfalfa seeds (Sheppard & Needham, 1980), *Fusarium oxysporum* f. sp. *lycopersici* in tomato seeds (Agarwal & Sinclair, 1996), and *Fusarium solani* f. sp. *phaseoli* in bean seeds (Nash & Snyder, 1964).

Soil is a natural environment for variety of human pathogens including *B. cereus*, *C. perfringens*, and *L. monocytogenes,* and the incidence of enteric pathogens, such as *Salmonella* and EHEC, significantly increases when untreated manure and animal wastes are applied to soil (Olaimat & Holley, 2012). Bacterial pathogens such as *Salmonella*

and EHEC persisted in soils up to several months (Barak & Liang, 2008; Ongeng, Muyanja, Geeraerd, Springael, & Ryckeboer, 2011; Semenov, Van Overbeek, & Van Bruggen, 2009). Vegetable seeds could come into contact with contaminated soil naturally at various stages during seed production. For example, alfalfa seedpods are close to the ground during harvesting, leading to an inevitable pick up of soil by alfalfa seeds (Sapers, 2014).

2.4. Growth and colonization of *Salmonella* and EHEC during sprout seed germination

Attachment of bacterial pathogens to vegetable seeds is the first step of colonization. The fate of pathogen cells after the initial attachment step also has a significant impact on fresh produce safety, especially sprout safety. Sprouting conditions, in terms of temperature and humidity, support the growth of human pathogens, which is a significant safety concern. Sprouting starts with soaking the seeds in water for 3 to 16 h to stimulate the germination process, followed by incubation at 18 to 28 °C for 3 to 6 days, with frequent irrigation (every 3-5 h), depending on the seed type (Sapers, 2014). During germination, the uptake of water by dry seeds is triphasic, with a rapid initial uptake (imbibition, phase I) followed by a plateau phase (phase II). A further resumption of water uptake and elongation of the embryo axis occurs, which is characterized as phage III (Schiltz et al., 2015). The influx of water into the cells of dry seeds during phase I results in rapid swelling and shape changes in seeds, leading to

temporary structural disruptions, particularly to cell membranes (Robert, Noriega, Tocino, & Cervantes, 2008). The water uptake of quiescent dry seeds also resumes metabolic activities, including respiratory activity and synthesis of proteins and mRNA (Bewley, 1997). During germination, nutrients stored in seeds are degraded by endogenous enzymes to several low-molecular-weight metabolites, a portion of which is released to the surroundings as seed or root exudates (Schiltz et al., 2015). These exudates may include large amounts of reducing sugars, amino acids, fatty acids, and other nutrients that could support the proliferation of human pathogens (Kylen & McCready, 1975). Indeed, it has been reported that levels of *Salmonella* and *E. coli* O157:H7 reached to 4 log CFU/g during the first 24 h of the sprouting process even when the pathogen contamination level on seeds was as low as 0.1 CFU/g (Stewart, Reineke, Ulaszek, & Tortorello, 2001). Human pathogens, such as *Salmonella* and *E. coli* O157:H7, can multiply rapidly during the germination of different vegetable seeds including alfalfa (Charkowski, Barak, Sarreal, & Mandrell, 2002; Gómez-Aldapa et al., 2013; Howard & Hutcheson, 2003; Liu & Schaffner, 2007), lettuce (Van der Linden et al., 2013), mung bean (Bari, Enomoto, Nei, & Kawamoto, 2010; Gómez-Aldapa et al., 2013; Warriner, Spaniolas, Dickinson, Wright, & Waites, 2003), and radish (Fransisca & Feng, 2012). The survival of bacterial pathogens on sprouts during storage has also been reported. Castro-Rosas and Escartin (2000) investigated the survival of *Salmonella* on alfalfa sprouts at 4 °C and found that only 0.3 log CFU/g reduction was achieved during a 15-day storage period. Similar findings were reported on the survival of *Salmonella* and *E. coli* O157:H7 on bean sprouts (Francis & O'beirne, 2001; Tian et al., 2012). However,

the population of *S*. Typhimurium was reported to grow 3.4 log CFU/g when mung bean sprouts were stored at 15 ºC for 7 days (Tian et al., 2012).

Salmonella and *E. coli* O157:H7 can not only attach to the surface of sprouts but also become established within sprout's vascular systems (Hirneisen, Sharma, & Kniel, 2012; K. Warriner, Spaniolas, Dickinson, Wright, & Waites, 2003). Once pathogens are internalized within the inner tissues of sprouts, simple washing is ineffective in inactivating them.

Many studies have been conducted in attempts to understand the growth of *S. enterica* and EHEC during seed germination, with most of them focusing on the germination of sprout seeds (Charkowski, Barak, Sarreal, & Mandrell, 2002). Besides sprouts, germination of other contaminated vegetable seeds might lead to contaminated seedlings, and eventually contaminated mature plants. Germination of *Salmonella* and EHEC contaminated lettuce seeds resulted in the contamination of seedlings and mature lettuce (Van der Linden et al., 2013). Deering, Jack, Pruitt, and Mauer (2015) also found that growing tomato seeds contaminated with *E. coli* O157:H7 led to the contamination of mature tomato plants and tomato fruits.

2.5. Molecular mechanisms involved in bacterial attachment and biofilm formation

Few genetic elements have been identified as essential for bacterial attachment to plant tissues (Yaron & Römling, 2014). Cell surface components, such as flagella, fimbriae, curli, and cellulose are important in the attachment of pathogens to fresh

produce (Barak & Schroeder, 2012). Flagella are long, thin surface appendages that help bacteria cells move along plant surfaces to a favorable attachment site (Wiedemann, Virlogeux-Payant, Chaussé, Schikora, & Velge, 2015). Flagella could potentially perform four roles, including enabling bacterial cells 1) to swim towards nutrients on a surface; 2) to initially reach a surface; 3) to spread along a surface; and 4) to adhere to a surface (Pratt & Kolter, 1998). It is reported that mutants of *fli*C (unable to synthesize flagellin) and *flh*D (inability to synthesize flagella) in *E. coli* were defective in the initial stages of biofilm formation. In addition, *E. coli* cells that possess paralyzed flagella (*ΔmotA, ΔmotB or ΔmotAB*) were found to be severely hindered in biofilm formation (Pratt & Kolter, 1998). Tan, White, Rahman, and Dykes (2016) reported that a *ΔfliC fljB* mutant of *S.* Typhimurium, which lacked expression of phase 1 and 2 flagellins, had a significantly lower level of attachment to plant cell walls compared to the wild type strain. Deletion of the flagella subunit encoding gene *fli*C in *E. coli* and *S.* Senftenberg significantly reduced their attachment levels to lettuce (Shaw, Berger, Pallen, Sjöling, & Frankel, 2011) and leaf epidermis of basil (Berger et al., 2009), respectively.

Strains of *E. coli* and *S. enterica* produce a diversity of pili and fimbriae that function as adhesion systems. Fimbriae are fine, hair-like protein appendages that carry adhesins on their tips with affinity to substratum (Yaron & Römling, 2014). Curli are a type of fimbriae that are composed of proteins called curlins (Epstein, Reizian, & Chapman, 2009). Curli usually have a role in attachment and it was found that the deletion of *csg*B and *csg*A (curli subunit) resulted in eight-fold and four-fold reductions in the attachment of *S.* Newport (Barak, Gorski, Naraghi-Arani, & Charkowski, 2005)

and *E. coli* O157:H7 (Torres, Jeter, Langley, & Matthysse, 2005) to alfalfa sprouts, respectively.

Cellulose, consisting of β (1–4)-linked D-glucose units secreted by bacterial cells, can interfere with flagellar rotation and limit bacterial motility (Zorraquino et al., 2013). Deletion of cellulose biosynthesis genes *bcs*A and *bcs*C in *S. enterica* decreased the level of attachment to alfalfa sprouts (Barak, Jahn, Gibson, & Charkowski, 2007) and tomato fruits (Shaw et al., 2011), respectively. A mutant of the cellulose production encoding gene *yhj*N, reduced the ability of *E. coli* O157:H7 to attach to alfalfa sprouts by 1.8 logs (Matthysse, Deora, Mishra, & Torres, 2008). In addition, the synergetic effects between thin aggregative fimbriae and cellulose played a crucial role in biofilm formation. Production of both fimbriae and cellulose is regulated by the CsgD, which is encoded by the *csg*DEFG operon in *E. coli* and *agf*D is the *Salmonella* homolog of *csg*D (Chirwa & Herrington, 2003). CsgD serves as the major hub of biofilm formation (Simm, Ahmad, Rhen, Le Guyon, & Römling, 2014). The extracellular matrix components, cellulose, curli fimbriae, and a biofilm-associated protein are positively regulated by CsgD. In addition, CsgD also regulates the O-antigen capsule encoding gene *yih* (Gibson et al., 2006). A mutant of *csg*D enhanced *E. coli* O157:H7 attachment to alfalfa sprouts (Torres, Jeter, Langley, & Matthysse, 2005).

Additionally, other genes that are involved in the production of extracellular carbohydrates may also have a significant effect on bacterial attachment to plant surfaces, and these include the poly-β-1,6-N-acetylglucosamine encoding gene *pga*C (Matthysse, Deora, Mishra, & Torres, 2008), colanic acid encoding gene *wca*D (Matthysse, Deora, Mishra, & Torres, 2008), and membrane protein encoding gene *omp*A in *E. coli* O157:H7

(Torres, Jeter, Langley, & Matthysse, 2005), and the putative membrane protein encoding gene *ycf*R and *yig*G in *S. enterica* (Salazar et al., 2013). *S. enterica* mutants lacking *yihO* which encodes the regulators for O-antigen capsule assembly and transport, have been shown to have reduced attachment to various plant surfaces (Barak, Jahn, Gibson, & Charkowski, 2007). Transposon insertion at *rpoS*, the gene for the global stress regulator, caused the *Salmonella* cells to attach poorly to alfalfa sprouts (Barak, Gorski, Naraghi-Arani, & Charkowski, 2005).

2.6. Control of bacterial pathogens on seeds and sprouts

2.6.1. Current recommendation and practice on sanitation of seeds and sprouts

Contaminated sprout seeds have been recognized as the primary source of pathogens for sprout-related outbreaks. The bacterial levels could increase from 2 log CFU/g to as high as 8 - 11 log CFU/g during sprouting (Gandhi & Matthews, 2003; Penas, Gomez, Frías, & Vidal-Valverde, 2008). Thus, seed treatment has been generally accepted as the most effective approach to improve the microbial safety of sprouts (NACMCF, 1999).

In the United States, the current industry seed disinfection follows the recommendation by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) (FDA, 1999; NACMCF, 1999). The use of 20,000 ppm calcium hypochlorite or a combination of treatments that would achieve a 5-log reduction in the microbial population is recommended for the treatment of seeds intended for sprout

production and interventions that lead to less than a 5-log reduction should be combined with microbiological testing of sprouts or irrigation water. However, the reduction of microbial populations by 20,000 ppm calcium hypochlorite treatment on sprout seeds varied from 1.0 - 6.5 log CFU/g, with an average of 2.8 log CFU/g reduction in EHEC and 3.2 log CFU/g reduction in *Salmonella* (Montville & Schaffner, 2004). Efficacy varies depending on several factors such as temperature (Beuchat & Scouten, 2002; Peñas, Gómez, Frías, & Vidal-Valverde, 2010; Scouten & Beuchat, 2002), duration of soaking (Taormina & Beuchat, 1999), and the type of seed (Fransisca & Feng, 2012). For example, alfalfa seeds have a uniform and relatively smooth surface with shallow valleys, whereas broccoli and radish seeds have more irregular surfaces with deep grooves, making it more difficult to inactivate resident pathogens (Fransisca & Feng, 2012). A successful seed treatment must inactivate microbial pathogens while preserving seed viability, germination rate, and vigor. The application of 20,000 ppm of calcium hypochlorite for 20 min could generally allow the seeds to achieve > 90% germination (Buchholz & Matthews, 2010; Fransisca, Park, & Feng, 2012).

The prevention or intervention steps might not only be taken on sprout seeds before sprouting, but also be applied during sprouting (*e.g.* to processing water or immature sprouts), and the finished product. The recommended concentration of chlorine for washing fresh produce is 50 - 200 ppm (Sapers, 2005). However, 200 ppm of chlorine was ineffective against bacterial pathogens on fresh produce due to the rapid depletion of free chlorine (Aruscavage, Lee, Miller, & LeJeune, 2006). Although the FDA encourages consumers to fully cook sprouts to eliminate bacterial pathogens (FDA, 2014; NACMCF, 1999), consumers consume sprouts raw or after minimal cooking to

preserve their nutritive value. Thus, the recommended chlorine-based interventions during sprout production have major limitations, and many studies were focused on the development of alternative treatments for sprout seeds and sprouts. These treatments may include physical, biological, and chemical treatments or combinations of these.

2.6.2. Physical intervention

Physical methods, especially thermal and high pressure processing, have been applied for microbial inactivation, such as juice processing (Ding, Fu, & Smith, 2013). Physical treatments may reach bacteria sheltered in scarified seed surfaces. In addition, physical methods are more environmentally friendly. Studies have been conducted to determine the efficacy of physical methods such as heat, high pressure, UV light, and irradiation to disinfect seeds and sprouts (M. Bari, K. Enomoto, D. Nei, & S. Kawamoto, 2010; G. Feng, Churey, & Worobo, 2007; Kim, Feng, Kushad, & Fan, 2006; Neetoo & Chen, 2010).

Treatment of alfalfa seeds in 58 °C hot water for 6 min led to a 5-log reduction in *Salmonella* and *E. coli* O157:H7 with no adverse effect on the germination ratio of alfalfa seeds (97%) (Weiss & Hammes, 2005). However, when it comes to commercial production, higher temperature is often needed due to the increased recovery of injured bacterial pathogen cells (Bari, Enomoto, Nei, & Kawamoto, 2010). This may have adverse effects on the textural and nutritional qualities of the sprouts, due to the sensitivity of plant tissues to heat (Fratamico & Bagi, 2001).

Irradiation treatment has been studied on a wide variety of seeds, and D_{10} -values for various pathogens have been calculated (Rajkowski, Boyd, & Thayer, 2003; Waje et al., 2009). Radiation dosage, which is a function of the energy of the radiation source and exposure time, was usually expressed in kilograys (kGy). The FDA has approved irradiation of sprout seeds at doses up to 8 kGy (FDA, 2008). Kim, Feng, Kushad, and Fan (2006) reported that an 8-kGy dose treatment of gamma radiation on alfalfa and broccoli seeds reduced the *E. coli* O157:H7 population by 5.03 log and 4.85 log CFU/g. However, the capital cost to build a commercial cobalt-60 food irradiation plant is $ 3-5 million (Cunningham, 2009). The commercial sprouting operations in the United States are usually small, with less than 10 employees (Thomas, Palumbo, Farrar, Farver, & Cliver, 2003), and the use of irradiation is not economically feasible. High pressure processing has been effective in the elimination of bacterial pathogens in vegetable seeds. Treatment of alfalfa seeds at 500 MPa at 45 °C for 2 min reduced the population of *Salmonella* and *E. coli* O157:H7 by 5.8 and 5.2 log CFU/g, respectively (Neetoo & Chen, 2010). However, low seed viability and delayed germination limited the application of high pressure for seed decontamination (Wuytack, Diels, Meersseman, & Michiels, 2003). In addition, the use of high pressure treatment may face some difficulty such as the control of high operating pressures, high equipment cost, and safety.

2.6.3. Biological intervention

Biological interventions provide another strategy for reducing microbial load on sprout seeds. It is generally achieved by the use of organisms such as bacteriophages

(Kocharunchitt, Ross, & McNeil, 2009; Ye, Kostrzynska, Dunfield, & Warriner, 2010) and protective bacteria, such as *Pseudomonas fluorescens* (Fett, 2006; Liao, 2008; Matos & Garland, 2005). These organisms may produce antimicrobial metabolites such as bacteriocins, organic acids, and enzymes that negatively affect the viability of pathogens (Hudson, Billington, & McIntyre, 2009). However, due to the complexity of their application, uncertainty about their efficacy on an industrial scale, and potential adverse health effects, whether these strategies will become a practical option in sprout production remains to be determined.

2.6.4. Chemical intervention

Chemical intervention is a cost-effective approach to disinfect seeds and sprouts. Other than treatment efficacy, another concern of chlorine-based disinfectant is the potential formation of carcinogenic disinfection by-products such as trihalomethanes and haloacetic acids (Lopez-Galvez et al., 2010; Van Haute, Sampers, Jacxsens, & Uyttendaele, 2015). The potential formation of these toxic disinfection by-products as well as potential future regulatory restrictions has motivated researchers to test alternative chemical agents such as hydrogen peroxide, ethanol, lactic acid, peroxyacetic acid, and fatty acids. However, the efficacies vary. Table 2.2 provides a summary of the efficacy of these chemical treatments on seed and sprout microbial safety.

Synthetic chemical disinfectants are hardly associated with the healthy image associated with fresh produce. This attitude is reflected in the ever-increasing consumer preference for the elimination of synthesized additives and the enhanced use of natural

preservatives in foods (Ding, Fu, & Smith, 2013). Thus, finding an alternative natural chemical, such as plant extract, to inactivate pathogen cells on sprouts seed might be beneficial.

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Table 2.1.
Summary of outbreaks associated with sprouts from 2007-2016 in the United States.

Etiology	Year	No. of outbreaks	No. of illnesses	Source
Salmonella enterica, *E. coli* O157	2016	5	104	Alfalfa sprouts, mung bean sprouts
Salmonella Nuenchen; Cubana; Kentuchy	2015	1	32 (1 death)	Alfalfa sprouts
Salmonella Enteritidis, EHEC, *L. monocytogens*	2014	4	141 (2 deaths)	Clover sprouts, mung bean sprouts
Salmonella Enteritidis	2013	1	3	Alfalfa sprouts
Salmonella Cubana, *E. coli* O145	2012	2	24 (1 death)	Sprouts
Salmonella Muenchen, Enteritidis, Agona; *E. coli* O26	2011	4	70	Alfalfa sprouts, clover sprouts
E. coli O104:H4	2011	1	3842 (53 deaths)	Fenugreek sprouts (Germany)
Salmonella Newport, Cuban	2010	5	200	Alfalfa sprouts, clover sprouts
Salmonella Saintpaul, Oranienburg, Cubana, Typhimurium	2009	4	297	Alfalfa sprouts
Salmonella Typhimurium, *E. coli* O157; *L. monocytogens*	2008	3	65	Alfalfa sprouts
Salmonella Montevideo, Mbandaka	2007	3	59	Alfalfa sprouts, bean sprouts

[a]Data compiled from CDC website on foodborne outbreak surveillance (https://wwwn.cdc.gov/foodborneoutbreaks/)

Table 2.2.

Efficacy of chemical intervention strategies for disinfection of seeds and sprouts

Chemical name	Disinfection condition	Bacterial reduction (log CFU/g); germination ratio	Target microorganism	Seed/Sprout	Reference
Chlorine based chemical					
Sodium hypochlorite (NaOCl)	NaOCl, 200 ppm, 8h, 30°C	1.0; -	Total aerobic count	Mung bean seeds	(Vandan Nagar, L. P. Godambe, & Shashidhar, 2016)
	NaOCl, 200 ppm, 1h, 30°C	2.5; NA	Total aerobic count	Mung bean sprouts	
	NaOCl, 170 ppm, 180 s	2.0; NA 1.5; NA	*E. coli* O157:H7, *Salmonella*	Mung bean sprouts	(Neo et al., 2013)
	NaOCl, 20000 ppm,	1.6; -	*E. coli* O157:H7	Radish seeds	(L. Fransisca, Zhou, Park, & Feng, 2011)
	NaOCl, 2000 ppm, 30 s	3.9; 91%	*Salmonella*	Alfalfa seeds	(Beuchat, 1997)
	NaOCl, 2000 ppm, 10 min	1.5; 95%	*E. coli* O157:H7	Alfalfa seeds	(Taormina & Beuchat, 1999)
Calcium hypochlorite $Ca(OCl)_2$	$Ca(OCl)_2$, 20000 ppm, 20 min	3.4; 92% 1.4; 95% 2.2; 96%	*E. coli* O157:H7	Alfalfa seeds Broccoli seeds Radish seeds	(Fransisca, Park, & Feng, 2012)
	$Ca(OCl)_2$, 20000 ppm, 15 min	1.1; 90%	*Salmonella*	Alfalfa seeds	(Buchholz & Matthews, 2010)
	$Ca(OCl)_2$, 20000 ppm, 20 min	2.7; - 2.5; -	*Salmonella* *E. coli* O157:H7	Mung bean seeds	(Bari, Enomoto, Nei, & Kawamoto, 2010)
	20000 ppm $Ca(OCl)_2$, 20 min + 2000 ppm Chlorine, 2 h	3.2; - 2.9; -	*Salmonella* *E. coli* O157:H7	Mung bean seeds	
	200 ppm $Ca(OCl)_2$, 5 min + Drying, 24 h	1.5; 96%	*E. coli* O157:H7	Radish seeds	(H. Kim, Kim, Bang, Beuchat, & Ryu, 2010)
	$Ca(OCl)_2$, 20000 ppm, 45 min	2.8; 74%	*Salmonella*	Alfalfa seeds	(Liao, 2009)
	$Ca(OCl)_2$, 20000 ppm, 15 min	6.9; 90%	*E. coli* O157:H7	Alfalfa seeds	(Lang, Ingham, & Ingham, 2000)
	$Ca(OCl)_2$, 20000 ppm, 10 min	2.6; 70%	*E. coli* O157:H7	Alfalfa seeds	(Taormina & Beuchat, 1999)
	$Ca(OCl)_2$, 1800 ppm, 30 s	3.9; 91%	*Salmonella*	Alfalfa seeds	(Beuchat, 1997)
Chlorine dioxide (ClO_2)	500 ppm ClO_2, 5 min + 45 °C air drying, 24 h + 70 °C dry heat, 48 h	5.9; 84.3%	*E. coli* O157:H7	Radish seeds	(Bang, Kim, Kim, Beuchat, & Ryu, 2011)

	ClO_2, 100 ppm, 5 min	3.0; NA 1.5; NA	*S.* Typhimurium *L. monocytogenes*	Mung bean sprouts	(Jin & Lee, 2007)
	200 ppm ClO_2 (5 min) + Drying (24 h)	3.8; 94%	*E. coli* O157:H7	Radish seeds	(H. Kim, Kim, Bang, Beuchat, & Ryu, 2010)
	50 ppm ClO_2 (10 min) + 0.5% fumaric acid	3.2; NA 4.1; NA 3.6; NA 3.7; NA	Total aerobic bacteria *E. coli* O157:H7 *S.* Typhimurium *L. monocytogenes*	Alfalfa sprouts	(Y. Kim, Kim, & Song, 2009)
	ClO_2 3 ppm, 5 min	1.5; NA 1.5; NA	*E. coli* *S.* Enteritidis	Alfalfa sprouts	(Millan-Sango, Sammut, Van Impe, & Valdramidis, 2017)
	ClO_2, 25.0 mg/L, 5 min	0.96; -	*E. coli* O157:H7	Alfalfa seeds	(Singh, Singh, & Bhunia, 2003)
Acidified sodium chlorite (ASC)	200 ppm ASC, 45 min	3.8; 91%	*Salmonella*	Alfalfa seeds	(Liao, 2009)
	1200 ppm ASC, 2h	2.7; 98%	*E. coli* O157:H7	Mung bean seeds	(Nei et al., 2010)
	1200 ppm ASC, 180 s	2.0; NA 2.0; NA 2.0; NA	*E. coli* O157:H7 *Salmonella* *L. monocytogenes*	Mung bean sprouts	(Phua, Neo, Khoo, & Yuk, 2014)
Organic acids					
Acetic acid (AA)	5% AA, 10 min, 42 °C	6.3; -	*E. coli* O157:H7	Alfalfa seeds	(Lang, Ingham, & Ingham, 2000)
	8.7 % gaseous AA, 2 h, 55 °C	5.0; 97%	*E. coli* O157:H7	Alfalfa seeds	(Nei, Latiful, Enomoto, Inatsu, & Kawamoto, 2011)
	8.7 % gaseous AA, 3 h, 55 °C	5.0; 98%	*Salmonella*		
	2% AA, 24 h, 24 °C 5% AA, 4 h, 24 °C	7.0-8.0; NA	*Salmonella*	Alfalfa sprouts	(Pao, Kalantari, & Khalid, 2008)
Lactic acid (LA)	5 % LA, 10 min, 42 °C	4.1; 93%	*E. coli* O157:H7	Alfalfa seeds	(Lang, Ingham, & Ingham, 2000)
	5% LA, 10 min, 42 °C + 2000 ppm chlorine, 15 min, 25 °C	6.1; -	*E. coli* O157:H7		
	2% LA, 10 min	3.0; NA 2.0; NA	*S.* Typhimurium *L. monocytogenes*	Mung bean sprouts	(Lee, Yun, Fellman, & Kang, 2002)
Malic acid (MA)	10% MA + 1% thiamine dilauryl sulfate (TDS), pH 1.5, 20 min	4.4; 89%	*E. coli* O157:H7	Alfalfa seeds	(Fransisca, Park, & Feng, 2012)

