Assessing Contamination Risk of Dust, Soil, Compost, Compost Amended Soil and Irrigation Water as Vehicles of Pathogen Contamination on Iceberg Lettuce Surfaces

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Introduction

The contamination of fresh produce by foodborne pathogens results in 9.5 million illnesses in the United States annually, causing $39 billion in medical losses (Schraff 2010). Iceberg lettuce and similar leafy greens are usually consumed raw with minimal processing or heat treatment. Hence, there is an increased risk of pathogen transmission from contaminated product. Fresh fruit and produce could be contaminated by spoilage or pathogenic microorganisms during pre-harvest and post-harvest procedures (Brandl, 2006). It has also been indicated that the produce might become contaminated during production in the field or in the packing house (Brandl, 2006; Lynch, Tauxe, & Hedberg, 2009). There has been an increase in produce related outbreaks in recent years.

Animals are the primary hosts of *Salmonella enterica* and the pathogen possesses genes to invade, survive host cells and resist immune defense mechanisms (Wallis & Galyov, 2000). *Salmonella* also has genes that confer fitness in non-host environments. The application of soil amended with manure produced by food animals could introduce foodborne pathogens in the farm environment (Hutchison, Walters, Moore, Crookes, & Avery, 2004). Several studies have demonstrated the persistence of *Salmonella* in the farm environment. The pathogen was detected in dairy farms, piggeries and slaughterhouse facilities both before and after slaughter (Baloda, Christensen, & Trajcevska, 2001; Hurd, McKean, Griffith, Wesley, & Rostagno, 2002; Millemann, Gaubert, Remy, & Colmin, 2000). Contamination of plant surfaces with *Salmonella* is also possible when manure (containing pathogen) amended soil is used to grow plants (Barak & Liang, 2008; Winfield & Groisman, 2003). Thus establishment of pathogens and their persistence in fields, water sources and agricultural soil could also increase the risk of produce contamination when grown in proximity to potential sources of *Salmonella*.

The field environment is a dynamic one, with an interplay of physical, chemical and biological factors. Contact of soil and water with produce surface is inevitable. Rain runoff, underground water, surface water currents can all aid in the dissemination of *Salmonella* in agricultural soil and sediment (Chao, 1987). Under certain conditions, percolation of contaminated water through soil filters out bacteria that can persist in the field environment. *Salmonella* has been isolated from soils in agricultural areas, and adhesion to soil particles might be related to cell surface hydrophobicity (Stenstrom, 1989). Soil and sediment could act a reservoir of organic molecules for bacterial nutrition or a substratum for attachment, and hence, serve as a niche for pathogenic bacteria (Chao, 1987; Thomason, Dodd, & Cherry, 1977; Winfield & Groisman, 2003).

The role of dust as a vehicle of transmission of *Salmonella* and other foodborne pathogens requires further evaluation. Dust storms occur frequently in semi-arid regions such as Arizona. Different environments could give rise to aerosols harboring bacteria. Animal and poultry bird confinement have been known to increase the overall microbial load in the immediate environment. Agricultural practices such as sprinkler irrigation of waste water and land application of biosolids or manure could lead to formation and dispersal of bioaerosols (Donnison et al., 2004; Millner, 2009). Water, soil, and manure are suspected as sources of contamination in the field. It has been shown that these can be aerosolized and can lead to the spread of pathogens (Brandl, 2006; Millner, 2009). The potential of airborne dust, water and soil associated *Salmonella* serotypes as a contamination threat in agricultural environments merits further evaluation to aid in better risk assessment.

The objectives of this study were to evaluate the transfer rate and potential risk of iceberg lettuce cross-contamination with environmental vehicles of *S. enterica* such as soil, water, compost

amended soil and dust. Imaging was also conducted to develop a spatial map to determine pathogen distribution upon soil, irrigation water and dust contact.

Materials and Methods

Bacterial culture preparation

Salmonella enterica serovar Newport was used. Antibiotic resistance was developed in this isolate in our laboratory by incremental increase in ampicillin and streptomycin exposure, resulting in resistance to both antibiotics. The antibiotic resistance pattern of the isolate provides efficient traceability in environmental samples such as soil, compost and irrigation water. Growth rate of this isolate was found to be similar to those of non-resistant serotypes. Bioluminescent imaging was performed using *Salmonella* Newport SN78 tagged with pAKlux1 plasmid. The pAKlux1 tagged *S*. Newport was cultured and isolated in media containing 25 µg/ml ampicillin to maintain selective pressure. For each experiment, a fresh overnight culture of each of these isolates was prepared in tryptic soy broth (TSB) containing 100µg/ml ampicillin and 25µg/ml streptomycin and incubated at 37°C in a shaking incubator at 200 rpm. The culture was centrifuged at 4000xg and the supernatant was discarded. The pellet was washed in phosphate buffered saline (PBS) twice and the final suspension of washed cells was used to inoculate various samples in the experiments.

Soil and compost amended soil – Preparation and inoculation

Soil from an organic farm, a conventional farm and compost amended soil were obtained from the iceberg lettuce farms in Yuma, Arizona. The soil samples were analyzed for background bacterial populations by conducting a total plate count on tryptic soy agar (TSA). The soil samples were screened for the presence of *Salmonella* spp. by plating on XLD agar. One hundred gram soil aliquots were stored at room temperature in Ziploc bags until use. For inoculation, 2 ml of PBS-

Salmonella suspension was injected into 10 g of soil in a sterile petri dish. The sample was mixed using a sterile rod to ensure homogenous distribution of the pathogen in soil.