	2% MA, 5 min	< 3; NA	*Shigella* spp.	Radish and mung bean sprouts	(Singla, Ganguli, & Ghosh, 2011)
Peroxyacetic acid (PAA)	1% PAA, 15 min	1.8; 91%	*S.* Stanley	Alfalfa seeds	(Buchholz & Matthews, 2010)
	3% PAA, 15 min	1.3; 88%	*S.* Stanley		
Ozone, ozonated water (OW)	2 ppm OW, 180 s	< 2; NA	*E. coli* O157:H7, *L. monocytogenes*,	Mung bean sprouts	(Phua, Neo, Khoo, & Yuk, 2014)
	2 ppm OW, 5 min	<3; NA	*Shigella* spp.	Radish and mung bean sprouts	(Singla, Ganguli, & Ghosh, 2011)
	2 ppm OW +2% MA, 5 min	4.4; NA	*Shigella* spp.	Radish sprouts	
	2 ppm OW +2% MA, 5 min	3; NA	*Shigella* spp.	Mung bean sprouts	
	Ozone gas, 24 h	1.5; -	*Salmonella*	Alfalfa seed	(Rajkowski & Ashurst, 2009)
	Ozone gas, 24 h + 1% PAA, 20 min	3; -	*Salmonella*	Alfalfa seed	
	Ozonated water, 14.3 mg/L, 3 min	0.6; -	*E. coli* O157:H7	Alfalfa seeds	(Singh, Singh, & Bhunia, 2003)
Natural extract	Roselle calyx aqueous extract, 24 h	6.5; NA > 6.8; NA	*L. monocytogenes* *E. coli* O157:H7	Alfalfa sprouts	(Jaroni & Ravishankar, 2012)
	Roselle leaf aqueous extract, 24 h	5.0; NA > 6.7; NA > 2.1; NA	*L. monocytogenes* *E. coli* O157:H7 *Salmonella*	Alfalfa sprouts	
	Carvacrol, 1500 ppm, 250 MPa	> 5.0; -	Total aerobic bacteria	Mung bean seeds	(Peñas, Gómez, Frías, & Vidal-Valverde, 2010)
	Thyme oil, 5.0 mL/L, 3 min	2.1; -	*E. coli* O157:H7	Alfalfa seeds	(Singh, Singh, & Bhunia, 2003)

CHAPTER 3

DIFFERENTIAL ATTACHMENT OF *SALMONELLA ENTERICA* AND ENTEROHEMORRHAGIC *ESCHERICHIA COLI* TO ALFALFA, FENUGREEK, LETTUCE, AND TOMATO SEEDS

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ABSTRACT

Vegetable seeds have the potential to disseminate and transmit foodborne bacterial pathogens. This study was undertaken to assess the abilities of selected *Salmonella* and enterohemorrhagic *Escherichia coli* (EHEC) strains to attach to fungicide-treated *vs*. untreated, and intact *vs*. mechanically-damaged seeds of alfalfa, fenugreek, lettuce, and tomato. Surface-sanitized seeds (2 g) were exposed to 4 individual strains of *Salmonella* or EHEC at 20^{o}C for 5 h. Contaminated seeds were rinsed twice, each with 10 ml of sterilized water before being soaked overnight in 5 ml of phosphate buffered saline at 4 ^{o}C. Seeds were then vortexed vigorously for 1 min, and pathogen populations in seed rinse water and soaking buffer were determined using standard plate count assay. In general, *Salmonella* cells had higher irreversible attachment ratios than the EHEC cells. Unit weight of lettuce seeds had the highest numbers of attached *Salmonella* or EHEC cells, followed by tomato, alfalfa, and fenugreek seeds. In contrast, individual fenugreek seeds had more attached pathogen cells followed by lettuce, alfalfa, and tomato seeds. Significantly more *Salmonella* and EHEC cells attached to mechanically-damaged seeds than to intact seeds ($P < 0.05$). Although on average, significantly more *Salmonella* and EHEC cells were recovered from untreated, than fungicide-treated seeds ($P < 0.05$), fungicide treatment did not significantly affect the attachment of individual bacterial strains to vegetable seeds ($P > 0.05$), with a few exceptions. This study fills gaps in the current body of literature and helps explain bacterial interactions with vegetable seeds with varying surface characteristics.

3.1. Introduction

Globally, fresh produce consumption has increased significantly in the last few decades because of the accrued health benefits (Esposito & Giugliano, 2011). Since most fresh produce receives minimal processing and is often eaten raw, it can be a vehicle for transmitting foodborne pathogens (Scharff, 2012). It is reported that in the United States, fresh produce was the most common vehicle for transmitting foodborne illness and 19% of the foodborne outbreaks and 24% of the illnesses that took place from 2004 to 2013 were associated with fresh produce (Fischer, Bourne, & Plunkett, 2015). In general, enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella enterica* are the major bacterial causes of foodborne illnesses (Warriner, Huber, Namvar, Fan, & Dunfield, 2009). Fresh produce that has been linked to the outbreaks of *Salmonella* and EHEC infections include lettuce, tomato, as well as alfalfa and fenugreek sprouts (Beutin & Martin, 2012; CDC, 2007; Cummings et al., 2001; Slayton et al., 2013).

Fresh produce contamination by pathogenic bacteria such as *Salmonella* and EHEC could occur at pre- or post-harvest stages (Olaimat & Holley, 2012). Vegetable seeds can be a potential source and efficient vector of human and plant pathogens. Unsanitized vegetable seeds could lead to the contamination of fresh produce, especially sprouts (Hanning, Ricke, & Nutt, 2009). According to the U.S. Food and Drug Administration (1999), most sprout outbreaks have been caused by seeds contaminated with bacterial pathogens before the sprouting process begins. Many pathogens can survive for months under the dry conditions used for seed storage. However, pathogen populations in the seeds are low and unevenly distributed, making them difficult to detect

by routine seed testing. Furthermore, seed sanitation treatments have been shown to be ineffective in eliminating bacterial pathogens, especially those that are located in surface cavities (Beuchat, 1997) or internal vegetable seed tissues (Fu, Reineke, Chirtel, & VanPelt, 2008).

Several studies have investigated the behavior of *Salmonella* and EHEC on various vegetable tissues, such as stems and leaves (Barak, Whitehand, & Charkowski, 2002; Takeuchi, Matute, Hassan, & Frank, 2000). However, only a few have assessed the attachment ability of bacterial pathogens on vegetable seeds (Charkowski, Sarreal, & Mandrell, 2001; Fransisca & Feng, 2012; Van der Linden et al., 2013), particularly lettuce, tomato, and fenugreek seeds. Furthermore, most of the earlier studies concerning bacterial pathogens and vegetable seeds have focused on the efficacy of chemical treatments in reducing seedborne bacterial pathogen populations (Buchholz & Matthews, 2010). Physical mechanisms of pathogen attachment to vegetable seeds have not been adequately addressed.

Numerous factors could affect bacterial interaction with seed surfaces. Seed coat characteristics have a significant impact on the levels of contamination by artificially inoculated bacterial pathogens (Rajkowski, 2009). Barak et al. (2002) observed fundamental differences between *Salmonella* and *E. coli* O157:H7 in the manner and degree of attachment to alfalfa sprouts. However, this differential attachment behavior has not been reported for alfalfa seeds nor other types of vegetable or sprout seeds. This study was undertaken to investigate differences in the abilities of two human enteric pathogens (*Salmonella enterica* and EHEC) to attach to seeds of four different types of

vegetables (alfalfa, fenugreek, tomato, and lettuce) and with different surface characteristics (fungicide-treated *vs*. untreated and intact *vs*. mechanically damaged).

3.2. Materials and methods

3.2.1. *Bacterial strains, growth media and vegetable seeds*

Four *S. enterica*, three *E. coli* O157:H7 and one *E. coli* O104:H4 strains were used in this study (Table 3.1). The bacterial strains were stored at -70 ºC and recovered on tryptic soy agar (TSA) at 37 ºC for 16 h. The resulting cultures were purified on bismuth sulfite agar (BSA; Becton Dickenson, Sparks, MD), sorbitol MacConkey (SMAC; Becton Dickenson, Sparks, MD) agar and MacConkey (MAC; Becton Dickenson, Sparks, MD) agar, respectively. Spontaneous mutant cells resistant to 50 µg/ml nalidixic acid (NA; MP Biomedicals, Santa Ana, CA) were selected and used in the experiments.

Thiram (dimethylcarbamothioylsulfanyl N,N-dimethylcarbamodithioate)-treated and untreated fenugreek (*Trigonella foenum-graecum*, cv. unidentified), lettuce (*Lactuca sativa* cv. Iceburg), and tomato (*Solanum lycopersicum* cv. Roma) seeds, as well as untreated alfalfa (*Medicago sativa*, cv. unidentified) seeds were obtained from a commercial source (Otis S. Twilley Seed Co. Inc., Hodges, SC) and stored at 10 ºC within a month before use. Commercial alfalfa seeds were treated with thiram 75 WP powder (Chemtura, Pekin, IL) in-house at a rate of 1.8 g per 500 g of seeds following instructions provided by Norac Concepts Inc (2008). In order to mechanically damage

seeds, 50 g of each seed type were blended in a 14-speed blender (Oster®, Milwaukee, WI) for 30 s, and seed debris was removed using a sterilized, fine mesh sieve (Walmart, Bentonville, AR; hole size: 0.05 mm). Thiram-treated intact, untreated intact, thiram-treated mechanically-damaged and untreated mechanically-damaged seeds of alfalfa, fenugreek, lettuce, and tomato were used in the study.

3.2.2. *Bacterial attachment to seed surfaces*

The experiment was performed based on the method decribed by Barak et al. (2002) and Darsonval et al. (2009) with modifications. Two grams of each type of seed (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; Becton Dickinson, Sparks, MD) at room temperature for 15 min with gentle mixing. Seeds were then neutralized with 10 ml of Dey-Engley neutralizing broth (Becton Dickinson) for 10 min with gentle mixing and rinsed twice, each with 10 ml of sterilized deionized water for 1 min. An overnight culture of each *Salmonella* and EHEC strain grown in Luria-Bertani no salt broth supplemented with NA (50 µg/ml) was diluted in sterilized water, and 20 ml of two concentrations (10^2 and 10^4 CFU/ml) of each inoculum was added to the centrifuge tubes with sanitized seeds. The precise inoculation levels were determined by plating 0.1 ml of appropriately diluted cell suspensions on TSA amended with NA. Vegetable seeds in the centrifuge tubes were agitated horizontally at 100 rpm in an orbital platform shaker (Model: 3520, Lab-line, IL, U.S.A) at 20 °C for 5 h. The inocula were then decanted to sterilized test tubes, and seeds were rinsed twice,

each with 10 ml of sterilized water for 30 s with gentle mixing. The rinse water from each seed type was collected into a sterilized test tube. Seeds were then soaked overnight at 4 °C in 5 ml of phosphate-buffered saline (pH 7.4) to release attached bacterial cells. Each sample in the experiemnt was duplicated and all experiments were conducted twice.

3.2.3. *Quantification of bacteria*

After being soaked at 4 °C overnight, seed samples were vortexed at 3,200 rpm (Fisher Scientific, Asheville, NC) for 1 min. The resulting samples were ten-fold serial diluted and appropriate dilutions of samples inoculated with *Salmonella* were plated on BSA. Those that were inoculated with *E. coli* O157 or O104 were plated on SMAC and MAC agar amended with NA, respectively. Additionally, all samples were plated on TSA amended with NA (NA-TSA). Bacterial populations in seed rinse water were also determined, as described previously. The ratio of the number of attached cells to the number of inoculated cells was reported as irreversible attachment ratio, whereas the ratio of the number of the cells recovered from seed rinse water to the number of inoculated cells was reported as reversible attachment ratio. The total population of unattached cells in spent inoculum suspension, attached cells recovered from vegetable seeds, and loosely attached cells in seed rinse water was compared with the bacterial counts in the original inocula. Significant difference between the sum population and cell counts in the inocula indicates bacterial growth during each experiment.

3.2.4. *Scanning electron microscopy*

To observe the surface morphology of dry vegetable seeds used in the study, scanning electron microscopy was performed according to the approach outline in the user's manual. Each type of dry seed was mounted directly on stubs using double-side adhesive tape, and sputter-coated with gold using an SPI module sputter coater (model 11428-AB, Structure Probe, Inc., West Chester, PA). The surface morphology of seeds were examined using a Scanning Electron Microscope Zeiss 1450EP (Carl Zeiss, Inc., Thornwood, NY). Digital images (65× [Fig. 3.2], 2000× [Fig. 3.3]) were captured using SmartSEM (Carl Zeiss, Inc., Thornwood, NY).

3.2.5. *Statistical analysis*

To estimate differences among irreversible attachment ratios of each tested bacterial strain on vegetable seeds, Fisher's least significant difference test in general linear model was used for means separation based on a 95% confidence level using SAS (version 9.4; SAS Institute Inc., Carey, N.C.). The same statistical test was also used to analyze the differences in pathogen attachment to seeds with varying integrity and fungicide treatments.

3.3. Results

3.3.1. *Irreversible attachment ratios of* Salmonella *and EHEC from different vegetable seeds*

Overall, the four *Salmonella* strains used in this study had similar abilities to attach to vegetable seeds (Table 3.2). However, among the EHEC strains; strain K4499 (12.5%) had a significantly higher ($P < 0.05$) irreversible attachment ratio than the other 3 EHEC strains. The irreversible attachment ratios of H1730 (1.5%) and BAA-2326 (0.2%) were similar, but they were significantly lower than those of K4499 and F4546 (5.2%). The mean EHEC irreversible attachment ratio from the 2 log CFU/ml inoculation level (5.8%) was significantly higher ($P < 0.05$) than from the 4 log CFU/ml inoculation level (3.9%), but *Salmonella* irreversible attachment ratios were similar from the two inoculation levels (Table 3.2). Unit weight (2 g) of lettuce seeds had the highest numbers of attached *Salmonella* cells (18.7%), followed by tomato (13.2%), alfalfa (11.3%), and fenugreek (6.0%) seeds (Table 3.2). A similar trend was observed with EHEC cells, except that there was no significant difference in the numbers of cells attached to alfalfa and fenugreek seeds. However, individual fenugreek seeds had the highest numbers of attached *Salmonella* and EHEC cells, followed by lettuce, alfalfa, and tomato seeds (Table 3.2). With regards to seed surface characteristics, significantly more *Salmonella* and EHEC cells attached to mechanically damaged seeds than to intact seeds (Table 3.2; $P < 0.05$). *Salmonella* irreversible attachment to untreated seeds was significantly higher ($P < 0.05$) than to thiram-treated seeds. In contrast, the numbers of

EHEC cells attached to thiram-treated *vs*. untreated seeds were not significantly different (Table 3.2).

3.3.2. *Differences in attachment between* Salmonella *and EHEC*

In general, the *Salmonella* strains used in this study displayed greater irreversible attachment ratios than the EHEC strains (Fig. 3.1). However, the irreversible attachment ratio of K4499 was comparable to those of the four *Salmonella* strains. For most of the bacterial strains included in the study, the irreversible attachment ratios to vegetable seeds were higher than the reversible attachment ratios, except for *S*. Stanley and *E. coli* ATCC BAA-2326.

For EHEC at both inoculum levels and *Salmonella* at the 2 log CFU/ml inoculation level, bacterial populations recovered from each type of seed were similar to the original inoculum levels, except for tomato seeds (Table 3.3). At the 4 log CFU/ml inoculation level, the number of inoculated *Salmonella* cells was also similar to those recovered from lettuce seeds, and the latter was not significantly different from the numbers of *Salmonella* cells recovered from alfalfa and fenugreek seeds ($P > 0.05$). For tomato seeds, the recovered pathogen levels were significantly lower than the original inoculum levels (Table 3.3). These data suggest that there was no significant bacterial growth during the 5 h attachment process at 20 ºC.

Although there were no significant differences in the mean irreversible attachment abilities of the four *Salmonella* strains used in this study, individual strains appeared to have a unique affinity to a specific type of vegetable seed (Table 3.4). Significantly more

S. Baildon (14.4%) and *S.* Cubana (17.7%) cells attached to alfalfa seeds than cells of *S.* Montevideo (5.8%) and *S.* Stanley (7.4%). Additionally, significantly more *S.* Montevideo (17.0%) and *S.* Stanley (19.9%) cells attached to tomato seeds than cells of the other two *Salmonella* strains (9.6% for *S.* Baildon and 6.5% for *S.* Cubana). *S.* Stanley (2.9%) displayed relatively low attachment ability to fenugreek seeds compared to other *Salmonella* serotypes (from 6.2% to 7.9%). Among the EHEC, strain K4499 had the highest irreversible attachment ratio on every vegetable seed type tested, followed by strain F4546. The irreversible attachment ratios of H1730 and BAA-2326 were significantly lower than the other two EHEC strains (Table 3.5). More F4546 cells attached to lettuce (8.9%) and tomato (7.1%) seeds than alfalfa (3.1%) and fenugreek (1.9%) seeds, while more K4499 cells attached to lettuce seeds (28.7%), followed by tomato (11.0%) and alfalfa (6.0%) seeds. The number of K4499 cells recovered from alfalfa seeds were not significantly different to those that attached to fenugreek seeds (4.3%). Significantly more H1730 cells attached to lettuce seeds (3.7%) than to the other three vegetable seed types. Similar number of ATCC BAA-2326 cells attached to tomato (0.2%), fenugreek (0.3%), and lettuce (0.4%) seeds, but the number of cells that attached to lettuce seeds was significantly higher than that recovered from alfalfa seeds (0.1%) (Table 3.5).

3.3.3. *Effect of vegetable seed surface characteristics on pathogen attachment*

Salmonella cells recovered from damaged alfalfa and tomato seeds, and *S.* Baildon and *S.* Cubana cells recovered from damaged fenugreek seeds were significantly

higher ($P < 0.05$) than those from the corresponding seeds without mechanical damage (Table 3.6). Furthermore, *Salmonella* cells recovered from damaged lettuce seeds, and *S.* Montevideo and *S.* Stanley cells recovered from damaged fenugreek seeds were similar to those from their undamaged counterparts, except for lettuce seeds inoculated with *S.* Cubana (Table 3.6).

EHEC cells recovered from damaged tomato seeds, F4546, K4499, and H1730 cells recovered from damaged alfalfa seeds, F4546, H1730, and ATCC BAA-2326 cells from damaged fenugreek seeds, and ATCC BAA-2325 cells from damaged lettuce seeds were significantly higher ($P < 0.05$) than those from their corresponding intact seeds. For the rest of the samples the number of EHEC cells on damaged and intact seeds was statistically similar, except for lettuce seeds that were inoculated with strain K4499 and plated on NA-TSA (Table 3.7). Damaged tomato seeds had the lowest number of attached bacterial pathogen cells, except for the samples inoculated with ATCC-BAA-2326 (Table 3.6 and 3.7).

Although on average significantly more *Salmonella* cells were recovered from untreated seeds than from thiram-treated seeds (Table 3.2), thiram treatment did not affect the attachment of individual bacterial strains to vegetable seeds, except for lettuce seeds inoculated with *S.* Cubana or *S.* Baildon (Table 3.8). Similarly, no significant difference in irreversible attachment ratio of EHEC cells was observed between thiram-treated seeds and untreated seeds, except for lettuce seeds inoculated with strain H1730 (Table 3.9).

3.3.4. *Surface morphology of intact and damaged vegetable seeds*

Fenugreek seeds had a cuboid-shape and were the largest of the four types of seeds used in this study. Lettuce seeds were long and thin and had granular structures on their surfaces (Fig. 3.2). Oval shaped alfalfa seeds had relatively smooth surfaces, while round-shaped tomato seeds had a rough surface texture (Fig. 3.2). The scanning electron micrographs of the seed surfaces revealed exposed cavities on mechanically damaged fenugreek and alfalfa seeds and seed debris on mechanically damaged lettuce and tomato seeds (Fig. 3.2). More detailed scanning electron micrographs of seed surface morphology revealed regular nodes and crevices on fenugreek seeds (Fig. 3.3A), crevices and irregular nodes on lettuce seeds (Fig. 3.3B), pubescent covering (fuzz) and crevices on tomato seeds (Fig. 3.3C), and slight cracks on alfalfa seeds (Fig. 3.3D).

3.4. Discussion

The present study revealed that on average *S. enterica* had greater irreversible attachment ratios than EHEC. Similar observations were reported in several previous studies involving alfalfa and bean sprouts (Barak, Whitehand, & Charkowski, 2002; Han, Klu, & Chen, 2014), cantaloupe rind surface (Ukuku & Fett, 2002), and food contact surfaces (Palmer, Flint, & Brooks, 2007). Difference in cell surface hydrophobicity between *Salmonella* and EHEC cells was believed to be one of the contributing factors for the observed phenomenon (Dickson & Koohmaraie, 1989; Palmer, Flint, & Brooks, 2007; Stenström, 1989; Ukuku & Fett, 2002). However, contradictory findings have also

been reported. Takeuchi et al. (2000) observed that more *E. coli* O157:H7 cells attached to lettuce leaf surface than *S.* Typhimurium cells. It is possible that cell surface hydrophobicity varies among bacterial species/serotypes as well as among individual strains within a bacterial species/serotype. Furthermore, intrinsic cell factors other than cell surface hydrophobicity may play important roles in the interaction between bacterial cells and contact surfaces (Macarisin, Patel, Bauchan, Giron, & Sharma, 2012).

Among the EHEC strains used in this study, *E. coli* O104:H4 ATCC BAA-2326 had the lowest attachment potential (Fig. 3.1). This bacterial strain was isolated from a fenugreek sprout associated outbreak in Germany in 2011, which affected 3,842 people in a dozen countries (Beutin & Martin, 2012). BAA-2326 evolved from an enteroaggregative *E. coli* (EAEC) that acquired the genes for Shiga-toxin production (Beutin & Martin, 2012). EAEC strains produce a large amount extracellular polymeric substances (EPS) (Okhuysen & DuPont, 2010). While EPS enhance cell aggregation and biofilm formation, they usually impair initial bacterial attachment to contact surfaces (Beloin, Roux, & Ghigo, 2008; Petrova & Sauer, 2012).

The numbers of the bacterial cells attached to vegetable seeds were generally higher than those that were recovered from seed rinsing waters, except for *S.* Stanley and *E. coli* ATCC BAA-2326 (Fig. 3.1). This suggests that more cells of these two bacteria were loosely associated with seed surfaces and more easily rinsed away. This observation was incongruent with a previous study by Barak et al. (2002) who reported that more *Salmonella* and *E. coli* O157: H7 cells were recovered from sprout rinse water than from sprout samples themselves. This inconsistency may be due to specific bacterial strains and experimental conditions used in the two studies. Additionally, seeds and seed

sprouts have different surface characteristics. Surfaces of seeds are rougher, and have irregular shapes and larger surface areas, while sprouts are thinner, longer, and smoother, and have smaller surface areas.

We observed that the irreversible attachment ratios of *Salmonella* cells ranged from 2.9% on fenugreek seeds contaminated with *S.* Stanley to 19.9% on lettuce seeds contaminated with *S.* Cubana and tomato seeds contaminated by *S.* Stanley. By comparison, EHEC irreversible attachment ratios ranged from 0.1% on alfalfa seeds contaminated with BAA-2326 to 28.7% on lettuce seeds contaminated with K4499 (Table 3.4 and 3.5). Most of the previous studies reported bacterial pathogen levels on vegetable seeds in log CFU/g, rather than in terms of irreversible attachment ratio. Fransisca et al. (2012) exposed 300 g alfalfa seeds to 300 ml of a 10^7 CFU/ml *E. coli* O157 culture for 2 min followed by rinsing for 20 min with water, and 3.16 CFU/g of *E. coli* O157 cells were recovered from alfalfa seeds; which was equivalent to an irreversible attachment ratio of 0.01%. This value was lower than the irreversible attachment ratios observed for alfalfa seeds contaminated with EHEC in the present study (Table 3.5). Fransisca et al. (2012) reported that each gram of seed was exposed to 10^7 CFU of *E. coli* cells, while in our study each gram of vegetable seeds was exposed to 10^3 to 10^5 CFU of pathogens cells. In addition, different bacterial strains and attachment conditions were used in the two studies. Higher cell concentrations were also used as inocula in several other studies (Bari, Enomoto, Nei, & Kawamoto, 2010; Hong & Kang, 2016; Neamati, Azizi, & Arouiee, 2010), and as expected, the attachment ratios calculated from these studies were lower than what was observed in the present study.

In the current study the inoculation level had no significant effect on *Salmonella* irreversible attachment ratios, indicating that the number of *Salmonella* cells attached to seed surfaces increased as inoculum concentration increased. However, EHEC irreversible attachment ratios from the 2 log CFU/ml inoculation level were significantly higher than those from the 4 log CFU/ml inoculation level. The precise reason for the observed phenomenon is not clear.

The surfaces of vegetable seeds are complex, and different types of seeds have different surface properties. As a result, they have different potentials to attract bacterial cells. Unit weight of lettuce seeds had the highest number of *Salmonella* and EHEC cells, followed by tomato, alfalfa, and fenugreek seeds (Table 3.2). According to SEM micrographs, lettuce seed surfaces have nodes and crevices, while tomato seeds are pubescent. These two types of seeds have a rougher surface than alfalfa seeds (Fig. 3.3), which in part, explains why pathogen irreversible attachment ratios from alfalfa seeds were lower (Table 3.2). This observation is supported by previous studies that demonstrated that wrinkled or rough seeds were likely to harbor more bacteria, and were more resistant to sanitizers compared to smooth seeds (Charkowski, Sarreal, & Mandrell, 2001; Fransisca & Feng, 2012). Additionally, in the current study alfalfa seeds clumped together in aqueous environment, which might have prevented bacterial cells from attaching to their surfaces. Among the four seed types used in the present study, unit weight of fenugreek seeds had the lowest *Salmonella* and EHEC irreversible attachment ratios. Fenugreek seeds are larger and heavier, and compared to other seeds, unit weight of fenugreek seeds have smaller surface areas to interact with bacterial cells. It is worth noting that the fact that the two types of sprout seeds used in this study did not have

higher number of *Salmonella* and EHEC cells attached did not make them microbiologically safer. The increase in bacterial population during seed germination and sprouting can still pose significant threats to consumer health.

Salmonella and EHEC irreversible attachment ratios from intact tomato seeds were the lowest among all the seed types tested. In addition, the total numbers of bacterial pathogens recovered from tomato seeds were significantly lower than the numbers of inoculated bacterial pathogen cells. Commercial tomato seeds are processed through a natural fermentation process to eliminate chemical compounds that inhibit germination (Neamati, Azizi, & Arouiee, 2010). Although tomato seeds are washed and dried after fermentation, residual fermentation compounds such as lycopene may be present in seed coat hairs, leading to an acidic seed surface (pH *ca*. 2.6; detailed data not shown). Furthermore, tomato seed fermentation mixtures may contain antimicrobial compounds that inhibit attachment and viability of bacterial pathogens. Arkoun et al. (2015) reported that lactic acid bacteria isolated from fermented tomato fruits produced a bacteriocin-like substance that had inhibited a variety of gram-negative bacteria, including *E. coli*.

Bacterial cells that attached to mechanically damaged seeds were significantly higher than those that attached to intact seeds, with a few exceptions. The micro-topography of tested seeds revealed that damaging seed coats resulted in cracks that can provide physical protection for bacterial cells. This may have made it more difficult for loosely attached bacterial cells to be rinsed away. This observation is in agreement with previous studies that showed cells of *L. monocytogenes* and *E. coli* O157:H7 were more likely to attach to cut edges of cabbage and lettuce (Ells & Hansen, 2006; Macarisin,

Patel, Bauchan, Giron, & Sharma, 2012; Takeuchi, Matute, Hassan, & Frank, 2000). One explanation for the recovery of higher number of bacterial cells from damaged seeds than intact seeds is that mechanical damage may result in increased uptake of water and increased leakage of solutes from seeds (Hwang, Gossen, Chang, Turnbull, & Howard, 2001), which may promote attachment and growth of bacterial pathogens. However, according the results shown in Table 3.3, significant bacterial multiplication did not occur during the attachment experiment.

Although significantly more *Salmonella* and EHEC cells were recovered from untreated seeds than from treated seeds, treatment of vegetable seeds with thiram did not affect the attachment of individual bacterial strains to vegetable seeds. The only exception was lettuce seeds. According to the U.S. Environmental Protection Agency, thiram is a non-systemic fungicide, seed protectant, and animal repellent. Thiram is applied to seeds prior to planting as a dust, wettable powder, or liquid. The chemical has antibacterial (Chaube & Pundhir, 2005) and antifungal activities (Xue et al., 2014); however, no significant difference in the level of bacterial attachment was found between thiram-treated and untreated seeds in the present study. This could be due to the fact that most of thiram might have been rinsed off during seed sanitization and subsequent rinsing.

3.5. Conclusion

In summary, we observed the interaction between two important bacterial pathogens, *i.e. Salmonella* and EHEC, and vegetable seeds with different surface

characteristics. The study provides a better understanding of whether seed surface structure, integrity, and fungicide treatment affect bacterial interaction with vegetable seeds surfaces. Bacterial pathogen attachment to vegetable seeds is the first step in colonization. The fate of pathogen cells after the initial attachment step also has a significant impact on fresh produce safety. A study is currently underway in our laboratory to assess whether *Salmonella* and EHEC cells could migrate from contaminated vegetable seeds to different tissues of seed sprouts and vegetable seedlings.

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Figure Legends

Fig. 3.1. Attachment ratios of *S. enterica* Montevideo (M), Stanley (S), Cubana (C), Baildon (B), *E. coli* O157:H7 F4546 (F), K4499 (K), H1730 (H), and *E. coli* O104:H4 strains ATCC BAA-2326 (G) to vegetable seeds.

Fig. 3.2. Scanning electron micrographs of selected vegetable seeds with different seed coat integrity properties. A: intact fenugreek seed, B: damaged fenugreek seed, C: intact lettuce seed, D: damaged lettuce seed, E: intact tomato seed, F: damaged tomato seed, G: intact alfalfa seed, H: damaged alfalfa seed. Bars = 200 μm.

Fig. 3.3. Scanning electron micrographs of surface morphology of fenugreek, lettuce, tomato and alfalfa seeds. A: fenugreek seed, B: lettuce seed, C: tomato seed, D: alfalfa seed. Bars = 10 μm.

Table 3.1.
Bacterial strains used in this study

Bacterial strains	Abbreviation	Description/source	Reference
S. Baildon	B	Tomato associated outbreak strain	(Cummings et al., 2001)
S. Cubana	C	Alfalfa sprout associate outbreak strain	(Mohle-Boetani et al., 2001)
S. Stanley	S	Alfalfa sprout associate outbreak strain	(Mahon et al., 1997)
S. Montevideo	M	Tomato associated outbreak strain	(Guo, Chen, Beuchat, & Brackett, 2000)
E. coli O157:H7 F4546	F	Alfalfa sprout associated outbreak strain	(Zansky et al., 2002)
E. coli O157:H7 K4499	K	Spinach associate outbreak strain	
E. coli O157:H7 H1730	H	Lettuce associate outbreak strain	
E. coli O104:H4 ATCC BAA-2326	G	Fenugreek associate outbreak strain	(Beutin & Martin, 2012)

Table 3.2.
Irreversible attachment ratios of *Salmonella* and EHEC on alfalfa, fenugreek, lettuce, and tomato seeds

Comparative catogories	Irreversible attachment ratio	
	Salmonella	EHEC
Strain		
S. Cubana/K4499	0.130A[a]	0.125A
S. Stanley/F4546	0.124A	0.052B
S. Montevideo/H1730	0.123A	0.015C
S. Baildon/ATCC BAA-2326	0.116A	0.002C
Inoculum level		
2 log CFU/ml	0.121A	0.058A
4 log CFU/ml	0.125A	0.039B
Integrity properties		
Damaged	0.174A	0.064A
Intact	0.072B	0.033B
Treatment practices		
Untreated	0.134A	0.053A
Thiram-treated	0.112B	0.044A
Seed types by unit weight (by individual seeds CFU/seed)		
Alfalfa	0.113C (7.4C)	0.025C (0.6C)
Fenugreek	0.060D (16.6A)	0.018C (2.5A)
Lettuce	0.187A (10.7B)	0.104A (1.9B)
Tomato	0.132B (4.0D)	0.048B (0.8C)

[a]Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4.
Values within each comparative category (bacterial strain, inoculatin level, and etc.) in the same column followed by the same letter were not significantly different ($P > 0.05$).

Table 3.3.
Total populations of *Salmonella* and EHEC recovered from vegetable seeds, seed rinse waters and spent inoculum suspensions compared to original inoculum concentrations

	Salmonella (log CFU/g)		EHEC (log CFU/g)	
Inoculum level	3.65±0.17A[a]	5.65±0.17A	3.13±0.38A	5.13±0.38A
Alfalfa	3.19±0.14A	4.94±0.15BC	2.26±1.12AB	4.52±0.29A
Fenugreek	3.41±0.17A	5.01±0.22BC	2.86±0.67A	4.61±0.57A
Lettuce	3.31±0.16A	5.30±0.09AB	2.58±0.81AB	4.70±0.37A
Tomato	2.14±1.42B	4.59±0.82C	1.53±1.27B	3.20±1.58B

[a]Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4.
Values within the same column followed by the same letter were not significantly different ($P > 0.05$).

Table 3.4.
Irreversible attachment of *Salmonella* to alfalfa, fenugreek, lettuce, and tomato seeds

	Irreversible attachment ratio (from BSA[a])				Irreversible attachment ratio (from NA-TSA[a])			
Seeds\Strains	Baildon	Cubana	Montevideo	Stanley	Baildon	Cubana	Montevideo	Stanley
Alfalfa	0.144A a[b]	0.177A a	0.058B b	0.074B b	0.159A a	0.192A a	0.065B b	0.091B b
Fenugreek	0.062B a	0.079B a	0.068B a	0.029B b	0.068B b	0.100B a	0.063B b	0.032B c
Lettuce	0.160A a	0.199A a	0.195A a	0.193A a	0.169A c	0.222A ab	0.177A bc	0.242A a
Tomato	0.096B b	0.065B b	0.170A a	0.199A a	0.107B bc	0.073B c	0.166A b	0.245A a

[a] BSA: bismuth sulfite agar; NA-TSA: tryptic soy agar amended with 50 µg/ml nalidixic acid
[b] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4.

Table 3.5.
Irreversible attachment of EHEC to alfalfa, fenugreek, lettuce, and tomato seeds

	Irreversible attachment ratio (from NA-SMAC[a])				Irreversible attachment ratio (from NA-TSA[a])			
Seeds\Strains	F4546	K4499	H1730	BAA-2326	F4546	K4499	H1730	BAA-2326
Alfalfa	0.031B b[b]	0.060BC a	0.008B c	0.001B c	0.036B b	0.079C a	0.008C c	0.005B c
Fenugreek	0.019B b	0.043C a	0.006B c	0.003AB c	0.031B b	0.049C a	0.014C c	0.005B c
Lettuce	0.089 A b	0.287A a	0.037A c	0.004A c	0.145A b	0.372A a	0.075A c	0.008AB d
Tomato	0.071A b	0.110B a	0.007B c	0.002AB c	0.129A a	0.150B a	0.047B b	0.010A b

[a] NA-SMAC: sorbitol MacConkey agar supplemented with 50 µg/ml nalidixic acid; NA-TSA: tryptic soy agar supplemented with 50 µg/ml nalidixic acid

[b] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4.

Table 3.6.

Irreversible attachment of *Salmonella* to mechanically damaged and intact alfalfa, fenugreek, lettuce, and tomato seeds [a]

	Irreversible attachment ratio (from BSA[b])				Irreversible attachment ratio (from NA-TSA[b])			
Seeds\Strains	Baildon	Cubana	Montevideo	Stanley	Baildon	Cubana	Montevideo	Stanley
DA-A	0.235A a [cd]	0.287A a	0.099C b	0.123C b	0.257A b	0.314A a	0.105C c	0.150C c
IN-A	0.053CD b	0.067CD a	0.017D c	0.025D c	0.060DE a	0.070C a	0.026E b	0.031D b
DA-Fe	0.083C ab	0.111BC a	0.070C b	0.034D c	0.087D b	0.151B a	0.060DE bc	0.031D c
IN-Fe	0.042D ab	0.047DE ab	0.067C a	0.023D b	0.049EF ab	0.050CD ab	0.067CD a	0.033D b
DA-L	0.155B bc	0.140B c	0.191B ab	0.226B a	0.162C b	0.162B b	0.165B b	0.264B a
IN-L	0.166B b	0.258A a	0.199B ab	0.161B b	0.175BC b	0.283A a	0.190B b	0.220B ab
DA-T	0.191B b	0.130B c	0.332A a	0.382A a	0.202B c	0.141B d	0.293A b	0.434A a
IN-T	0.001E b	0.001E b	0.008D ab	0.015D a	0.013F b	0.005D b	0.039ED ab	0.057D a

[a] DA: damaged, IN: intact; L: lettuce seed, T: tomato seed, A: alfalfa seed, Fe: fenugreek seed

[b] BSA: bismuth sulfite agar; NA-TSA: tryptic soy agar supplemented with 50 µg/ml nalidixic acid

[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4.

[d] Underlined: Significantly higher counts on damaged seeds than intact seeds.

Table 3.7.

Irreversible attachment of EHEC to mechanically damaged and intact alfalfa, fenugreek, lettuce, and tomato seeds [a]

	Irreversible attachment ratio (from NA-SMAC[b])				Irreversible attachment ratio (from NA-TSA[b])			
Seeds\Strains	F4546	K4499	H1730	BAA-2326	F4546	K4499	H1730	BAA-2326
DA-A	0.049CD b [cd]	0.096C a	0.016B c	0.001C c	0.056C b	0.126C a	0.012B c	0.006B c
IN-A	0.013E b	0.024D a	0.001C c	0.000C c	0.016CD b	0.031D a	0.003C c	0.003B c
DA-Fe	0.031D ab	0.056CD a	0.009B c	0.005AB c	0.050C a	0.052CD a	0.022B b	0.007A b
IN-Fe	0.007E b	0.031CD a	0.002C b	0.001C b	0.011D b	0.046D a	0.006C b	0.004B b
DA-L	0.099B b	0.252AB a	0.033A bc	0.008A c	0.138B b	0.354B a	0.065A bc	0.013A c
IN-L	0.079BC b	0.323A a	0.040A bc	0.001C c	0.151B b	0.390A a	0.086A c	0.002B d
DA-T	0.133A b	0.217B a	0.014B c	0.003B c	0.227A b	0.281B a	0.094A c	0.017A d
IN-T	0.009E a	0.004D ab	0.001C b	0.001C b	0.031CD a	0.019D ab	0.001C b	0.003B b

[a] DA: damaged, IN: intact; L: lettuce seed, T: tomato seed, A: alfalfa seed, Fe: fenugreek seed

[b] NA-SMAC: sorbitol MacConkey agar amended with 50 µg/ml nalidixic acid; NA-TSA: tryptic soy agar amended with 50 µg/ml nalidixic acid

[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4.

[d] Underlined: Significantly higher counts on damaged seeds than intact seeds.

Table 3.8.

Irreversible attachment of *Salmonella* to thiram (dimethylcarbamothioylsulfanyl N, N-dimethylcarbamodithioate)-treated and untreated alfalfa, fenugreek, lettuce, and tomato seeds [a]

	Irreversible attachment ratio (from BSA[b])				Irreversible attachment ratio (from NA-TSA[b])			
Seeds\Strains	Baildon	Cubana	Montevideo	Stanley	Baildon	Cubana	Montevideo	Stanley
TR-A	0.136AB a[cd]	0.150BC a	0.059B b	0.063B b	0.152ABC ab	0.180B a	0.066B c	0.081B bc
UN-A	0.152AB ab	0.204AB a	0.058B c	0.085B bc	0.165AB ab	0.203AB a	0.064B c	0.100B bc
TR-Fe	0.062C ab	0.089CD a	0.058B b	0.026B c	0.073D b	0.130BC a	0.068B bc	0.031B c
UN-Fe	0.063C ab	0.069D a	0.079B a	0.031B b	0.063D ab	0.070C a	0.059B ab	0.033B b
TR-L	0.129B b	0.136BCD ab	0.173A ab	0.181A a	0.146ABC b	0.174B ab	0.171A ab	0.207A a
UN-L	0.191A a	0.261A a	0.217A a	0.205A a	0.191A bc	0.271A ab	0.183A c	0.277A a
TR-T	0.097BC ab	0.066D b	0.179A a	0.188A a	0.115BCD bc	0.088C c	0.186A ab	0.265A a
UN-T	0.096BC b	0.064D b	0.160A ab	0.209A a	0.100D b	0.058C b	0.146A ab	0.226A a

[a] TR: thiram treated, UN: thiram untreated; A: damaged, IN: intact; L: lettuce seed, T: tomato seed, A: alfalfa seed, Fe: fenugreek seed

[b] BSA: bismuth sulfite agar; NA-TSA: tryptic soy agar supplemented with 50 µg/ml nalidixic acid

[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4.

[d] Underlined: similar counts on thiram treated and untreated seeds.

Table 3.9.
Irreversible attachment of EHEC to thiram (dimethylcarbamothioylsulfanyl N, N-dimethylcarbamodithioate)-treated and untreated alfalfa, fenugreek, lettuce, and tomato seeds [a]

	Irreversible attachment ratio (from NA-SMAC[b])				Irreversible attachment ratio (from NA-TSA[b])			
Seeds\Strains	F4546	K4499	H1730	BAA-2326	F4546	K4499	H1730	BAA-2326
TR-A	0.029DE ab[cd]	0.045B a	0.011BC bc	0.000A c	0.038B b	0.076BCD a	0.008D c	0.004B c
UN-A	0.032CDE b	0.075B a	0.006C b	0.000A b	0.034B b	0.081BCD a	0.007D b	0.005B b
TR-F	0.017E b	0.040B a	0.005C bc	0.004A c	0.034B a	0.039D a	0.013CD b	0.007AB b
UN-F	0.021E b	0.046B a	0.006C bc	0.002A c	0.027B b	0.058CD a	0.015CD bc	0.004B c
TR-L	0.072ABC b	0.260A a	0.025B bc	0.003A c	0.122A b	0.323A a	0.046BC c	0.007AB c
UN-L	0.106A b	0.315A a	0.049A bc	0.005A c	0.168A b	0.421A a	0.105A bc	0.008AB c
TR-T	0.063BCD b	0.113B a	0.010BC c	0.002A c	0.131A ab	0.156B a	0.060B bc	0.009AB c
UN-T	0.079AB a	0.108B a	0.005C b	0.001A b	0.126A a	0.144BC a	0.034BCD b	0.011A b

[a] TR: thiram treated, UN: thiram untreated; L: lettuce seed, T: tomato seed, A: alfalfa seed, Fe: fenugreek seed
[b] NA-SMAC: sorbitol MacConkey agar amended with 50 µg/ml nalidixic acid; NA-TSA: tryptic soy agar amended with 50 µg/mL nalidixic acid
[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4.
[d] Underlined: similar counts on thiram-treated and untreated seeds.

Fig. 3.1.

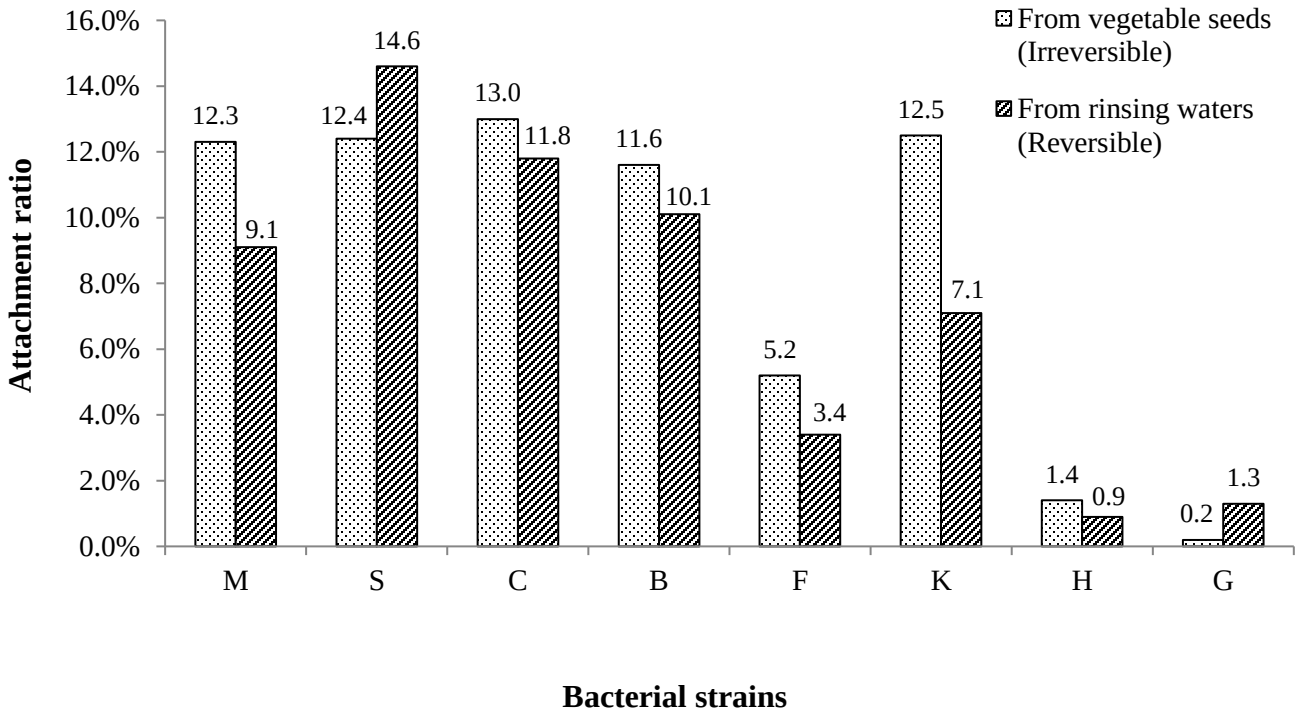

16.0%
14.0%
12.0%
10.0%
8.0%
6.0%
4.0%
2.0%
0.0%
Attachment ratio
From vegetable seeds (Irreversible)
From rinsing waters (Reversible)
12.3
9.1
12.4
14.6
13.0
11.8
11.6
10.1
5.2
3.4
12.5
7.1
1.4
0.9
0.2
1.3
M
S
C
B
F
K
H
G
Bacterial strains

Fig. 3.2.

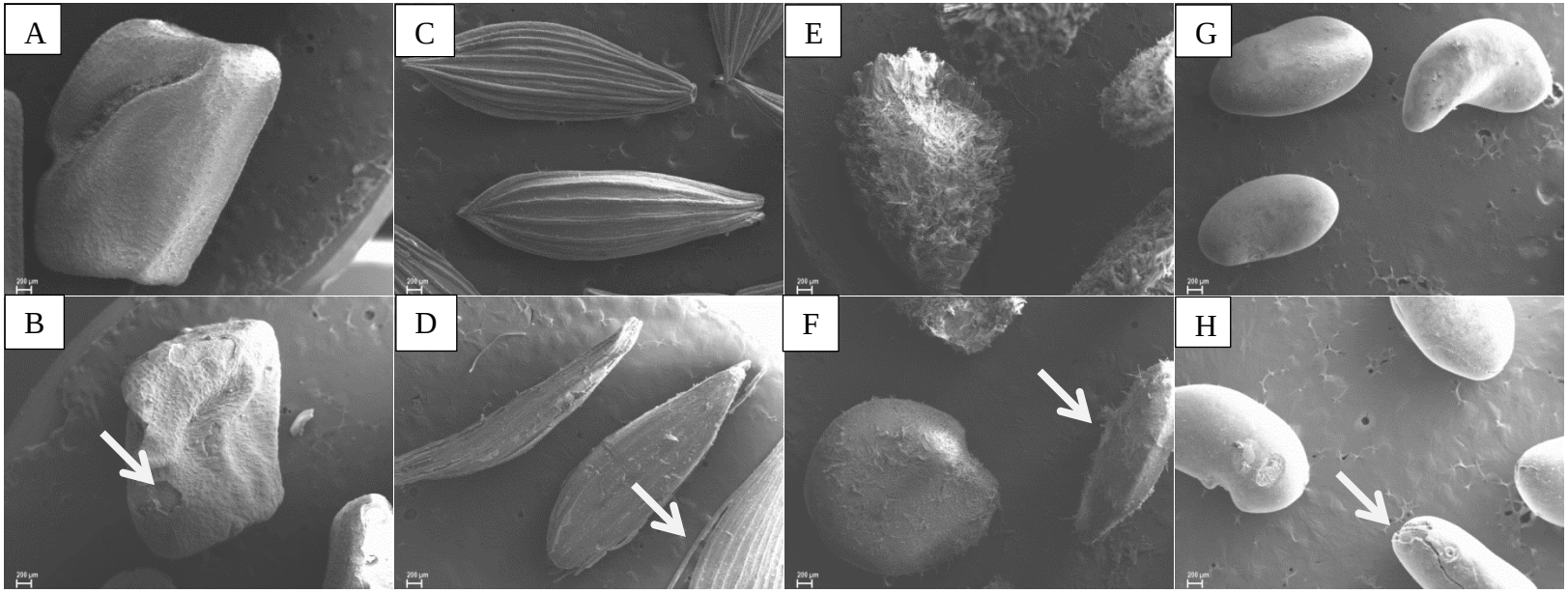
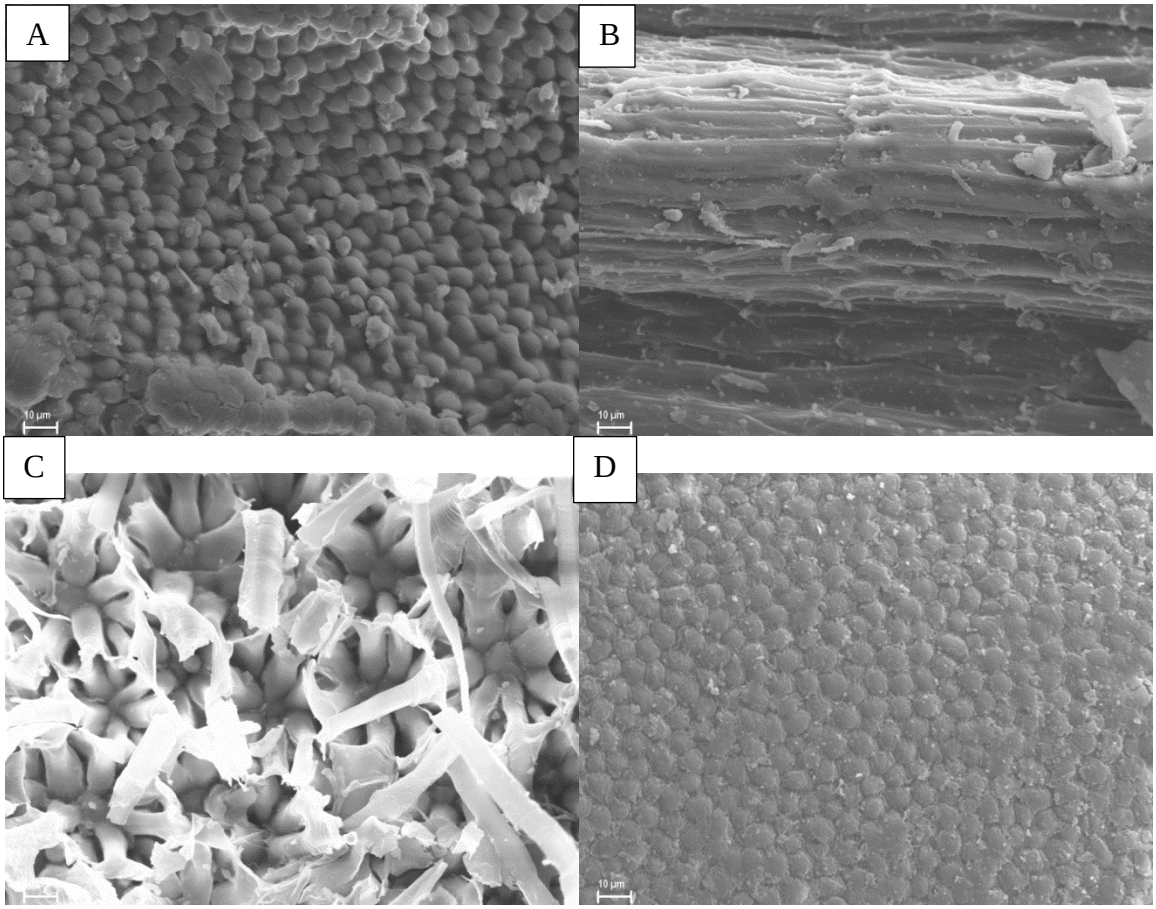


Fig. 3.3.



CHAPTER 4

FATE OF VARIOUS *SALMONELLA ENTERICA* AND ENTEROHEMORRHAGIC *ESCHERICHIA COLI* ON SELECTED VEGETABLE SEEDS, CONTAMINATED BY IMMERSION INTO BACTERIAL SUSPENSIONS AND BY CONTACT WITH ARTIFICIALLY-INOCULATED SOIL, DURING GERMINATION

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ABSTRACT

Contaminated vegetable seeds have been identified as a potential source of foodborne pathogens. This study was undertaken to examine the physiological behavior of various *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) cells on alfalfa, fenugreek, lettuce, and tomato seeds, contaminated by two inoculation methods [immersion into bacterial suspensions (IM) *vs*. contact with artificially-inoculated soil (SO)] during germination. Surface-sanitized seeds of alfalfa, fenugreek, lettuce, and tomato were exposed to four individual strains of *Salmonella* or EHEC. For inoculation by IM, 20 mL overnight cultures of each pathogen (10^4 CFU/mL) was co-incubated with the sanitized seeds (2 g) at 20 ^{o}C for 1 h. For inoculation by SO, lyophilized cells (10^5 CFU/g) of each pathogen inoculated in sterile, sandy soil were mixed with sanitized vegetable seeds (2 g) at 20 ^{o}C for 1 h. Contaminated vegetable seeds were germinated on 1% water agar at 25 ^{o}C in the dark. Populations of *Salmonella* and EHEC on various tissues of sprouts/seedlings (seed coat, root, cotyledon, stem, *etc*.) were determined every other day over a 9-day germination period. Both the incidence of pathogen-positive samples and the mean cell populations of the pathogens on sprout/seedlings tissues developed from seeds contaminated by IM (*ca.* 92.0%, 4.5-4.7 log CFU/0.01 g) were significantly higher than those from seeds contaminated by SO (*ca.* 71% %, 3.0 log CFU/0.01 g). Seed coats had the highest ($P < 0.05$) bacterial counts, followed by the root, cotyledon, and stem tissues. When seeds were contaminated by IM, lettuce seedlings and alfalfa sprouts had significantly higher ($P < 0.05$) numbers of *Salmonella* and EHEC cells, followed by fenugreek sprouts and tomato seedlings; while tissue section of fenugreek sprouts and lettuce seedlings had higher ($P < 0.05$) number of

Salmonella and EHEC cells than that of alfalfa sprouts and tomato seedlings when seeds were contaminated by SO. The *E. coli* O104:H4 strain established the lowest ($P < 0.05$) cell population on sprout/seedling tissues among all EHEC strains used in the study. These data suggested that the growth and dissemination of *Salmonella* and EHEC cells on alfalfa, fenugreek, lettuce, and tomato sprout/seedling tissues were influenced by seed inoculation method, as well as bacterial strains, plant hosts, and sprout/seedling tissues involved.

Keywords: Enterohemorrhagic *Escherichia coli*; *Salmonella*; sprouts/seedlings; vegetable seeds

4.1. Introduction

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

Fresh produce contamination by bacterial pathogens such as *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) could occur at both pre- and post-harvest stages in the production chain (Jiang, Chen, & Dharmasena, 2014). Vegetable seeds are prone to microbial contamination, and un-sanitized vegetable seeds tainted with human enteric pathogens could lead to the contamination of fresh produce, especially vegetable sprouts (Hanning, Nutt, & Ricke, 2009). According to the U.S. Food and Drug Administration (1999), most sprout-associated outbreaks have been caused by seeds contaminated with bacterial pathogens before the sprouting process begins because currently available seed sanitation treatments are not always effective in eliminating

bacterial pathogens associated with sprout seeds (Ding, Fu, & Smith, 2013; Fu, Reineke, Chirtel, & VanPelt, 2008; Hong & Kang, 2016). Contamination of sprout seeds by *Salmonella* and EHEC has been confirmed as the cause of several sprout-associated outbreaks of infections (Breuer et al., 2001; CDC, 2016). In a previous study of our laboratory, cells of individual *Salmonella* and EHEC strains exhibited a differential attachment behavior on the surface of alfalfa, fenugreek, lettuce, and tomato seeds (Cui, Walcott, & Chen, 2017). Bacterial attachment to vegetable seeds is the initial step of vegetable sprout/seedling contamination by pathogenic bacteria. The fate of pathogen cells during seed germination could have a significant impact on microbial safety of fresh produce, especially seed sprouts.

Vegetable seeds were usually contaminated in microbiological laboratories by immersion into bacterial suspensions (Charkowski, Barak, Sarreal, & Mandrell, 2002; Gomez-Aldapa et al., 2013). This approach mimics the natural process of sprout seed contamination by sprouting waters. In addition to this approach, vegetable seeds could become contaminated by pathogens through direct contact with infested soils. Up till this point, it is not known if human pathogens carried by vegetable seeds contaminated by the two methods would behave in a similar fashion during seed germination. This study was undertaken to observe the physiological behavior of selected *Salmonella* and EHEC cells on alfalfa, fenugreek, lettuce, and tomato seeds contaminated by two different inoculation methods, during germination.

4.2. Materials and methods

4.2.1. Vegetable seeds

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

4.2.2. Bacterial strains

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

4.2.3. Seed sanitation

Vegetable seeds were sanitized to inactivate the background microflora according to a protocol described previously (Cui, Walcott, & Chen, 2017). Briefly, each type of vegetable seeds (2 g) was placed in a 50 ml Falcon centrifuge tube (Fisher Scientific, Asheville, NC) and sanitized with 10 ml of 20,000 ppm sodium hypochlorite solution (pH 6.8; Becton Dickenson, Sparks, MD) at room temperature for 15 min with gentle mixing. Residual NaOCl on vegetable seeds was neutralized with 10 ml of Dey-Engley neutralizing broth (Becton Dickenson, Sparks, MD) for 10 min with gentle mixing and then rinsed twice, each with 10 ml of sterile deionized water for 1 min. The seeds were air-dried in the biological safety cabinet (Class II type A/B 3, Nuaire, Plymouth, MN) overnight at room temperature.

4.2.4. Seed contamination

4.2.4.1. By IM

An overnight culture of individual *Salmonella* and EHEC strain grown in Luria-Bertani no salt broth supplemented with NA (50 µg/ml) was ten-fold serially diluted in sterilized water and 20 ml of each inoculum with a cell population of *ca*. 10^4 CFU/ml was added to the centrifuge tubes with sanitized seeds. The numbers of bacterial cells used for vegetable seed inoculation were determined by plating 0.1 ml of appropriately diluted cultures on TSA containing NA. Vegetable seeds in the centrifuge tubes were agitated

horizontally at 100 rpm in an orbital platform shaker (Model: 3520, Lab-line, IL, U.S.A) at 20 °C for 1 h and the inocula were then discarded. Pathogen cells on each type of contaminated seeds were enumerated to determine the initial level of bacterial contamination on vegetable seeds.

4.2.4.2. By SO

Ten ml overnight culture of each *Salmonella* and EHEC strain grown in tryptic soy broth (Becton Dickenson, Sparks, MD) supplemented with NA (50 µg/ml) was centrifuged at 5,000 *g* for 5 min. The cell pellets were washed with 10 ml sterilized deionized water and then re-suspended in 20 ml 10% sterilized skim milk (Walmart, Bentonville, AR) (8 log CFU/ml). Each bacterial suspension (5 ml) was transferred into a 10-ml glass test tube (Fisher Scientific, Asheville, NC) and was frozen at -20 °C in a static state for 24 h. After overnight storage, samples were desiccated in a Free Zone Benchtop Freeze Dry System (Labconco, Kansas City, MO) at a condenser temperature of -40 °C and a chamber pressure of <0.05 mbar for 10 h. The end temperature of the samples was 28 °C. After freeze-drying, the test tubes with freeze-dried bacterial cells were sealed immediately and stored at -20 °C until use.

Twenty g of sandy soil (Mosser Lee Co., Millston, WI) sterilized at 121 °C for 15 min were mixed with 0.01 g (*ca.* 10^6 CFU) of each freeze-dried bacterial culture in a Whirl-Pak bag (1 oz., Nasco, Fort Atkinson, WI) at 200 rpm on an orbital platform shaker (Model: 3520, Lab-line, IL, U.S.A) at room temperature for 12 h. The precise numbers of bacterial cells in the inocula were determined as described previously. The

contaminated soils were subsequently mixed with sanitized vegetable seeds at 200 rpm on an orbital platform shaker at room temperature for 1 h.

4.2.5. Seed germination and sprout/seedling growth

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

4.2.6. Sample preparation and microbiological analyses

On the first day of germination, pathogen counts on vegetable seeds (inoculated seeds with no differentiated tissue sections) were determined whereas on the third day of

germination, developed sprouts/seedlings were carefully dissected using sterilized forceps and scissors, and the seed coat/cotyledon (combination of seed coat and cotyledon due to difficulty in separating the two tissue sections), stem, and root tissues were collected. The roots were tissues with fibrils and the boundary between root and stem tissues was determined by their positions in relation to the surface of water agar. The portion above the surface of water agar was taken as stem tissues and that beneath the surface of agar was the root tissue. On the fifth day and forward, seed coat and cotyledon tissues were analyzed separately. Different tissue sections of sprouts/seedlings sampled in the study are shown in Fig. 1.

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

4.2.7. Statistical analysis

Fisher's least significant difference test in the general linear model was conducted, using the Statistical Analysis Software (version 9.4; SAS Institute Inc., Carey,

N.C.), to determine the difference in the cell population (log CFU/0.01 g of seedling/sprout tissues) of various *Salmonella* or EHEC strains recovered from different sprout/seedling tissues developed from vegetable seeds by each contamination method. In addition, pathogen counts recovered at different sampling points during germination, from all sprout/seedling tissue sections developed from a single seed type, and from individual tissue sections of all 4 types of sprouts/seedlings were compared. All the tests were performed with a significant level of 0.05.

4.3. Results

4.3.1. Overall statistical analysis

Among the total of 544 tissue samples analyzed in the entire germination process involving each pathogen (Table 4.1 and 4.2), both the incidence of pathogen-positive samples and the mean population of pathogens on tissues from seeds contaminated by IM (*ca.* 92.0%, 6.5-6.7 log CFU/g) were significantly higher than those from seeds by SO (*ca.* 71% %, 5.2 log CFU/g). In both circumstances, the mean populations of bacteria recovered from seed coat samples were significantly ($P < 0.05$) higher than those from the cotyledon and root samples, followed by those from stem samples. *Salmonella* and EHEC populations from the pre-germinated seeds were significantly lower than other samples ($P < 0.05$, Table 4.1 and 4.2). When seeds were contaminated by IM, lettuce seedling and alfalfa sprout tissues had significantly higher ($P < 0.05$) mean EHEC populations than the tissues of fenugreek sprouts and tomato seedlings. When seeds were

contaminated by SO, however, fenugreek sprouts and lettuce seedings had significantly higher ($P < 0.05$) *Salmonella* and EHEC populations than alfalfa sprouts and tomato seedlings (Table 4.1 and 4.2).

When seeds were contaminated by IM, the mean population of *S.* Cubana was significantly higher ($P < 0.05$) than the populations of other 3 *Salmonella* strains used in this study (Table 4.1). When seeds were contaminated by SO however, the four *Salmonella* strains recovered from sprout/seedling tissues were significantly different ($P < 0.05$); *S.* Baildon had the highest mean cell population, followed by *S.* Stanley, *S.* Cubana, and *S.* Montevideo. For both inoculation methods, *E. coli* O104:H4 BAA-2326 established the lowest cell population on sprout/seedling tissues among all EHEC strains used in the study (Table 4.2). Mean populations of the three *E. coli* O157:H7 strains were significantly different ($P < 0.05$) with F4546 had the highest mean cell population. Mean population of H1730 was significantly higher than K4499 when seeds were contaminated by IM, however this order was reversed when seeds were contaminated by SO (Table 4.2).

4.3.2. *Pathogen population change over time during germination*

When seeds were contaminated by IM, the mean populations of *Salmonella* on tissues of all sprouts/seedlings increased significantly ($P < 0.05$) with germination time (Table 4.1). A similar trend was observed with samples contaminated by SO, except that the population on Day 5 was statistically ($P > 0.05$) similar to that on Day 7. The mean population of EHEC on tissues of all sprouts/seedlings recovered from seeds

contaminated by both inoculation methods increased significantly ($P < 0.05$) as the prolongation of germination time except that the population on Day 3 was statistically ($P > 0.05$) similar to that on Day 5 (Table 4.2).

Detailed *Salmonella* and EHEC population change on sprouts/seedlings developed from individual seed types contaminated by IM and SO are shown in Fig. 4.2 and 4.3, respectively. When seeds were contaminated by IM, the mean populations of the four *Salmonella* or EHEC strains from alfalfa, fenugreek, and lettuce tissues increased *ca*. 3.6-4.7 log CFU/g during the first three days of germination and no significant population increase was noticed thereafter. The mean *Salmonella* populations on tomato samples increased *ca*. 2.5 log CFU/1 g on the first day, and another significant population increase was not seen until Day 7 to Day 9 (Fig. 4.2a). The mean EHEC populations from all sprout/seedling tissues were stable after a significant increase on the first day of germination except an unexpected population drop on tomato seedling tissues on Day 5 (Fig. 4.2b). However, when the seeds were contaminated by SO, cell populations of both *Salmonella* and EHEC on tissues of fenugreek and lettuce increased over time (Fig. 4.3). The mean *Salmonella* population on alfalfa sprouts increased to *ca.* 5.7 log CFU/g on the 5^{th} day of germination and then decreased gradually thereafter. A significant increase in *Salmonella* population on tomato seedlings did not occur until Day 7 (Fig. 4.3a). The mean EHEC population recovered from alfalfa sprouts tissues was stable after a significant growth on the first day of germination. A significant increase in EHEC population on tomato seedlings took place after Day 5 to Day 7 and remained relatively stable thereafter (Fig. 4.3b).

4.3.3. Mean populations of bacterial pathogens *on individual tissue sections of each type of sprouts/seedlings*

4.3.3.1. Mean populations of Salmonella *and EHEC on individual tissue sections of each type of sprouts/seedlings developed from seeds contaminated by IM*

When seeds were contaminated by IM, *Salmonella* and EHEC counts from seed coats of alfalfa and fenugreek sprouts as well as lettuce seedlings were significantly higher ($P < 0.05$) than those from all other tissue samples (Table 4.3 and 4.4). The average cell populations from the root, seed coat/cotyledon, and cotyledon samples of the three types of sprouts/seedlings were significantly higher ($P < 0.05$) than those from the stem samples. Pre-germinated alfalfa, fenugreek, and lettuce seeds had the lowest average cell counts. No significant difference ($P > 0.05$) was observed among *Salmonella* and EHEC populations from tomato seed coat, root, seed coat/cotyledon, cotyledon, and stem samples, but cell populations on these tissues were significantly higher than the population of pre-germinated seeds (Table 4.3 and 4.4).

4.3.3.2. Mean populations of Salmonella *and EHEC on individual tissue sections of each type of sprouts/seedlings developed from seeds contaminated by SO*

Pre-germinated vegetable seeds of all types contaminated by SO had the lowest mean populations of *Salmonella* (Table 4.5). *Salmonella* counts from fenugreek and lettuce seed coats were significantly higher than those recovered from other fenugreek

tissues. The average *Salmonella* populations from fenugreek seed coat/cotyledon, cotyledon, and root samples were statistically similar but they were significantly higher than the cell population from the stem samples. Similar *Salmonella* populations were also associated with all alfalfa tissue samples. Tomato seed coat/cotyledon tissues had significantly lower *Salmonella* populations than other tomato tissues.

Similar to *Salmonella*-contaminated samples, all four types of pre-germinated vegetable seeds had the lowest EHEC counts (Table 4.6). EHEC counts from seed coats of fenugreek sprouts and lettuce seedlings were significantly higher than those from other samples of the same seed types and the average cell populations from the seed coat/cotyledon and root samples were significantly higher than those from the cotyledon samples and stem samples. Similar to what was observed with *Salmonella*, there was no significant difference among EHEC populations recovered from the tested alfalfa tissue samples. The populations of EHEC on tomato seed coat, root, and cotyledon were not significantly different, but the recovery population from the seed coat was significantly higher than the populations from the stem tissues, and seed coat/cotyledon tissue samples.

4.3.4. Mean populations of individual Salmonella *and EHEC strains on sprout/seedling tissues developed from individual seed type*

4.3.4.1. Mean populations of individual Salmonella *and EHEC strains on sprout/seedling tissues developed from individual type of seeds contaminated by IM*

When seeds were contaminated by IM, *S.* Baildon and *S.* Montevideo had similar ($P > 0.05$) mean cell populations on alfalfa, fenugreek, and lettuce tissues and these populations were significantly higher than ($P < 0.05$) the population on tomato tissues (Table 4.7). *S.* Cubana and *S.* Stanley established significantly higher ($P < 0.05$) cell populations on alfalfa and lettuce tissues compared to fenugreek and tomato tissues. In addition, the mean populations of the four *Salmonella* strains associated with lettuce and alfalfa tissues were not significantly different ($P < 0.05$). *S.* Baildon had significantly higher ($P < 0.05$) mean populations than *S.* Stanley on fenugreek tissues. *S.* Cubana had the highest mean population on tomato tissues, followed by *S.* Montevideo and S. Stanley, and then *S.* Baildon.

Populations of the three *E. coli* O157:H7 strains on alfalfa, fenugreek, and lettuce tissues were not significantly different ($P > 0.05$), and these cell populations were significantly higher ($P < 0.05$) than those from tomato tissues (Table 4.8). Populations of *E. coli* BAA-2326 on alfalfa and lettuce tissues were significantly higher ($P < 0.05$) than those on fenugreek and tomato tissues. Furthermore, the mean population of the three *E. coli* O157:H7 strains on alfalfa, fenugreek, and lettuce tissues were similar ($P > 0.05$), and significantly higher ($P < 0.05$) than the mean population of BAA-2326 (Table 4.8).

Strain F4546 had a similar ($P > 0.05$) mean population to that of H1730 but had a significantly higher ($P < 0.05$) mean population than the populations of K4499 on tomato tissues.

4.3.4.2. Mean populations of individual Salmonella *and EHEC strains on sprout/seedling tissues developed from individual type of seeds contaminated by SO*

The average cell populations of four individual *Salmonella* or EHEC strains from all tissue samples of each type of vegetable seeds contaminated by SO over the 9-day germination period are shown in Table 4.9 and 4.10, respectively. *S.* Baildon and *S.* Stanley had the highest mean populations on tissues of fenugreek sprouts and lettuce seedlings, followed by alfalfa sprouts, and then tomato seedlings (Table 4.9). Cell populations of *S.* Montevideo on lettuce seedlings were significantly higher than fenugreek sprouts, followed by alfalfa sprouts and tomato seedlings. . *S.* Cubana established the highest cell population on fenugreek sprouts, followed by lettuce seedings; those associated with alfalfa sprouts and tomato seedlings were significantly lower. In addition, the mean populations of the four individual *Salmonella* strains associated with tomato tissues were not significantly different. *S.* Montevideo had the lowest mean populations on tissues of the other 3 types of sprouts/seedlings. *S.* Baildon and *S.* Stanley had higher mean populations than *S.* Cubana on alfalfa and lettuce tissues. No significant differences were noticed among the mean populations of *S.* Baildon, *S.* Stanley, and *S.* Cubana on fenugreek tissues.

All four individual EHEC strains established significantly higher cell populations on fenugreek and lettuce tissues than alfalfa and tomato tissues (Table 4.10). *E. coli* O104:H4 BAA-2326 had the lowest mean populations on all tested vegetable tissues than the three *E. coli* O157:H7 strains. Strains F4546 and K4499 had significantly higher mean populations than those of H1730 on fenugreek and lettuce tissue. Strain F4546 also had a significantly higher mean population than those of K4499 on alfalfa and tomato tissues, followed by those of H1730.

4.4. Discussion

In the present study, vegetable seeds were contaminated by two inoculation methods, IM and SO, which may introduce different levels of pathogen cells into different areas of a vegetable seed. Rajkowski (2009) reported that when seeds were immersed into a bacterial suspension, cracks or breaks on seed coats became more pronounced and curled away from the cotyledon; thus, bacterial cells became trapped in the cracks or under the seed coat upon drying. In addition, exposure of bacterial cells to a liquid bacterial culture allowed more homogeneous contact between bacterial cells and seed. In general, bacterial cells are more attracted to hydrophobic than hydrophilic surfaces (Absolom et al., 1983; Tuson & Weibel, 2013). Therefore, more bacterial cells are likely associated with seed surface when seeds are inoculated by IM than SO. These could partially explain why a higher incidence of pathogen-positive tissues and mean pathogen populations were associated with tissue developed from seeds contaminated by IM than by SO. We observed that bacterial growth and moved along the surfaces of

sprouts/seedlings developed from seed contaminated by IM was relatively faster than those by SO. For example, after 5 days of germination, a 4.0 log CFU/1g increase in the mean population of EHEC was observed on tissues developed from seeds contaminated by IM while only 2.9 log CFU/1 g increase was observed on tissues developed from seeds contaminated by SO. The submersion of seeds into a bacterial suspension allowed them to be in contact with water, which enables the influx of water into dry seeds, triggering germination. This theory was supported by the observation that no visible budding was observed after 24 h of germination of lettuce seeds contaminated by SO. However, with IM, seedling shoots grew to as long as *ca.* 2 mm in the same time period (data not shown).

For both inoculation methods, the mean populations of the four *Salmonella* and EHEC strains on tissues of fenugreek and lettuce sprouts/seedlings as well as *Salmonella* on alfalfa sprouts increased significantly (*ca.* 3.5-5.0 log CFU/g) during the initial stage of germination and population increase was within 1.0 log CFU/g thereafter (Fig. 4.2 and 4.3). This growth trend was similar to what was observed by Castro-Rosas and Escartin (2000) who found that the populations of *S.* Typhi and *E. coli* O157:H7 increased from 3.0 to 6.0 log CFU/g during the initial stage of alfalfa seed germination and remained relatively stable in the next 10 days. Similar observations were made with diarrheagenic *E. coli* and *S.* Montevideo during mung bean seed germination (Gomez-Aldapa et al., 2013; Warriner, Spaniolas, Dickinson, Wright, & Waites, 2003), *Salmonella* and *E. coli* O157:H7 during butterhead lettuce seed germination (Van der Linden et al., 2013), and *Salmonella* during alfalfa seed germination (Howard & Hutcheson, 2003; Jaquette, Beuchat, & Mahon, 1996). At early stage of germination, sprouting seeds produce

nutrient exudates into the surrounding environment (Koizumi et al., 2008). These nutrients include low-molecular-weight metabolites consisting of carbohydrates, amino acids, flavonoids, sterols, and salts that provide essential nutrients for the growth of bacterial cells (Nelson, 1991; Schiltz et al., 2015).

E. coli O104:H4 BAA-2326 had the lowest number of cells compared to other EHEC strains on sprout/seedling tissues developed from seeds inoculated by both IM and SO (Table 4.2). BAA-2326 was isolated from a fenugreek sprout-associated outbreak of infections in Germany in 2011, which affected 3,842 people in several countries (Beutin & Martin, 2012). It evolved from enteroaggregative *E. coli* (EAEC) and acquired the genes for Shiga-toxin production from EHEC (Beutin & Martin, 2012). Although EAEC in general has the ability to produce aggregative adherence fimbriae I and a large amount of extracellular polymeric substances that mediate cell aggregation and biofilm formation, these substances could impair the initial bacterial adherence to their contact surfaces (Petrova & Sauer, 2012) including the surface of vegetable seeds (Cui, Walcott, & Chen, 2017). Compared to other bacterial strains used in this study, the lowest number of cells of BAA-2326 attached to pre-germinated vegetable seeds (data not shown), which might have a significant impact on the prevalence of pathogen-positive samples as well as the level of cell populations on sprout/seedling tissues.

The growth and movement of bacterial cells from vegetable seeds, contaminated by both IM and SO, to different tissues of sprouts/seedlings was demonstrated in the present study. Results indicated that seed coats had the highest number of pathogen cells (Table 4.1 and 4.2). Although some bacterial cells might have gained entry into vegetable seeds through the cracks, most of the bacterial cells were likely associated with

the surface of seeds, explaining the highest recovery cell populations on seat coats. In addition to seed coats, higher pathogen populations were also associated with the root and cotyledon tissues compared to the stem tissues. This observation agreed with a previous study by Warriner, Spaniolas, Dickinson, Wright, and Waites (2003) who found that the root tissues of mung bean sprouts had higher *E. coli* and *S.* Montevideo populations than the stem tissues. Another study reported that *S.* Newport and *E. coli* O157:H7 populations associated with *Arabidopsis thaliana* roots were approximately 2 log-higher than those associated with the stem tissues (Cooley, Miller, & Mandrell, 2003). As primary exudate-releasing organs, cotyledon and root tissues contain a large amount of nutrients such as carbohydrates, amino acids, and salts which could attract, and support the growth of, bacterial cells during germination (Rovira, 1969; Wolswinkel, 1992). It has been reported that bacterial cells tend to move toward nutrient-rich regions in capillary tubes, in soil (Scher, Kloepper, & Singleton, 1985), and in plants (Klerks, Franz, van Gent-Pelzer, Zijlstra, & Van Bruggen, 2007; Zhang et al., 2014), a phenomenon known as chemotaxis. For example, *S.* Typhimurium cells were attracted to nutrients produced by photosynthetically active cells on lettuce leaves under light, and mutations affecting chemotaxis significantly inhibited pathogen movement (Kroupitski et al., 2009).

Results of the present study revealed that pathogen growth on tissues of fenugreek sprouts and lettuce seedlings were more profound than those on tomato seedlings (Table 4.7-4.10). This could be partially caused by the lower initial level of pathogen cells attached to tomato seeds. When seeds were contaminated by IM, the average numbers of cells of the four EHEC strains and four *Salmonella* strains attached to tomato seeds were significantly lower than those of the other three types of seeds (Fig. 4.2). A positive

correlation between the initial contamination level of *Salmonella* on alfalfa seeds and the final population on alfalfa sprouts was reported by Liao and Fett (2003). Another factor that may affect the differential pathogen growth on seedlings from different seed types could be the nutrition profiles in seed exudates. Some germinating seeds contain natural antimicrobials that could adversely impact the growth of bacterial cells. Tomato exudates reportedly contain organic acids and a range of polyphenols (Urbonaviciene, Viskelis, Bartkiene, Juodeikiene, & Vidmantiene, 2015) and had a weakly acidic pH, which might adversely affect the growth of bacterial cells. In addition, a previous study by Tu (1972) reported that trace amounts of reducing sugars and amino acids that could be used by bacterial pathogen from tomato seedlings after 5 days of germination, which can explain why a significant increase of mean populations of *Salmonella* and EHEC cells on tomato tissues occurred after 5 days of growth (Fig. 4.2 and 4.3).

Salmonella and EHEC cells attached to pre-germinated vegetable seeds were recovered from tissues of lettuce and tomato seedlings in the present study. The eventual fate of recovered pathogens cells during later stage of plant development will determine the impact of seedling contamination on produce safety. The exposure of vegetable seedlings to environmental conditions such as fluctuating temperature, poor nutrient, desiccation, UV radiation, and competition from other microorganisms will adversely impact pathogen viability (Bodenhausen, Horton, & Bergelson, 2013). However, a previous study by Franz et al. (2007) found that lettuce leaves grown from 15% of the contaminated seedlings under greenhouse growth conditions (15 ºC with a relative humidity of 60%) tested positive for *Salmonella* and the leaves from 28% of the seedlings tested positive for *E. coli* O157:H7 with an average cell population of 2-3 log CFU/g.

4.5. Conclusions

Results of the study suggest that the growth and movement of *Salmonella* and EHEC cells on alfalfa, fenugreek, lettuce, and tomato sprout/seedling tissues are influenced by seed inoculation approach as well as bacterial strains, plant hosts, and sprout/seedling tissues involved. For example, the incidence of pathogen-positive samples and mean *Salmonella* and EHEC populations on tissues developed from seeds contaminated by IM were significantly higher than those from seeds by SO. *E. coli* O104:H4 BAA-2326 established the lowest cell population on sprout/seedling tissues; both *Salmonella* and EHEC cells had the lowest level of cell population on tomato seedlings; and seed coat, root, and cotyledon tissues had significantly higher counts of *Salmonella* and EHEC than the stem tissue. The study provides knowledge on the differential behavior of seed-borne bacterial pathogens on various tissues of sprouts/seedlings during germination and fills the knowledge gaps in the current body of literature.

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Figure Legends

Fig. 4.1. Different tissue sections of sprouting seeds sampled in the study.

Fig. 4.2. Mean cell populations of the four *Salmonella* (a) and four EHEC (b) strains recovered from tissues developed from alfalfa, fenugreek, lettuce, and tomato seeds, contaminated by immersion into bacterial suspensions, during germination.

Fig. 4.3. Mean cell populations of the four *Salmonella* (a) and four EHEC (b) strains recovered from tissues developed from alfalfa, fenugreek, lettuce, and tomato seeds, contaminated by contact with artificially-inoculated soil, during germination.

Table 4.1.
Mean populations of *Salmonella* recovered from all tested tissue samples at different sampling points, from all sprouts/seedlings tissues developed from a single seed type, and from each tissue section of all 4 types of sprouts/seedlings

	Salmonella populations (log CFU/0.01g)	
Comparative category	IM	SO
Sampling time during germinating (Day)		
0 (n = 32)	2.89 F [a]	1.83 E
1 (n = 32)	5.61 E	3.35 D
3 (n = 96)	6.59 D	4.61 C
5 (n = 128)	6.88 C	5.43 B
7 (n = 128)	7.04 B	5.68 B
9 (n = 128)	7.32 A	6.15 A
Bacterial strains		
S. Cubana (n = 136)	7.01 A	5.01 C
S. Stanley (n = 136)	6.63 B	5.54 B
S. Baildon (n = 136)	6.45 B	6.16 A
S. Montevideo (n = 136)	6.54 B	4.01 D
Tissue section of sprouts/seedlings		
Seed coat (n = 96)	7.84 A	6.44 A
Root (n = 128)	6.99 B	5.51 B
Cotyledon (n = 96)	7.11 B	5.58 B
Seed coat/cotyledon (n = 32)	6.97 B	5.19 C
Stem (n = 128)	6.22 C	4.90 C
Seed (n = 64)	4.25 D	2.59 D
Seed type from which sprouts/seedlings developed		
Lettuce (n = 136)	8.04 A	6.36 A
Alfalfa (n = 136)	7.93 A	4.58 B
Fenugreek (n = 136)	7.47 B	6.58 A
Tomato (n = 136)	3.20 C	5.20 C

[a] Statistical comparisons were based on Fisher's least significant difference test using the Statistical Analysis Software version 9.4. Values within each comparative parameter in the same column followed by the same letter were not significantly different ($P > 0.05$).

Table 4.2.
Mean populations of EHEC recovered from all tested tissue samples at different sampling points, from all sprouts/seedlings tissues developed from a single seed type, and from each tissue section of all 4 types of sprouts/seedlings

	EHEC populations (log CFU/0.01g)	
Comparative category	IM	SO
Sampling time during germinating (Day)		
0 (n = 32)	2.27 E [a]	1.29 E
1 (n = 32)	5.85 D	4.20 D
3 (n = 96)	6.42 C	4.90 C
5 (n = 128)	6.65 C	5.20 C
7 (n = 128)	7.00 B	5.56 B
9 (n = 128)	7.28 A	6.09 A
Bacterial strains		
F4546 (n = 136)	7.15 A	6.31 A
H1730 (n = 136)	6.97 A	4.72 C
K4499 (n = 136)	6.76 B	5.59 B
BAA-2326 (n = 136)	5.23 C	4.06 D
Tissue section of sprouts/seedlings		
Seed coat (n = 96)	7.77 A	6.38 A
Root (n = 128)	7.02 B	5.49 B
Cotyledon (n = 96)	6.75 B	5.39 B
Seed coat/cotyledon (n = 32)	7.04 B	5.62 B
Stem (n = 128)	6.03 C	4.73 C
Seed (n = 64)	4.06 D	3.05 D
Seed type from which sprouts/seedlings developed		
Lettuce (n = 136)	7.82 A	6.88 A
Alfalfa (n = 136)	7.74A	3.33 B
Fenugreek (n = 136)	7.46 B	6.84 A
Tomato (n = 136)	3.08 C	3.62 B

[a] Statistical comparisons were based on Fisher's least significant difference test using the Statistical Analysis Software version 9.4. Values within each comparative parameter in the same column followed by the same letter were not significantly different ($P > 0.05$).

Table 4.3.
Salmonella populations from various tissues of sprouts/seedlings developed from alfalfa, fenugreek, lettuce, and tomato seeds, contaminated by immersion into bacterial suspensions, during 9 days of germination

	BSA [a] (log CFU/g)				NATSA [b] (log CFU/g)			
	Alfalfa	Fenugreek	Lettuce	Tomato	Alfalfa	Fenugreek	Lettuce	Tomato
Cotyledon	8.25 Ba [c]	8.06 Ba	8.04 Ca	4.09 Ab	8.31 Ba	8.14 Ba	8.17 Ca	4.11 Ab
Root	8.45 Ba	7.61 Cb	8.66 Ba	3.24 Ac	8.47 Ba	7.56 Cb	8.72 Ba	3.22 Ac
Seed	5.29 Dab	4.67 Eb	5.80 Ea	1.25 Bc	5.21 Dab	4.76 Eb	5.92 Ea	1.20 Bc
Seed coat/cotyledon	8.32 Ba	7.89 BCa	8.41 Ba	3.28 Ab	8.41 Ba	7.85 BCa	8.45 Ba	3.15 Ab
Seed coat	9.27 Aa	9.50 Aa	9.45 Aa	3.12 Ab	9.32 Aa	9.56 Aa	9.52 Aa	3.32 Ab
Stem	7.38 Ca	6.66 Db	7.39 Da	3.46 Ac	7.39 Ca	6.74 Db	7.44 Da	3.48 Ac

[a] BSA: Bismuth sulfite agar
[b] NATSA: Tryptic soy agar supplemented with 50 µg/ml of nalidixic acid
[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$).

Table 4.4.
EHEC cell populations from various tissues of sprouts/seedlings developed from alfalfa, fenugreek, lettuce, and tomato seeds, contaminated by immersion into bacterial suspension, during 9 days of germination

	NASMAC [a] (log CFU/g)				NATSA [b] (log CFU/g)			
	Alfalfa	Fenugreek	Lettuce	Tomato	Alfalfa	Fenugreek	Lettuce	Tomato
Cotyledon	7.94 Ba [c]	8.00 Ba	7.94 Ba	3.14 Ab	7.98 Ba	8.10 Ba	8.00 Ba	3.29 Ab
Root	8.31 Ba	7.72 Bb	8.46 Ba	3.59 Ac	8.34 Ba	7.75 Bb	8.47 Ba	3.73 Ac
Seed	5.14 Da	4.61 Da	4.76 Da	1.73 Bb	5.35 Da	4.85 Da	4.60 Da	1.74 Bb
Seed coat/cotyledon	7.96 Ba	7.95 Ba	8.38 Ba	3.88 Ab	8.02 Ba	7.95 Ba	8.55 Ba	3.96 Ab
Seed coat	9.23 Aa	9.36 Aa	9.48 Aa	3.01 Ab	9.27 Aa	9.40 Aa	9.55 Aa	3.04 Ab
Stem	7.16 Ca	6.67 Cb	7.25 Ca	3.05 Ac	7.22 Ca	6.73 Cb	7.29 Ca	3.35 Ac

[a] NASMAC: Sorbitol MacConkey agar supplemented with 50 µg/ml of nalidixic acid;
[b] NATSA: Tryptic soy agar supplemented with 50 µg/ml of nalidixic acid
[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$).

Table 4.5.
Number of *Salmonella* cells recovered from various tissues of sprouts/seedlings developed from alfalfa, fenugreek, lettuce and tomato seeds, contaminated by direct contact with artificially-inoculated soil, during 9 days of germination

	Log CFU/0.01 g (recovered from BSA [a])				Log CFU/0.01 g (NATSA [b])			
	Alfalfa	Fenugreek	Lettuce	Tomato	Alfalfa	Fenugreek	Lettuce	Tomato
Cotyledon	4.92 Ac [c]	7.37 Ba	6.52 Bb	3.53 Ad	4.94 Ac	7.41 Ba	6.61 Bb	3.55 Ad
Root	5.02 Ab	6.75 Ba	6.88 Ba	3.39 Ac	5.10 Ab	6.84 Ba	6.98 Ba	3.39 Ac
Seed	2.16 Bc	3.25 Da	3.02 Db	1.93 Cc	2.33 Bc	3.27 Da	3.11 Db	1.93 Cc
Seed coat/ cotyledon	4.49 Ab	7.24 Ba	6.36 Ba	2.68 Bc	4.54 Ab	7.22 Ba	6.51 Ba	2.67 Bc
Seed coat	5.51 Ab	8.49 Aa	8.04 Aa	3.71 Ac	5.51 Ab	8.52 Aa	8.10 Aa	3.71 Ac
Stem	4.41 Ab	5.91 Ca	6.13 Ca	3.15 Ac	4.46 Ab	5.91 Ca	6.19 Ca	3.11 Ac

[a] BSA: Bismuth sulfite agar
[b] NATSA: Tryptic soy agar supplemented with 50 µg/ml nalidixic acid
[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$).

Table 4.6.
Number of EHEC cells recovered from various tissues of sprouts/seedlings originated from alfalfa, fenugreek, lettuce and tomato seeds, contaminated by direct contact with artificially-inoculated soil, during 9 days of germination

	Log CFU/0.01 g (recovered from NASMAC [a])				Log CFU/0.01 g (NATSA [b])			
	Alfalfa	Fenugreek	Lettuce	Tomato	Alfalfa	Fenugreek	Lettuce	Tomato
Cotyledon	3.63 Ab [c]	7.21 Ca	6.65 Ca	4.07 ABb	3.64 Ac [c]	7.30 Ca	6.95 Ca	4.12 ABb
Root	3.49 Ab	7.44 Ba	7.39 Ba	3.61 ABb	3.49 Ab	7.54 Ba	7.48 Ba	3.64 ABb
Seed	2.31 Bc	3.43 Eb	4.33 Ea	2.15 Dc	2.39 Bb	3.57 Ea	4.50 Ea	2.35 Db
Seed coat/ cotyledon	4.17 Ab	7.94 Ba	7.76 Ba	2.61 Cb	4.17 Ab	8.05 Ba	7.92 Ba	2.65 Cb
Seed coat	3.57 Ab	8.57 Aa	8.58 Aa	4.77 Ab	3.59 Ab	8.80 Aa	8.71 Aa	4.76 Ab
Stem	3.06 Ab	6.11 Da	6.34 Da	3.43 Bb	3.09 Ab	6.23 Da	6.37 Da	3.50 Bb

[a] NASMAC: Sorbitol MacConkey agar supplemented with 50 µg/ml nalidixic acid
[b] NATSA: Tryptic soy agar supplemented with 50 µg/ml nalidixic acid
[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$).

Table 4.7.
Mean populations of individual strains of *Salmonella* cells from all sprouts/seedlings tissues developed from alfalfa, fenugreek, lettuce, and tomato seeds, contaminated by immersion into bacterial suspension, during 9 days of germination

	BSA [a] (log CFU/g)				NATSA [b] (log CFU/g)			
	S. Baildon	*S.* Cubana	*S.* Montevideo	*S.* Stanley	*S.* Baildon	*S.* Cubana	*S.* Montevideo	*S.* Stanley
Alfalfa	7.99 Aa [c]	7.95 Aa	7.81 Aa	7.97 Aa	8.04 Aa	7.97 Aa	7.77 Aa	8.04 Aa
Fenugreek	7.79 Aa	7.54 Bab	7.43 Aab	7.12 Bb	7.81 Aa	7.59 Bab	7.46 Aab	7.18 Bab
Lettuce	8.12 Aa	8.12 Aa	7.92 Aa	8.01 Aa	8.21 Aa	8.21 Aa	7.99 Aa	8.06 Aa
Tomato	1.91 Bc	4.42 Ca	2.99 Bb	3.43 Cb	1.89 Bc	4.38 Ca	3.15 Bb	3.43 Cb

[a] BSA: Bismuth sulfite agar
[b] NATSA: Tryptic soy agar supplemented with 50 µg/ml of nalidixic acid
[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$).

Table 4.8.
Mean populations of individual strains of EHEC cells from all sprouts/seedlings tissues developed from alfalfa, fenugreek, lettuce, and tomato seeds, contaminated by immersion into bacterial suspension, during 9 days of germination

	NASMAC [a] (log CFU/g)				NATSA [b] (log CFU/g)			
	BAA-2326	F4546	H1730	K4499	BAA-2326	F4546	H1730	K4499
Alfalfa	6.56 Ab [c]	8.20 Aa	8.20 Aa	8.01 Aa	6.70 Ab	8.24 Aa	8.22 Aa	8.06 Aa
Fenugreek	5.96 Bb	7.99 Aa	8.06 Aa	7.82 Aa	6.15 Bb	8.01 Aa	8.08 Aa	7.90 Aa
Lettuce	6.69 Ab	8.25 Aa	8.22 Aa	8.12 Aa	6.82 Ab	8.22 Aa	8.26 Aa	8.10 Aa
Tomato	1.70 Cc	4.17 Ba	3.38 Bab	3.06 Bb	1.74 Cc	4.40 Ba	3.53 Bab	3.23 Bb

[a] NASMAC: Sorbitol MacConkey agar supplemented with 50 µg/ml of nalidixic acid
[b] NATSA: Tryptic soy agar supplemented with 50 µg/ml of nalidixic acid
[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$).

Table 4.9.
Number of *Salmonella* cells recovered from samples originated from alfalfa, fenugreek, lettuce, and tomato seeds, contaminated by direct contact with artificially-inoculated soil, during 9 days of germination

	Log CFU/0.01 g (recovered from BSA [a])				Log CFU/0.01 g (NATSA [b])			
	S. Baildon	*S.* Cubana	*S.* Montevideo	*S.* Stanley	*S.* Baildon	*S.* Cubana	*S.* Montevideo	*S.* Stanley
Alfalfa	6.32 Ba [c]	3.74 Cc	3.05 Cd	5.21 Bb	6.36 Ba [c]	3.79 Cc	3.06 Cd	5.32 Bb
Fenugreek	7.75 Aa	7.07 Aa	4.52 Bb	7.00 Aa	7.76 Aa	7.15 Aa	4.52 Bb	7.05 Aa
Lettuce	7.30 Aa	6.12 Bb	5.28 Ac	6.75 Aa	7.38 Aa	6.18 Bb	5.34 Ac	6.88 Aa
Tomato	3.29 Ca	3.13 Ca	3.18 Ca	3.20 Ca	3.28 Ca	3.10 Ca	3.19 Ca	3.21 Ca

[a] BSA: Bismuth sulfite agar
[b] NATSA: Tryptic soy agar supplemented with 50 µg/ml nalidixic acid
[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$).

Table 4.10.
Number of EHEC cells recovered from samples originated from alfalfa, fenugreek, lettuce, and tomato seeds, contaminated by direct contact with artificially-inoculated soil, during 9 days of germination

	Log CFU/0.01 g (recovered from NASMAC [a])				Log CFU/0.01 g (NATSA [b])			
	BAA-2326	F4546	H1730	K4499	BAA-2326	F4546	H1730	K4499
Alfalfa	2.58 Bc [c]	4.78 Ba	3.05 Bb	2.91 Bb	2.60 Bc	4.83 Ba	3.05 Bb	2.90 Bb
Fenugreek	5.42 Ac	7.60 Aa	6.59 Ab	7.76 Aa	5.61 Ac	7.71 Aa	6.72 Ab	7.86 Aa
Lettuce	5.72 Ac	7.74 Aa	6.22 Ab	7.86 Aa	6.01 Ac	7.83 Aa	6.30 Ab	7.93 Aa
Tomato	2.50 Bc	5.14 Ba	2.98 Bc	3.83 Bb	2.53 Bc	5.24 Ba	3.02 Bc	3.92 Bb

[a] NASMAC: Sorbitol MacConkey agar supplemented with 50 µg/ml nalidixic acid
[b] NATSA: Tryptic soy agar supplemented with 50 µg/ml nalidixic acid
[c] Values within the same column followed by the same uppercase letter were not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter were not significantly different ($P > 0.05$).

Fig. 4.1.

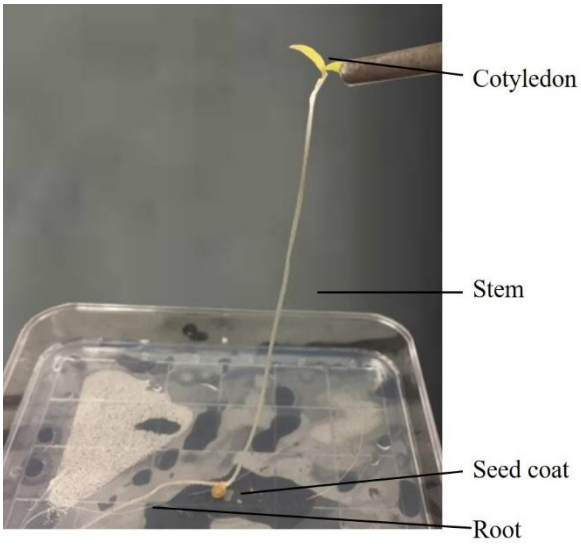
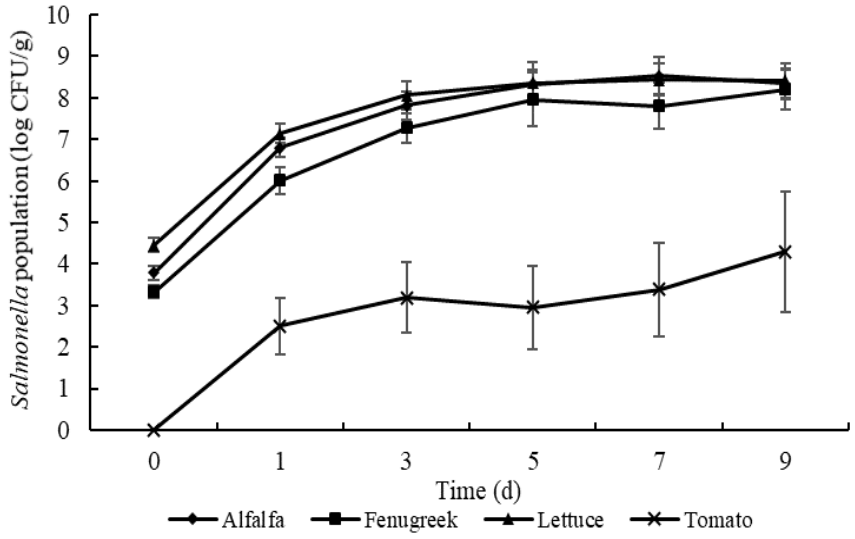


Fig. 4.2.

a.


Salmonella population (log CFU/g)
10
9
8
7
6
5
4
3
2
1
0
0
1
3
5
7
9
Time (d)
Alfalfa
Fenugreek
Lettuce
Tomato

b.

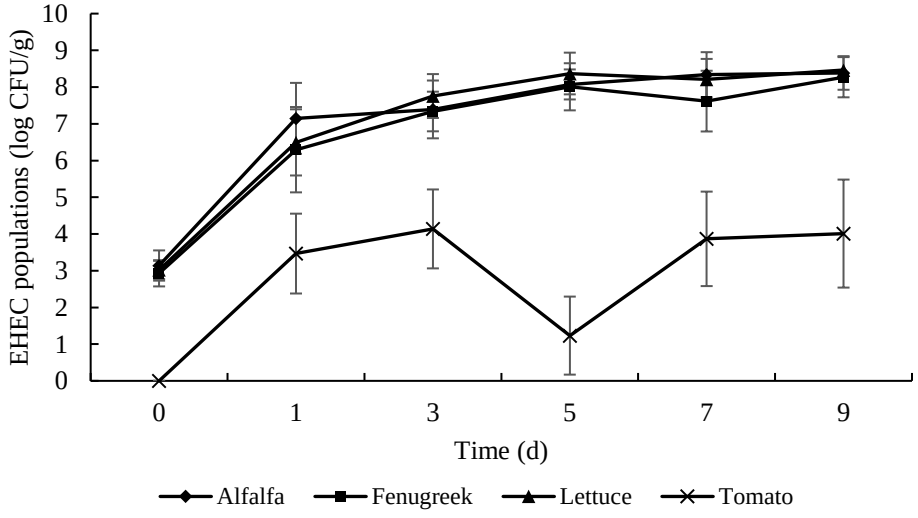
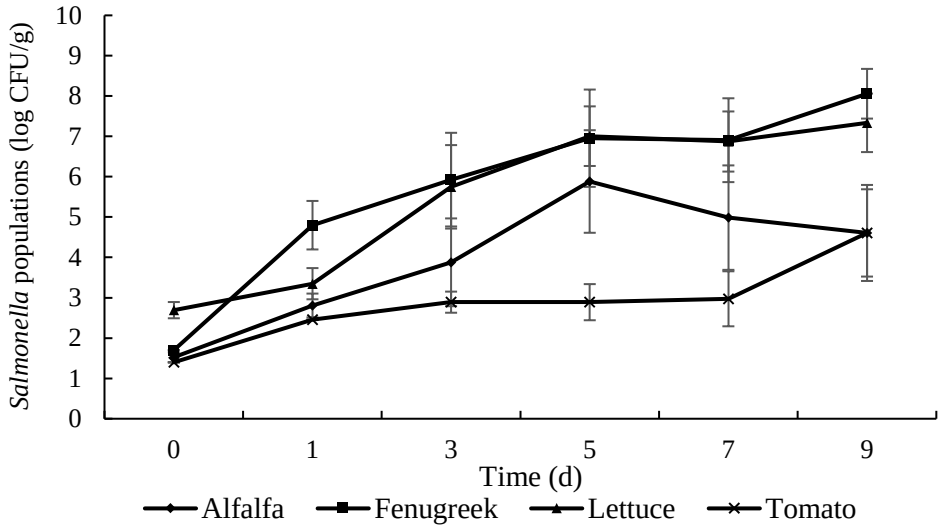
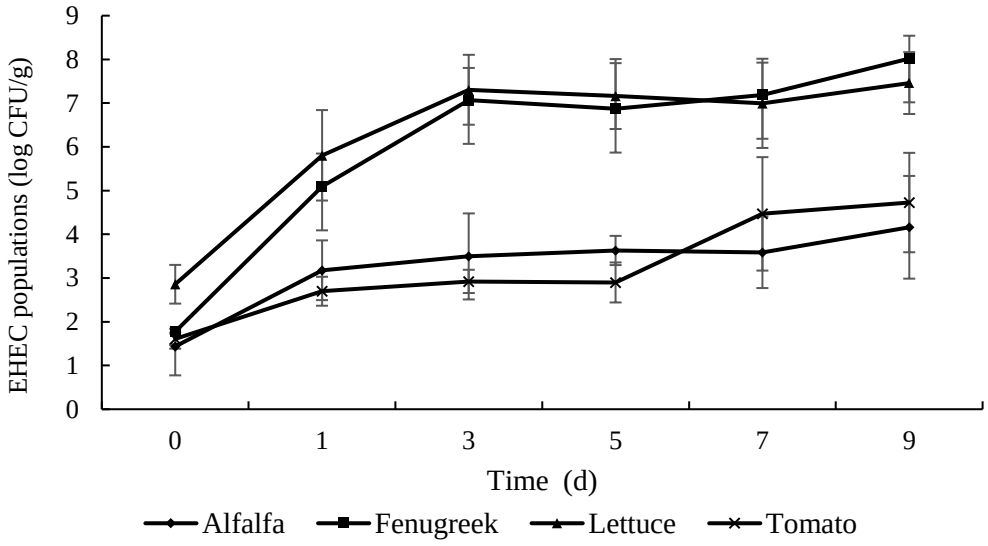

EHEC populations (log CFU/g)
10
9
8
7
6
5
4
3
2
1
0
0
1
3
5
7
9
Time (d)
Alfalfa
Fenugreek
Lettuce
Tomato

Fig. 4.3.

a.


Salmonella populations (log CFU/g)
10
9
8
7
6
5
4
3
2
1
0
0
1
3
5
7
9
Time (d)
Alfalfa
Fenugreek
Lettuce
Tomato

b.


EHEC populations (log CFU/g)
9
8
7
6
5
4
3
2
1
0
0
1
3
5
7
9
Time (d)
Alfalfa
Fenugreek
Lettuce
Tomato

CHAPTER 5

CONTROLS OF *SALMONELLA* AND *ESCHERICHIA COLI* O157:H7 ON ALFALFA SEEDS AND SPROUTS USING PROBIOTIC TOMATO FERMENTS

To be submitted to *Food Control.*

ABSTRACT

This study was undertaken to evaluate the efficacy of probiotic tomato ferments on the inactivation of *S. enterica* and *E. coli* O157:H7 on alfalfa seeds and sprouts. Fully ripened, disease-free, Roma tomatoes were rinsed, cut, and manually homogenated aseptically. Two hundred ml of the tomato homogenate were mixed with 1 ml of 9 log CFU/ml probiotic culture of *L. rhamnosus* (G) or a probiotic mixture (M) or 1 ml of sterilized water. After fermenting at 21 ºC for 4 days, pulp, supernatant, and the entire content of the probiotic tomato ferments were tested for their antimicrobial efficacies towards *S.* Cubana and *E. coli* F4546 in a microtiter plate assay. Supernatant of tomato ferment prepared with probiotic M (Su-M) was selected to disinfect alfalfa seeds and sprouts artificially inoculated with the overnight cultures of *S.* Cubana and *E. coli* F4546 at *ca*. 6 log CFU/g. Inoculated alfalfa seeds (2 g) and alfalfa sprouts (1 g) were treated with 10 ml of Su-M at room temperature for 12 h. Bacterial populations were determined at 8 different time intervals. Germination ratio of Su-M-treated alfalfa seeds was also determined. Probiotic tomato ferments were more effective than spontaneous ferment in reducing the pathogen populations in the microtiter plate assay. Furthermore, supernatants of the ferments had significantly higher antimicrobial potentials than the pulps. Su-M was significantly more effective against *S.* Cubana than *E. coli* F4546 on alfalfa seeds and sprouts. *S.* Cubana and *E. coli* F4546 populations on alfalfa seeds decreased 3.4 and 2.3 log CFU/g, respectively at the 240 min sampling point; this treatment did not seem to have compromised the germination percentage of the alfalfa seeds. A 6.4 log CFU/g reduction in the population of *S.* Cubana on alfalfa sprouts were achieved with a 60 min treatment whereas the same treatment only reduced the population of *E. coli* O157:H7 by 1.6 log CFU/g. Results suggested that probiotic tomato ferments could be used to disinfect vegetable seeds and sprouts.

Keywords: *Salmonella*; *E. coli* O157:H7; probiotic tomato ferments; alfalfa seed/sprouts

5.1. Introduction

Seed sprouts are considered as healthy foods because they are low in calories and fat and high in protein, vitamins, minerals, and antioxidants (Marton, Mandoki, Csapo-Kiss, & Csapo, 2010). Behind these healthy images, sprouts are one of the most common vehicles for transmitting foodborne bacterial diseases (Callejón et al., 2015). During the period of 2007-2016, there were at least 32 sprout-associated outbreaks in the United States (CDC, 2017). Alfalfa sprouts were implicated in most of these outbreaks and other outbreaks have been linked to the consumption of bean and clover sprouts (Ding, Fu, & Smith, 2013). The bacterial pathogens responsible for these outbreaks include *S. enterica* and *E. coli* O157:H7.

According to the FDA (2012), most sprout-related outbreaks have been due to seeds contaminated with bacterial pathogens. Many pathogens can survive for months under the dry conditions used for seed storage (Van der Linden et al., 2013). Although the number of bacterial cells in/on sprout seeds is exceptionally low, the number of cells on sprouts is high since sprouting condition favors the proliferation of bacterial cells. Research has shown that the levels of *Salmonella* and *E. coli* O157:H7 can reach 4 log CFU/g during the first 24 h of sprouting even when the level of pathogen contamination on sprout seeds was as low as 0.1 CFU/g (Stewart, Reineke, Ulaszek, & Tortorello, 2001). Thus, seed treatment is an effective intervention approach used by the sprout industry (NACMCF, 1999).

Currently, the National Advisory Committee on Microbiological Criteria for Foods recommends the use of 20,000 ppm calcium hypochlorite on sprout seeds (NACMCF, 1999), but this treatment does not guarantee the removal of all types of pathogens (Beuchat, 1997; Fu, Reineke, Chirtel, & VanPelt, 2008; Montville & Schaffner, 2004). In addition, chlorine-based

sanitation treatments may lead to the formation of carcinogenic by-products (Van Haute, Sampers, Jacxsens, & Uyttendaele, 2015). Therefore, the development of alternative treatments for sprout seeds and seed sprouts has drawn the attention of seed safety specialists and researchers.

Commercial tomato seeds are processed through a natural fermentation process in order to eliminate the chemical compounds that inhibit germination (Nemati, Nazdar, Azizi, & Arouiee, 2010). The residue of the ferments has been shown to prevent the attachment of *Salmonella* and *E. coli* O157:H7 to tomato seeds (Cui, Walcott, & Chen, 2017). The objective of this study was to evaluate the efficacy of probiotic tomato ferments in the inactivation of selected bacterial pathogens artificially inoculated onto alfalfa seeds and sprouts.

5.2. Materials and methods

5.2.1. Bacterial strains and growth conditions

The bacterial strains used in the study included *S. enterica* serovar Cubana, and *E. coli* O157:H7 F4546, both of which were involved in alfalfa sprout associated outbreaks of infections. The bacterial strains were retrieved from frozen storage at -70ºC and grown on tryptic soy agar (TSA; BD Diagnostic Systems, Sparks, MD) at 37ºC for 16 h. The resulting cultures were purified on xylose lysine tergitol-4 agar (XLT4), and sorbitol MacConkey (SMAC), respectively. Spontaneous mutant cells of each bacterial strain, resistant to 50 µg/ml of nalidixic acid (MP Biomedicals, Santa Ana, CA) were selected and used throughout the study.

Two commercial probiotic starters, designated as G and M, respectively were used in the study. Probiotic G (Culturelle®, Cromwell, CT) had a single probiotic strain of *L. rhamnosus* GG and each capsule was claimed by the manufacturer to have 10 billion live cells. Probiotic M (Flora Source® Multi-Probiotic®) was a probiotic mixture that had a manufacturer's claim of 16 billion live cells per capsule and contained 16 different bacterial strains including *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium lactis*, *Bifidobacterium lactis* Bif Relief 24-7™, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, *Lactococcus lactis* and *Streptococcus thermophilus*. The exact strain ratio of this product is not available since they are considered proprietary information by the manufacturers.

5.2.2. Preparation of probiotic tomato ferments

Fully ripened, disease-free, Roma tomatoes (*Solanum lycopersicum*) were purchased from a local grocery store in Griffin, GA. The tomatoes were rinsed in water to remove dirt and then were cut aseptically, using a sterile kitchen knife (Walmart, Bentonville, AR). The tomatoes were squeezed manually, with a gloved hand, in a sterilized stainless-steel mixing bowl (7.6 L; Walmart, Bentonville, AR), to obtain a homogenate with the size of the pulps being smaller than *ca*. 1 cm^3. Two hundred ml of the tomato homogenate was transferred to a 250 ml sterilized beaker aseptically. For the two treatment groups, 9 log CFU of G or M in 1 ml of distilled water was added. One ml of sterilized water was added to the tomato homogenate in the control group. The tomato homogenates were incubated at 21 ± 1 ºC for 4 days. The ferments

were stirred once or twice a day to submerge the pulp and to prevent the build-up of mold. After 4 days, 50 ml of tomato ferments (TF) were centrifuged at 5,000 *g* for 10 min to separate the pulps and supernatants. The pulps (TF-Pu), supernatants (TF-Su), and entire content of the ferments (TF-SuPu) were used to inactivate *S*. cubana and *E. coli* F4546 in a microtiter plate assay and on alfalfa seeds and sprouts. Three independent trials were conducted.

5.2.3. Sensitivity of S. *Cubana and* E. coli *F4546 to probiotic TF*

An overnight culture of *S.* Cubana and *E. coli* F4546 grown in tryptic soy broth (TSB; BD Diagnostic Systems, Sparks, MD) supplemented with nalidixic acid (50 µg/ml) was serially diluted in sterile water, to *ca.* 5×10^6 CFU/ml. The precise numbers of bacterial cells used for vegetable seed inoculation were determined by plating 0.1 ml of appropriately diluted cultures on TSA containing nalidixic acid. The TF-Su, TF-Pu, or TF-SuPu described above (0.9 ml or 0.9 g) was mixed with 0.1 ml of each bacterial culture in separate wells of a 24-well tissue culture plate (Corning Incorporated, Durham, NC). The control group had the same composition except that the ferment was replaced with 0.9 ml of distilled water. The tissue culture plate was agitated horizontally at 100 rpm in an orbital platform shaker (Model: 3520, Lab-line, IL) at 20 ± 1 ^{o}C for 24 h. For *S.* Cubana, aliquots were removed after 0, 2, 5, 30, 50, 120, 240, and 480 min of incubation while the treatment times for F4546 were 0, 30, 120, 240, 480, 1080, and 1440 min. Appropriate ten-fold serial dilutions of each sample were plated onto XLT4 and SMAC with nalidixic acid to quantify the populations of *S.* Cubana and *E. coli* F4546, respectively. When the numbers of cells dropped below the detection limit, enrichment was performed according to protocols outlined in Bacteriological Analytical Manual (FDA, 2011a, 2011b).

5.2.4. Efficacy of treatment with TF-Su on S. *Cubana and* E. coli *F4546 on alfalfa seeds and sprouts*

5.2.4.1. Inoculation of alfalfa seeds and sprouts with S. *Cubana and* E. coli *F4546*

Alfalfa (*Medicago sativa*) seeds were obtained from a commercial source (Otis S. Twilley Seed Co. Inc., Hodges, SC) and were stored at 10 ºC before being used in the study. Alfalfa sprouts were obtained from a local grocery store. An overnight culture of individual *S.* Cubana and *E. coli* F4546 strain grown in TSB supplemented with nalidixic acid (50 µg/ml) was serially diluted in sterilized water, to *ca.* 10^7 - 10^8 CFU/ml. Five hundred ml of each prepared bacterial culture was mixed with 100 g of alfalfa seeds or sprouts with gentle agitation at 50 rpm at 20 ± 1 ºC for 30 min. The cell suspension was drained completely, and the seeds were placed onto tissue paper and air-dried in the biological safety cabinet (Class II type A/B 3, Nuaire, Plymouth, MN) overnight at room temperature. Inoculated seeds (*ca.* 10^6 CFU/g) were then placed in plastic bags and stored at 4°C until used.

5.2.4.2. *Treatment protocols*

TF-Su prepared with probiotic M (Su-M) was used in the study. Two gram of contaminated alfalfa seeds and 1 g of contaminated sprouts prepared as described above were treated with 10 ml of Su-M and samples were taken at different time intervals: 0, 15, 30, 60, 120, 240, 480, 720 min. Alfalfa seeds and sprouts treated with 10 ml of sterilized water were used as control. The alfalfa seeds or sprouts with Su-M or sterilized water were agitated horizontally at

100 rpm in an orbital platform shaker (Model: 3520, Lab-line, IL, U.S.A) at 20 ± 1 °C. For microbial analysis, the Su-M was discarded at each sampling time, and treated samples were transferred to a Whirl-Pak bag (Nasco, Fort Atkinson, WI) containing 8 ml of Dey-Engley broth and homogenized for 2 min with a stomacher. After homogenization, approximate 10 - fold serial dilutions were made in 0.1 M phosphate buffered saline (PBS, pH 7.4) and selected dilutions were spread-plated onto nalidixic acid amended XLT4 and SMAC plates, respectively, for quantification of *S.* Cubana and *E. coli* F4546. When the numbers of cells dropped below the detection limit, enrichment for each of the bacterial pathogens was performed as described previously.

5.2.5. Germination percentage of alfalfa seeds treated with Su-M

Approximately 0.5 g (~ 150) uninoculated alfalfa seeds, treated with Su-M for different lengths of time were placed on a sterilized deionized water-saturated filter paper in a sterilized petri plate. The petri dishes with the seeds were placed in a plastic box in the dark at 25 ^{o}C for 3 days, with a 12-h periodic application of 10-15 ml of sterile water. The number of germinated seeds was counted, and germination percentage was calculated. The experiment was done in triplicate.

5.2.6. Statistically analysis

Fisher's least significant difference test in the general linear model was conducted, using the Statistical Analysis Software (version 9.4; SAS Institute Inc., Carey, N.C.), to determine the

difference in the cell population (log CFU/ml or CFU/g) of *S.* Cubana or *E. coli* F4546 treated with different TFs. Pathogen counts recovered at different sampling points were also compared. All the tests were performed with a significant level of 0.05.

5.3. Results

5.3.1. Sensitivity of S. *Cubana and* E. coli *F4546 to TFs in the microtiter plate assay*

The mean surviving populations of *S.* Cubana and *E. coli* F4546 after exposure to TF-Su, TF-Pu, and TF-SuPu of all three tomato ferment preparations are shown in Table 5.1. The populations of *S.* Cubana and *E. coli* F4546 decreased with increases in treatment time (Table 5.1). On average, the population of *S.* Cubana was reduced by 5.1 log CFU/ml during an 8 h treatment with the probiotic TFs while the population of *E. coli* F4546 was reduced by *ca*. 2.7 log CFU/ml during a 24 h treatment. The cell populations of both *S.* Cubana and *E. coli* F4546 treated with TF-Su were significantly ($P < 0.05$) lower than those treated with TF-Pu and TF-SuPu. Significantly lower bacterial cell populations were recovered from the TF prepared with probiotic M compared to the and ferment prepared with probiotic G.

The antimicrobial efficiency of different portions of TF prepared with different probiotic starters on *S.* Cubana and *E. coli* F4546 are shown in Fig. 5.1 and 5.2, respectively. In TF prepared with probiotic M, *S.* Cubana populations decreased from 5.4 log CFU/ml to below the detection limit (< 0.3 log CFU/ml) within a 2 min treatment with TF-Su and TF-SuPu, respectively (Fig. 5.1a, b); the enrichment procedure failed to recover cells with a 120 min treatment. Although the population of *S.* Cubana treated with TF-Pu was also reduced below the

detection limit at the 240 min sampling point, *Salmonella* cells were found in the enriched samples until the end of experiment (Fig. 5.1c). Similar results were found using the ferment prepared with probiotic G, except that the population of *Salmonella* treated with TF-Su and TF-SuPu dropped below the detection limit within a 5 min treatment and those treated with TF-Pu reduced below the detection limit at the 480 min sampling point (Fig. 5.1). When treated with the TF-Su and TF-SuPu of spontaneous tomato ferments, *S.* Cubana population dropped below the detection limit at the 120 and 240 min sampling point, respectively; but the pathogen could be recovered from the enrichment utill the end of the experiment. Population of *Salmonella* treated with TF-Pu was reduced by 3.1 log CFU/ml at the 240 min sampling point (Fig. 5.1c).

E. coli F4546 treated with TF-Su, prepared with probiotic M, could not be recovered at the 24 h sampling point. The pathogens treated with TF-SuPu and TF-Pu for 24 h had a 4.9 and 2.8 log CFU/ml reduction, respectively (Fig. 5.2). *E. coli* F4546 treated for 24 h with TF-Su, TF-SuPu, and TF-Pu prepared with starter G had a 4.1, 2.0, and 2.2 log CFU/ml reduction, respectively. *E. coli* F4546 population treated with all three preparations of spontaneous TF had *c.a.* 1.0 log CFU/ml reduction after a 24 h of treatment.

5.3.2. Effectiveness of Su-M on S. *Cubana and* E. coli *F4546 on alfalfa seeds*

The efficacy of Su-M treatments on *Salmonella* and *E. coli* populations on alfalfa seeds is shown in Fig. 5.3. Significant reductions in *Salmonella* and EHEC population were observed during the entire course of the experiment. *S.* Cubana or *E. coli* F4546 population on alfalfa seeds had a 3.4 or 2.3 log CFU/g reduction at the 240 min sampling point; and 4.9 and 4.1 log CFU/g reduction at the 720 min sampling point, respectively. The germination percentage of

treated alfalfa seeds was only significantly reduced with treatments longer than 240 min (Table 5.2).

5.3.3. Efficacy of the Su-M on S. *Cubana and* E. coli *F4546 on alfalfa sprouts*

The efficacy of Su-M treatments on *Salmonella* and *E. coli* populations on alfalfa sprouts is shown in Fig. 5.4. Both *S.* Cubana and *E. coli* F4546 populations on alfalfa sprouts decreased significantly between each of the two sampling points. A 3.0 or 6.4 log CFU/g reduction in *S.* Cubana population was achieved with a 30 and 60 min treatment, respectively. No viable cells were recovered at the 120 min sampling point. *E. coli* F4546 population on alfalfa sprouts was reduced by 1.6 and 3.8 log CFU/g with a 60 and 240 min treatment, respectively.

5.4. Discussion

Seeds used for sprouting have been identified as the sources of sprout-related outbreaks, and pathogen such as *Salmonella* and *E. coli* O157:H7 have been isolated from naturally contaminated sprout seeds (Breuer et al., 2001; Ferguson et al., 2005). Substantial research effort has been devoted to evaluate the effect of synthetic chemicals to inactivate bacterial pathogens on sprout seeds (Buchholz & Matthews, 2010; Gandhi & Matthews, 2003; Hong & Kang, 2016; Nei, Latiful, Enomoto, Inatsu, & Kawamoto, 2011; Scouten & Beuchat, 2002). Fewer studies have focused on the use of biological products as potential antimicrobial agents against bacterial pathogens on vegetable seeds.

We found that the antimicrobial effect of TF-Su was significantly higher than that of TF-Pu (Table 5.1). This could be due to the fact that major end products from lactic acid bacteria fermentation, such as lactate, acetate, and alcohol are water soluble and these products have antimicrobial effect (Gänzle, 2015). Furthermore, chemicals in liquid state allow a more homogeneous contact with bacterial cells.

Treatments with spontaneous TF caused a 4.3 and 1.5 log CFU/ml reduction in the population of *S.* Cubana and *E. coli* F4546 in the microtiter plate assay; in comparison, significantly higher levels of reductions in bacterial cell populations were achieved when using treatment with probiotic TFs (Table 5.1). The probiotic TFs had a pH value of 3.0 which was lower than the pH level of 3.9 in the spontaneous TF. Similar observation were made by Urbonaviciene, Viskelis, Bartkiene, Juodeikiene, and Vidmantiene (2015) who found that tomato products treated with lactic acid bacteria had pH values that were 7.2% lower and titratable acidity values 17.3 % higher than the corresponding values of spontaneous TF. Acid production depends on the available number of viable bacteria cells to utilize the carbohydrates in the substrate (Steinkraus, 1997). Spontaneous fermentation typically relies on the activity of natural microflora, the number of which is presumably lower than the number of artificially inoculated starter cultures.

Su-M had an antimicrobial effect against *S.* Cubana and *E. coli* F4546 on alfalfa seeds and sprouts (Fig. 5.3 and 5.4). The precise mechanism behind the observed phenomenon was unknown, however, several fermentation by products, such as organic acid, ethanol, hydrogen peroxide, bacteriocins, as well as natural phytochemicals have been suggested as the functional components (Barbieri et al., 2017; Omodamiro & Amechi, 2013; Šušković et al., 2010). Bartkiene, Vidmantiene, Juodeikiene, Viskelis, and Urbonaviciene (2013) reported that a mixture

of L- and D- lactic acid was found in natural fermented tomato product and the accumulation of lactic acid could lead to the inactivation of *Salmonella* and *E. coli* O157:H7 (De Keersmaecker et al., 2006; Lee, Yun, Fellman, & Kang, 2002). In addition, the lactic acid bacteria fermentation could lead to the production of other potential antimicrobial compound, such as acetic acid (Gänzle, 2015). These organic acids may play an important role in the inactivation of bacterial pathogens on alfalfa seeds and sprouts. Preliminary composition analysis revealed that Su-M contained 12,000 ppm lactic acid, 2,770 ppm acetic acid, and 580 ppm malic acid. The mixture of organic acids could exert a greater antimicrobial activity than a single organic acid (Theron & Lues, 2010). Ethanol (0.04%) and total polyphenols (320 ppm) were also found in Su-M and these chemicals have also been reported to be effective in elimination of the bacterial pathogens (Daglia, 2012). In addition, other metabolites of lactic acid bacteria metabolites might also exist. Arkoun, Abbas, and Zighen (2015) reported that lactic acid bacteria isolated from fermented tomato fruits produced a bacteriocin-like substance that could inhibit a variety of gram-negative bacteria such as *Salmonella* and *E. coli*.

Treatments with probiotic TFs resulted in a significantly higher reduction in *S.* Cubana than *E. coli* F4646 population on alfalfa seeds (Fig. 5.3 and 5.4). This might be because the *E. coli* O157:H7 strain used in this study is more acid tolerant than *S.* Cubana. Acid tolerance of *E. coli* O157:H7 was reported (Leyer, Wang, & Johnson, 1995). Tsai and Ingham (1997) found that *E. coli* O157:H7 cells could survive in ketchup, an acidic condiment, after a 7-days storage at 5°C, whereas no *Salmonella* strains could be recovered after 2 days of storage at the same temperature. Koutsoumanis and Sofos (2004) found the mean populations of *E. coli* O157:H7 reduced by only 1 log CFU/ml after 2 h exposure to acidified TSB (pH 3.5) whereas more than 7 log CFU/ml of reduction in *Salmonella* population was observed under the same condition.

Treatments with probiotic TFs resulted in a significantly lower reduction in bacterial pathogens on alfalfa seeds than on sprouts (Fig. 5.3 and 5.4). This observations was similar to a previous study conducted by Sharma and Demirci (2003) who reported a 2.7 log CFU/g reduction in *E. coli* O157:H7 populations on alfalfa sprouts treated with 50 ppm acidic electrolyzed water for 64 min whereas a 1.5 log CFU/g reduction was observed on alfalfa seeds treated under the same condition. This might be due to the fact that seeds and seed sprouts have different surface characteristics. Surfaces of alfalfa seeds are rougher and have irregular shapes, which can protect the bacterial pathogen cells against sanitizers.

Treatment with probiotic TFs led to a 3.4 or 2.3 log CFU/g reduction in *S.* Cubana and *E. coli* F4546 populations on alfalfa seeds (Fig. 5.3 and Table 5.2), which is similar to the antimicrobial efficacy of 20,000 ppm calcium hypochlorite treatment (Ding, Fu, & Smith, 2013). The reduction in microbial populations caused by 20,000 ppm of calcium hypochlorite treatment on sprout seeds varied from 1.0 - 6.5 log CFU/g, with an average of 3.2 log CFU/g reduction in *Salmonella* population and 2.5 log CFU/g reduction in EHEC population (Montville & Schaffner, 2004). The recommended concentration of chlorine for washing fresh produce is from 50 to 200 ppm and treatment with 200 ppm of chlorine could only reduce bacterial cell population by 1-3 logs due to rapid depletion of free chlorine (Aruscavage, Lee, Miller, & LeJeune, 2006). This level of reduction was lower than the 6.4 log CFU/g reduction in *S.* Cubana population with a 60 min probiotic TF treatment and the 3.8 log CFU/g reduction in *E. coli* F4546 with a 240 min treatment, respectively. Furthermore, production of TFs does not require substantial equipment costs and the probiotics used are considered as beneficial microorganisms that are “generally recognized as safe” for human consumption (Silva, Carvalho, Teixeira, & Gibbs, 2002). Observations from the current study suggest that probiotic TFs could

be used as potential sanitizing agents for alfalfa seeds and sprouts. The results could improve the microbiological safety of seeds and sprouts. Future studies should focus on the identification of the active compounds in probiotic TFs. Mechanisms of action of the active compounds should also be explored in future studies.

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Figure Legends

Fig. 5.1. Surviving populations of *S.* Cubana after treatment with the entire content [TF-SuPu (a)], supernatant [TF-Su (b)], and pulp [TF-Pu (c)] of probiotic tomato ferment prepared using different starters. Detection limit: 2 CFU/ml

Fig. 5.2. Surviving populations of *E. coli* F4546 after treatment with the entire content [TF-Supu (a)], supernatant [TF-Su (b)], and pulp [TF-Pu (c)] of probiotic tomato ferment prepared using different starters. Detection limit: 2 CFU/ml

Fig. 5.3. Antimicrobial efficacy of the supernatant of probiotic tomato ferments using the 16-strain mixture of the probiotic strains as a starter on *S.* Cubana and *E. coli* F4546 artificially inoculated on alfalfa seeds. Detection limit: 0.5 CFU/g

Fig. 5.4. Antimicrobial efficacy of the supernatant of probiotic tomato ferments using the 16-strain mixture of the probiotic strains as a starter on *S.* Cubana and *E. coli* F4546 artificially inoculated on alfalfa sprouts. Detection limit: 1 CFU/g ** Bacterial pathogen not detected in the enrichment

Table 5.1.
Mean populations of *Salmonella* and *E. coli* F4546 surviving the treatments with tomato ferments in microtiter plates

Comparative category	Log CFU/ml	
	S. Cubana	*E. coli* O157:H7 F4546
Time		
0 min	5.410 A	5.489 A
2 min/ 30 min	2.247 B	5.357 B
5 min/2 h	1.619 C	4.975 C
30 min/4 h	1.413 D	4.728 D
1 h/8 h	1.181 E	4.244 E
2 h/18 h	0.881 F	3.290 F
4 h/24 h	0.366 G	2.775 G
8 h	0.354 G	-
Composition		
Ferment pulp (TF-Pu)	2.356 A	4.487 A
Ferment mixture (TF-SuPu)	0.621 B	4.374 B
Ferment supernatant (TF-Su)	0.479 C	3.824 C
Probiotic Starter		
Control	5.410 A	5.489 A
No starter (spontaneous)	2.124 B	4.976 B
Single strain starter G	0.730 C	4.151 C
16 strains starter M	0.601 D	3.558 D

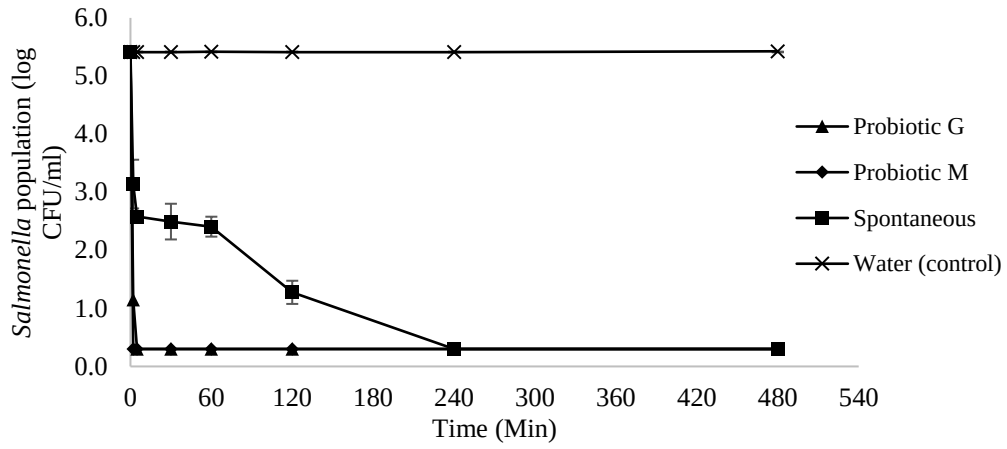
[a] Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4. Values within each comparative parameter in the same column followed by the same letter were not significantly different ($P > 0.05$).

Table 5.2.
Germination percentage of alfalfa seeds treated for various times with supernatant of tomato fermented using the 16-strain probiotic mixture (M)

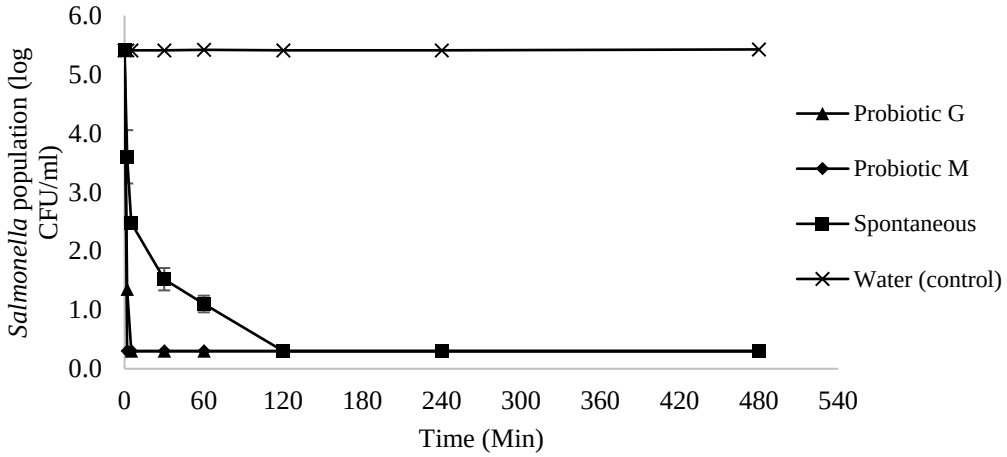
Treatment	Germination percentage
0 min	91.3% ± 0.6% A
15 min	91.3% ±1.1% A
30 min	91.5% ± 0.5% A
1 h	91.2% ± 1.6% A
2 h	90.3 % ± 0.7% A
4 h	89.2% ± 0.9% A
8 h	72.4% ± 2.9% B

Fig.5.1.

a.


6.0
5.0
4.0
3.0
2.0
1.0
0.0
Salmonella population (log CFU/ml)
0
60
120
180
240
300
360
420
480
540
Time (Min)
Probiotic G
Probiotic M
Spontaneous
Water (control)

b.


6.0
5.0
4.0
3.0
2.0
1.0
0.0
Salmonella population (log CFU/ml)
0
60
120
180
240
300
360
420
480
540
Time (Min)
Probiotic G
Probiotic M
Spontaneous
Water (control)

c.

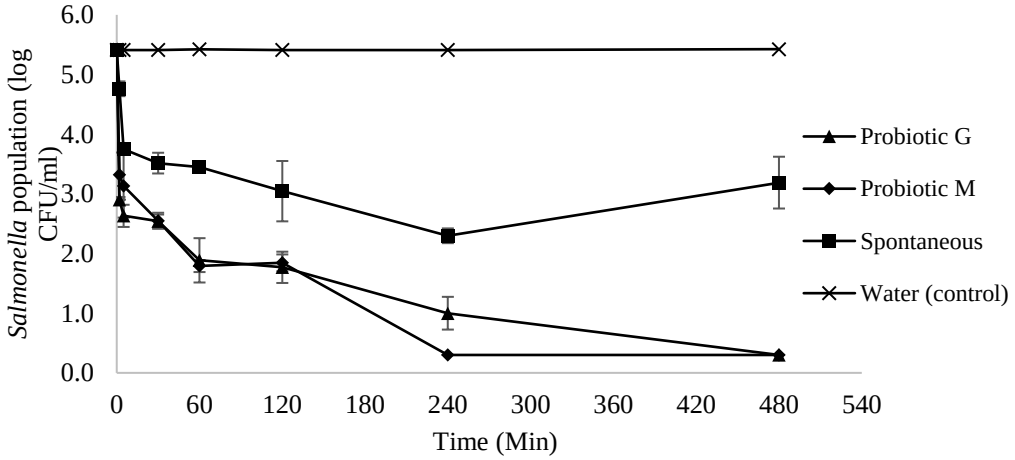
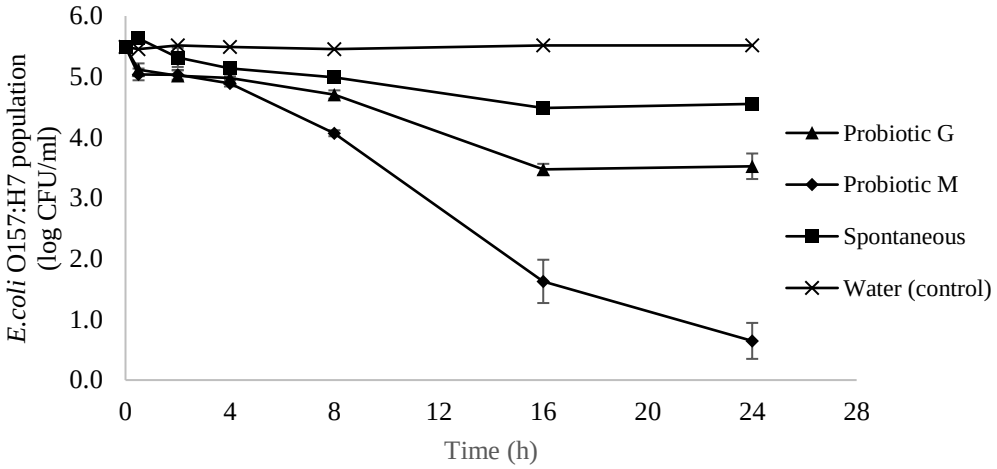
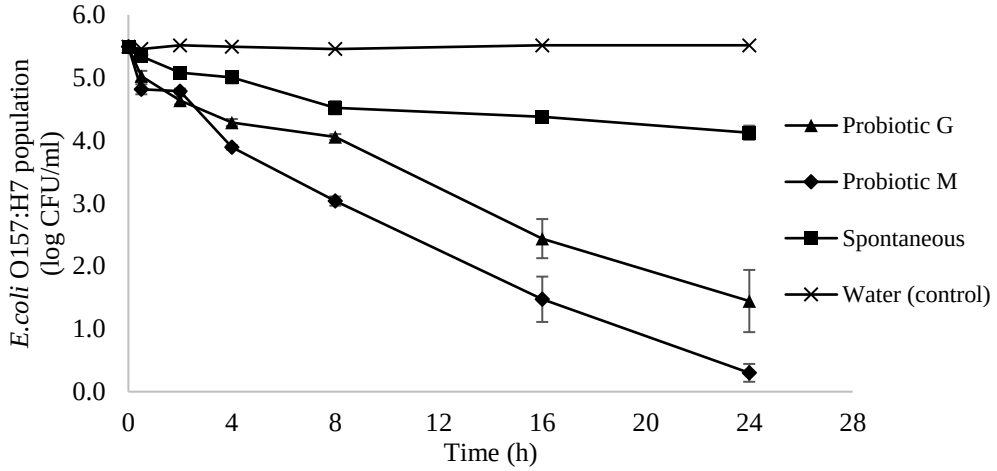

6.0
5.0
4.0
3.0
2.0
1.0
0.0
Salmonella population (log CFU/ml)
0
60
120
180
240
300
360
420
480
540
Time (Min)
Probiotic G
Probiotic M
Spontaneous
Water (control)

Fig. 5.2.

a.


6.0
5.0
4.0
3.0
2.0
1.0
0.0
E.coli O157:H7 population (log CFU/ml)
0
4
8
12
16
20
24
28
Time (h)
Probiotic G
Probiotic M
Spontaneous
Water (control)

b.


6.0
5.0
4.0
3.0
2.0
1.0
0.0
E.coli O157:H7 population (log CFU/ml)
0
4
8
12
16
20
24
28
Time (h)
Probiotic G
Probiotic M
Spontaneous
Water (control)

c.

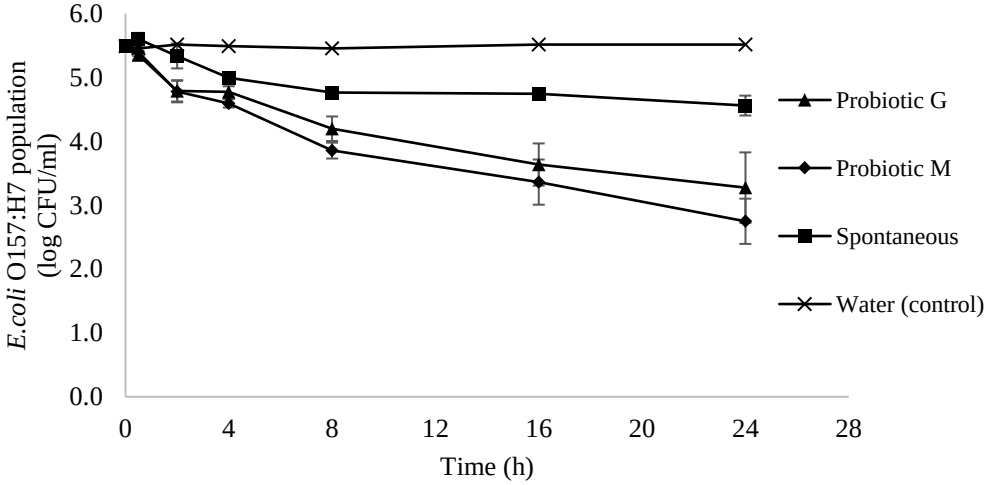

6.0
5.0
4.0
3.0
2.0
1.0
0.0
E.coli O157:H7 population (log CFU/ml)
0
4
8
12
16
20
24
28
Time (h)
Probiotic G
Probiotic M
Spontaneous
Water (control)

Fig. 5.3.

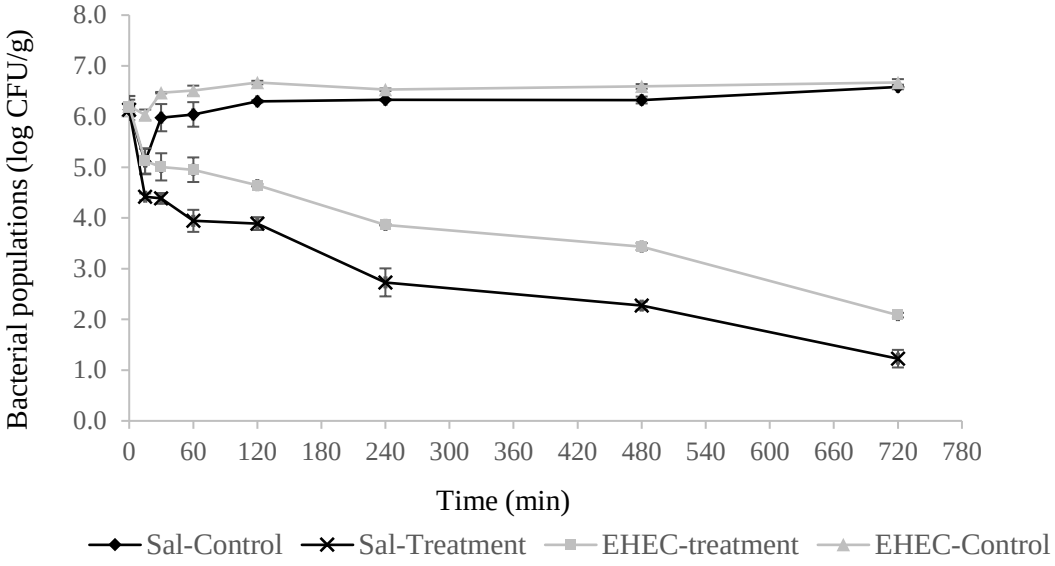
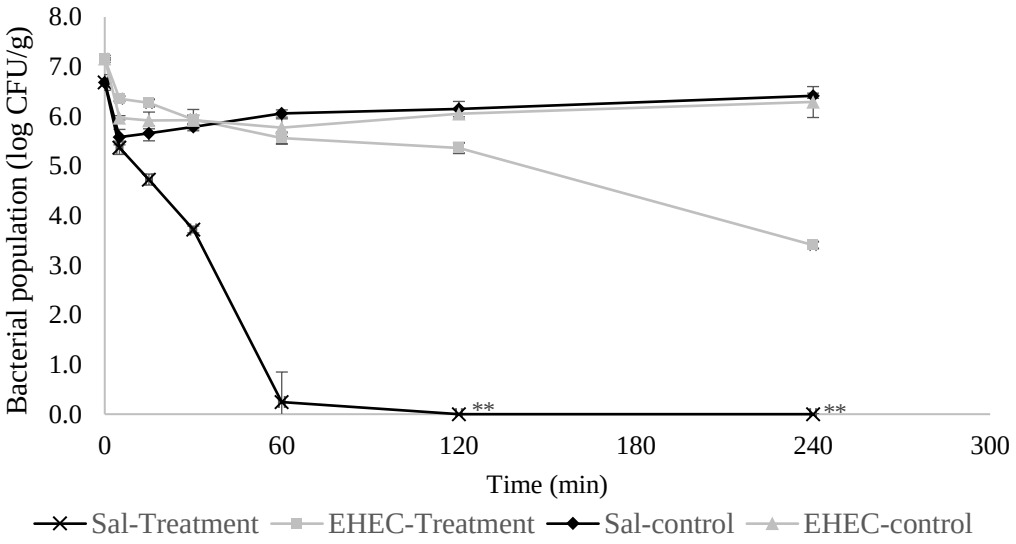

Bacterial populations (log CFU/g)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
60
120
180
240
300
360
420
480
540
600
660
720
780
Time (min)
Sal-Control
Sal-Treatment
EHEC-treatment
EHEC-Control

Fig. 5.4.


Bacterial population (log CFU/g)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
60
120
180
240
300
Time (min)
**
**
Sal-Treatment
EHEC-Treatment
Sal-control
EHEC-control

CHAPTER 6

CONCLUSIONS

Following findings were derived in this study,

1). Cells of selected *Salmonella* strains had a greater attachment ability than those of EHEC strains. Unit weight of lettuce seeds had the highest numbers of attached *Salmonella* or EHEC cells, followed by tomato, alfalfa, and fenugreek seeds. In contrast, individual fenugreek seeds had more attached pathogen cells followed by lettuce, alfalfa, and tomato seeds. Significantly more *Salmonella* and EHEC cells attached to mechanically-damaged seeds than to intact seeds ($P < 0.05$). Fungicide treatment did not significantly affect the attachment of individual bacterial strains to vegetable seeds ($P > 0.05$), with a few exceptions.

2). The incidence of pathogen-positive sprout/seedling samples and mean *Salmonella* and EHEC populations on sprout/seedling tissues developed from the seeds contaminated by immersion into bacterial suspensions were significantly higher than those developed from the seeds contaminated by contacting with artificially-inoculated soil. *Salmonella* and EHEC counts on seed coats were significantly higher than those from the root and cotyledon tissues, followed by those on stem tissue samples. The *E.*

coli O104:H4 strain used in the study established the lowest cell population on sprout/seedling tissues; both *Salmonella* and EHEC cells had the lowest level of cell population on tomato seedlings.

3). Probiotic tomato ferments were more effective in reducing the pathogen populations than the spontaneous ferment. Supernatants of the ferments had significantly higher antimicrobial potentials than the pulps. Probiotic tomato ferments are more effective in inactivating *Salmonella* than *E. coli* O157:H7 cells. Without jeopardizing the germination ratio, treatment with filter-sterilized supernatants of probiotic tomato ferments prepared with 16-strain probiotic mixture (Su-M) reduced the populations of *S.* Cubana and *E. coli* F4546 on alfalfa seeds by 3.4 log CFU/g and 2.3 log CFU/g, respectively. No viable *S.* Cubana cells were recovered on alfalfa sprouts after a 2 h treatment with Su-M. With a 4 h treatment of Su-M, *E. coli* F4546 population on alfalfa sprouts was reduced by 3.8 log CFU/g.

This study elucidated the mechanisms of interaction between human pathogens and vegetable seeds, examined the physiological behavior of pathogen cells on germinating vegetable seeds, and evaluated the performance of a novel sanitation approach, tomato ferments, for sprout seeds and seed sprouts. The information and knowledge generated from the study may help reduce the incidence of fresh produce-associated outbreaks of infection, and subsequently the economic losses associated with

vegetable-related outbreaks of infections, especially sprouts-related outbreaks of infections.

APPENDIX A

IDENTIFICATION OF GENES CRITICAL FOR ATTACHMENT AND BIOFILM FORMATION OF *SALMONELLA ENTERICA*

A.1. Introduction

The number of outbreaks of infection associated with the consumption of fresh produce increased dramatically over the last two decades (Sapers, 2014). A wide spectrum of microorganisms are involved in these outbreaks (Lynch, Tauxe, & Hedberg, 2009). *Salmonella enterica* is one of the bacterial pathogens that are frequently involved in fresh produce-associated outbreaks (Callejón et al., 2015). Emerging evidence suggests that *S. enterica* is able to adhere to and to form biofilms on plant tissues. Bacterial cells embedded within biofilms are more difficult to remove and are more resistant to disinfection treatment. Consumption of inadequately-decontaminated fresh produce may lead to human infections and foodborne outbreaks (Yaron & Römling, 2014).

Specific genetic elements that play a role in bacterial attachment and biofilm formation on plant tissues were identified by different experimental approaches (Yaron & Römling, 2014), such as transposon insertion (Barak, Gorski, Naraghi-Arani, & Charkowski, 2005) or expression analysis (Macarisin, Patel, & Sharma, 2014). These studies have shown that the cell surface components, such as flagella, fimbriae, curli, and cellulose are important in pathogen attachment and biofilm formation on their contact surface (Barak & Schroeder, 2012). Tan, White, Rahman, and Dykes (2016) reported

that a *ΔfliC fljB* mutant of *S.* Typhimurium which lacks the expression of phase I and 2 flagellins had significantly lower level of attachment to plant cell walls compared to the wild type strain. Deletion of *csgB* and *csgA* genes (curli subunit) resulted in an eightfold reduction in the attachment of *S.* Newport to alfalfa sprouts (Barak, Gorski, Naraghi-Arani, & Charkowski, 2005). The deletion of cellulose biosynthesis genes in *S. enterica* resulted in 1 log reduction in the attachment to alfalfa sprouts (Barak, Jahn, Gibson, & Charkowski, 2007) and tomato fruits (Shaw et al., 2011), respectively. However, inconclusive results were reported by other studies. Berger et al. (2009) reported that the deletion of *fli*C in *S.* Senftenberg resulted in reduced attachment on the surface of basil leaves; however, the deletion of same gene in *S.* Typhimurium had no effect on cell attachment to basil leaves surface. Another study found that the deletion of *fli*C in *S.* Senftenberg had no effect on cell attachment to tomato fruits (Shaw et al., 2011).

The objective of this study was to use transposon mutagenesis to identify genes that are critical for the attachment and biofilm formation of selected *Salmonella* strains on vegetable seeds.

A.2. Materials and methods

A.2.1. Donor and recipient strains

Three *Salmonella* outbreak strains, Baildon (tomato outbreak strain), Cubana (alfalfa sprout outbreak strain), and Enteritidis were used in the study as recipients. The bacterial strains were stored at -70 ºC and recovered on tryptic soy agar (TSA; Becton Dickenson, Sparks, MD) at 37 ºC for 16 h. Spontaneous mutant cells resistant to 100 μg/ml nalidixic acid (MP Biomedicals, Santa Ana, CA) were selected. Sensitivity of the selected nalidixic acid-resistant mutants to ampicillin was confirmed on TSA supplemented with 100 μg/ml ampicillin (MP Biomedicals, Santa Ana, CA).

E. coli SM 10 λpir with a pUT *lux*AB vector was used as the donor strain (Biomedal, 2006). The pUT *lux*AB vector contains the *mini-Tn5 (luxAB-tet^r)* as well as an ampicillin resistant gene, *bla*. The donor cells were maintained on Luria-Bertani (LB; Becton Dickenson, Sparks, MD) agar supplemented with 100 μg/ml of ampicillin and 12.5 μg/ml of tetracycline.

A.2.2. Transposon mutagenesis

One ml of overnight cultures of the donor strain grown in tryptic soy broth (TSB; Becton Dickenson, Sparks, MD) supplemented with 100 μg/ml of ampicillin and 12.5 μg/ml of tetracycline as well as recipient strains grown in TSB supplemented with 100 μg/ml nalidixic acid were centrifuged at 10,000 *g* for 2 min. The harvested pellets were re-suspended in 1.0 ml of plain TSB. One hundred μl of the resulting cultures were diluted in 400 μl of TSB. Cells of diluted donor and each recipient were mixed at 1:1 ratio and the mixtures were spot-inoculated on TSA plates. The inoculated plates were incubated at 37 °C for 24 h. Cells on TSA plates were collected and suspended in 10 ml of TSB supplemented with 100 μg/ml of nalidixic acid and 12.5 μg/ml of tetracycline and incubate at 37 °C for 6 h with constant agitation at 100 rpm. The transconjugants that grew in TSB with nalidixic acid and tetracycline were pre-selected to comprise the mutant pools which were used in the selection of attachment deficient mutants described below.

A.2.3. Selection of attachment deficient mutants

The selection of attachment deficient mutants was performed using a previous protocol described by Barak, Gorski, Naraghi-Arani, and Charkowski (2005) with modifications. Specifically, 2.0 ml of each transconjugant mixture grown in LB no-salt (LBNS) broth supplemented with 100 μg/ml of nalidixic acid and 12.5 μg/ml of tetracycline was transferred to a single well of a 24-well tissue culture plate (Corning Incorporated, Durham, NC) and incubated for 3 h at 25 °C with constant agitation at 40 rpm on an orbit shaker (Orbit Shaker, Lab-Line Instruments, INC.). Suspending cells in each well were transferred to different wells of the tissue culture plate and incubated for another 3 h. The transfer of suspending cells was repeated for three times a day. At the end of the day, the cultures were transferred to fresh LBNS broth supplemented with 100 μg/ml of nalidixic acid and 12.5 μg/ml of tetracycline and incubated overnight at 37 °C. This selection procedure was continued for 10 consecutive days. After 10 days, the cell suspensions were streaked on TSA plates supplemented with 100 μg/ml of nalidixic acid and 12.5 μg/ml of tetracycline and single mutant colonies were selected and their sensitivity to ampicillin tested. The colonies sensitive to ampicillin were selected 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 (Jain & Chen, 2007) with modifications. 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 a 24-well tissue culture plate. After incubation for 7 days at 28 °C, the broth cultures were withdrawn and the wells were rinsed twice with 2 ml of fresh sterile broth to remove loosely attached cells. 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 Bunsen burner (Fisher Scientific, Asheville, NC). The fixed cells were then stained with 2 ml of 1% crystal violet for 15 min at room temperature. The stain was then rinsed off 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 by measuring the A_{550} of the extraction solutions with a spectrophotometer (Thermo Scientific, MA).

A.2.5. DNA extraction, PCR amplification, and sequencing

Total cellular DNA was extracted from the selected mutants. Specifically, 1 ml of overnight culture was centrifuged at 10,000 *g* for 5 min and the resulting pellets were washed twice, each with 1 ml sterilized distilled water, and then re-suspended in 100 µl of sterilized distilled water. The cells were lysed by boiling for 10 min, and cellular debris was removed by centrifugation at 10,000 *g* for 10 min. The aqueous supernatant was used as the template for PCRs.

The PCR was conducted using a transposon-specific primer directed toward the transposon-chromosomal junction, paired with a primer with the recognizing sequence of a restriction enzyme toward the transposon insertion site. The information on selected primers used to amplify parts of the transposon and its downstream sequence is shown in Table A1. PCRs were carried out in a 25 µl PCR mixture contained 1×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.1 µM of each, 0.5 U *Taq* DNA polymerase (Thermo Scientific Inc., MA), and 2 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 stained with 1% ethidium bromide and visualized using a Gel Doc System 2000 (Bio-Rad

Laboratories, Hercules, CA) after electrophoresis on 1% agarose (GIBCO BRL, Rockville, MD). PCR products with specific amplifications were sent to Eurofins Genomics for sequencing and the DNA sequence data were analyzed and aligned with those deposited in Genbank using BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

A.2.6. Confirmation of the identity of Salmonella mutants *using a DNA-based typing method*

Enterobacterial repetitive intergenic consensus (ERIC)-PCR patterns of the mutants and recipient strains were determined according to a protocol described previously (Guo, Chen, Brackett, & Beuchat, 2001). The primer used in the PCR reaction was (5'-3') AAG TAA GTG ACT GGG GTG AGC G, based on a highly conserved, ERIC sequence (Hulton, Higgins, & Sharp, 1991). The 25 µl PCR mixture contained PCR buffer, dNTP (0.2 mM each), primers (0.1 µM), *Taq* polymerase (0.5 U), and 5 µl DNA template. PCRs were performed in a DNA thermal cycler 480 apparatus (Perkin Elmer, Norwalk, Conn.) 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, with a final extension at 72°C for 20 min. The PCR amplicons were analyzed by gel electrophoresis as described previously.

A.2.7. Plasmid extraction and restriction digestion analysis

Selected mutants grown in Luria-Bertani broth supplemented with 100 μg/ml of nalidixic acid and 12.5 μg/ml of tetracycline were incubated in 37 ºC at 150 rpm for 16 h. Plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, MA) according to the manufacture's instruction. Extracted plasmid DNA was digested with *EcoRI* (Thermo Scientific) according to the manufacturer's instructions. Both digested and un-digested plasmid DNA was analyzed by gel electrophoresis as described previously.

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 are shown in Fig. A.1. Chromosomal DNA was extracted from 80 transconjugants that were sensitive to ampicillin and with altered ability in forming biofilms compared to the recipient strains. Specific PCR products were amplified using the FendTc as a forward primer and *EcoRI* recognizing sequence as a backward primer and DNA sequence data showed that the *mini-Tn5*-borne *tet*^{r} had been introduced into the mutant cells (Fig. A2a). Further analysis revealed a part sequence of

ampicillin resistance gene, *bla* (Fig. A2b), although the phenotype of the mutant is ampicillin sensitive, which indicate the presence of donor plasmid in the mutant cells. However, ERIC fingerprints of mutant cells (Fig. A.3.) confirmed that the mutants were derived from the recipient rather than donor cells. It is possible that *Salmonella* recipient cells might have gained the plasmid from donor during conjugation, however, DNA rearrangement of the plasmid might have taken place during the process (Fig A.4), making the *bla* malfunction.

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Figure legends

Fig. A.1 Biofilm formation by recipient strain *S.* Cubana and selected mutants from *S.* Cubana on 24-well tissue culture plates incubated at 28°C for 7 days. Biofilm-forming ability was quantified by crystal violet staining and absorbance measurement at 550 nm. The bars represent standard deviations (n = 4).

Fig. A.2. Selected specific PCR products used for DNA sequencing. a) Forward primer: FendTc; backward primer: *EcoRI*; Lanes 1-3, PCR amplicons from mutants C27, C27, C110, respectively; lane 4, 100-bp DNA; products from lane 1, 3 were sent for sequencing analysis. Sequence alignment data matched parts of the sequence downstream the tetracycline resistant gene on donor cell; b) Forward primer: 2Sm-Spec 10F; backward primer: *PstI*; Lanes 1-3, PCR amplicons from mutants C27, C110, C1133, respectively; lane 4, 1-kb DNA ladder; products from lane 1-3 were sent for sequencing analysis.

Fig. A.3 DNA-based typing of selected mutants, recipient and donor strains. Lane 1, 1-kb DNA ladder (Thermo Scientific); lanes 2-7: DNA profiles of selected mutants from *S.* Cubana; lane 8: recipient strain *S.* Cubana; lane 9: donor strain; lane 10: *S.* Baildon

Fig. A.4 Plasmid profile of mutant cells, recipient and donor cells (a) Lane 1-6, Plasmid of selected mutants from *S.* Cubana; lane 7: plasmid from recipient - *S.* Cubana; lane 8: plasmid from donor; lane 9: 1-kb DNA ladder. Plasmid of selected mutant cells and donor cells digested with EcoRI (b). Lane 1: plasmid of C27 digested with EcoRI; Lane 2: plasmid of C110 digested with EcoRI; Lane 3: plasmid of donor digested with EcoRI; Lane 4: plasmid of C27; Lane 5: plasmid of C110; Lane 6: plasmid of donor; lane 7: 1-kb DNA ladder.

Table A.1.

Selected primers used for PCR amplification and sequencing

Primer	Sequence (5'-3')
Transposon-specific primer forward:	
FendTc	CCGCCCTATACCTTGTCTGC
2Sm-Spec 1F	AACCAACCCTTGGCAGAACA
2Sm-Spec 10F	TCGCTAACGGATTCACCACT
Restriction enzyme primer backward:	
EcoRI	GAATTC
PstI	CTGCAG

Fig. A.1.

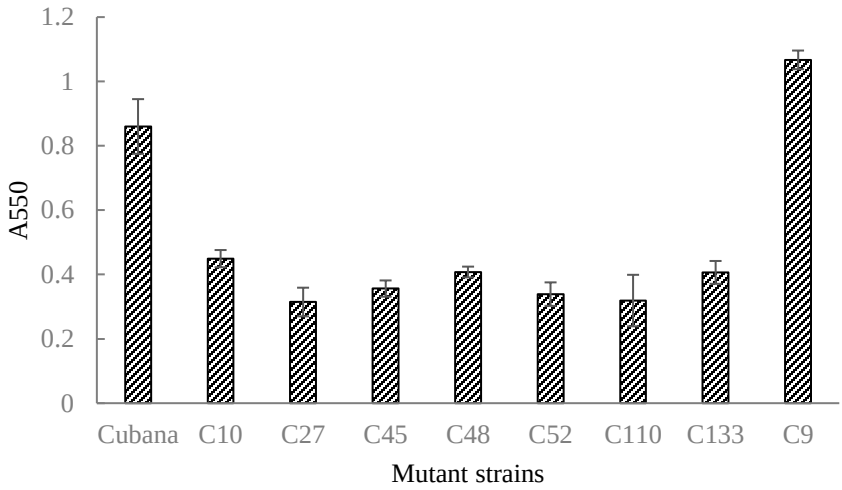
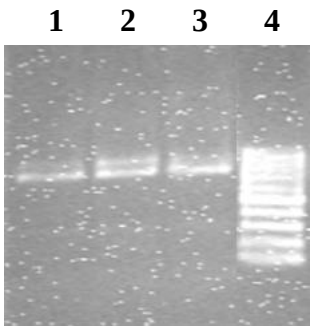

1.2
1
0.8
0.6
0.4
0.2
0
A550
Cubana
C10
C27
C45
C48
C52
C110
C133
C9
Mutant strains

Fig. A.2.

a.




```
Mutant            1 GGCGGCACCTNGCT-ACGGATTCACCACTCCNAGAATTGNAGCCAATCAA     49
                    |||||||||| ||| ||||||||||||||| ||||||| ||||||||||
Donor             1 GGCGGCACCTCGCTAACGGATTCACCACTCCAAGAATTGGAGCCAATCAA     50

Mutant           50 TTCTTGCGGAGAACTGTGAATGCGCAAACCAACCCTTGGCAGAACATATA     99
                    |||||||||||||||||||||||||||||||||||||||||||||||||.
Donor            51 TTCTTGCGGAGAACTGTGAATGCGCAAACCAACCCTTGGCAGAACATATC    100

Mutant          100 CATCGCGTCCGCTATCTCCAGCAACCGCAAGCGATGAATCTCGTGCAG--    147
                    ||||||||||||.||||||||||.|||||.|||..|.||| ||.||||
Donor           101 CATCGCGTCCGCCATCTCCAGCAGCCGCACGCGGCGCATC-CGCGCAGAT    149
```

b.

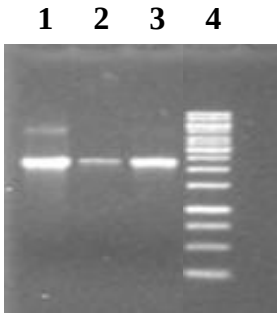


Fig. A.3.

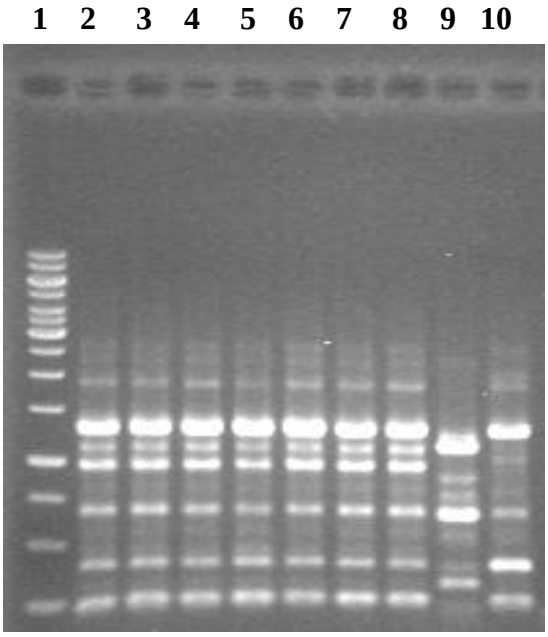
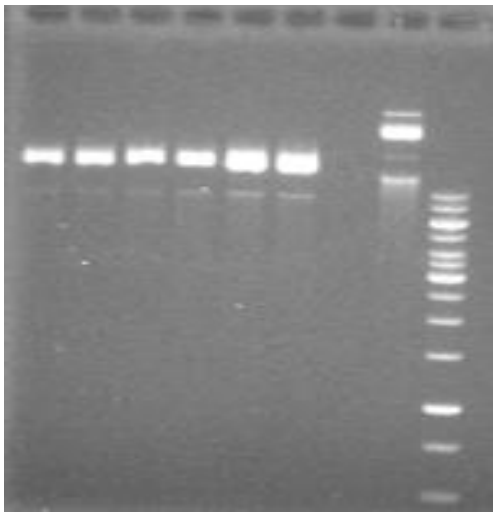


Fig.A.4.

a.



b.