Dust– Preparation and inoculation

Soil from an organic farm and a conventional farm were obtained from the iceberg lettuce fields in Yuma, Arizona. The soil samples were passed first through a No. 20 sieve and then a No. 100 sieve to form fine dust particulate. One gram dust sample portions were inoculated with 100 µL of *S*. Newport-PBS suspension. The samples were mixed using a sterile scalpel and allowed to dry for 30 min before contact with leaf surface.

Irrigation water– Preparation and inoculation

Irrigation water was obtained from irrigation canals in the Yuma and Maricopa counties, Arizona. For inoculation, 10 ml of *S*. Newport-PBS suspension was added to 90 ml of irrigation water and stored in a sterile spray bottle.

Preparation of leaf samples for soil transfer study

Iceberg lettuce heads were purchased from local grocery stores in Tucson, AZ. The two outer leaves of the iceberg lettuce head were removed and discarded. The third outermost leaf of the lettuce head was used to obtain 16 discs of 1 cm diameter. The discs were cut out from the abaxial portion of the leaf using a sterile cork borer. The leaf was divided into 8 quadrants and 2 discs were obtained from each quadrant. The discs were placed in a sterile petridish until contact with the soil was facilitated.

Preparation of leaf samples for water transfer study

Iceberg lettuce heads were purchased from local grocery stores in Tucson, AZ. The two outer leaves of the iceberg lettuce head were removed and discarded. Thirty gram leaf samples were obtained from the third and inner leaves. The leaf sample was stuck to a cutting board using tape and the adaxial surface was used for water contact and transfer study.

Preparation of leaf samples for dust transfer study

Iceberg lettuce heads were purchased from local grocery stores in Tucson, AZ. The two outer leaves of the iceberg lettuce head were removed and discarded. Ten gram leaf samples were obtained from the third leaf. The leaf sample was stuck to a cutting board using tape and the adaxial surface was used for dust contact and transfer study.

Transfer of pathogen by conventional soil and organic soil contact

Ten gram aliquots of conventional or organic soil- *S*. Newport SN78 mixture was placed in a sterile petridish. Contact with conventional soil/organic soil was facilitated by placing iceberg lettuce discs on the *S*. Newport SN78 inoculated conventional soil or organic soil for durations of 5 seconds, 5 minutes and 60 minutes. Post contact, leaves were analyzed for retention of *S*. Newport after contact event using spread plating.

Transfer of pathogen by contact with irrigation water

A 12 inch distance between the leaf and sprayer was maintained. The sprayer contained 5 ml of *S*. Newport SN78 inoculum and the water was sprayed on the leaf surface until all 5 ml was in contact with leaf. Post contact, leaves were analyzed for retention of *S*. Newport after contact event using spread plating.

Transfer of pathogen by contact with contaminated dust

A 6 inch distance was maintained between the leaf and dust. Dust was placed in a 50 ml centrifuge tube that was sliced into half to allow dispersion. Pressurized air from a 10 oz cleaning duster can (Office max) was used to propel the dust to leaf surface. Leaves were sampled immediately after contact.

Microbiological analysis of lettuce leaves, leaf discs, soil, water and dust samples

Soil, disc samples, leaf samples, water and dust were enumerated for *S*. Newport populations after each contact event. Attached bacterial cells from these matrices were removed by adding buffered peptone water to the samples in stomacher bags and pummeling the samples in a stomacher. The mixed aliquots were serially diluted using 0.1% peptone water and plated on xylose lysine desoxycholate (XLD) containing ampicillin and streptomycin. Plates were incubated at 37°C for 24-48 hrs and colony forming units were counted for enumeration of *Salmonella*.

Determination of Transfer Rates

The transfer rates of *Salmonella* from conventional soil and organic soil to lettuce discs were calculated using a formula obtained from Ravishankar *et al.,* (2010):

% of transfer rate =   Population of *Salmonella* on destination X 100

                              (Pop. of *Salmonella* on source + Pop. of *Salmonella* on destination)

Imaging

Bioluminescent imaging of leaves after contact with dust, water and soil was performed using the AMI 1000 advanced molecular imager (Spectral Instruments Imaging, Tucson, Arizona). The leaf samples were placed in the imager and were imaged after a 60 second exposure period for the

detection of photon emission. To determine viability of cells, GFP expression was imaged using UV light excitation.

Results and Discussion

Soil associated cross transfer to lettuce surfaces

Analysis of conventional and organic soils obtained from Yuma, Arizona indicated physical differences between the two soil types. Organic soil had a higher moisture content and retained moisture whereas the conventional soil was drier. Organic soil had slightly higher bacterial microbiota than conventional soil. Organic soil had 6.96±0.120 log CFU/g of background microbiota while conventional soil had a background of 6.57±0.028 log CFU/g of background microbiota. The pH of the organic soil was 8.80 while that of the conventional soil was 8.66. The differences between conventional and organic soil might have influenced the rates of transfer of *S*. Newport SN78 from the soil to the leaf. In conventional soil, the transfer rates of *S*. Newport from soil to leaf increased with time. The same trend was observed with organic soils, but the rate of water loss in conventional soils was higher. The transfer rates of *S*. Newport SN78 from conventional soil and organic soil to leaf surface after 5 seconds of exposure was 3.84% and 0.85% respectively (Tables 1 & 2). In conventional soils, 5 second contact time resulted in 5.09±0.48 log CFU to iceberg lettuce leaf surface while in organic soils the same contact time resulted in a transfer of 6.33±0.28 log CFU (Tables 1 & 2). The initial concentration of *S*. Newport in conventional and organic soils were 6.49±0.40 and 8.4±0.18 log CFU, respectively (Tables 1 & 2).

Exposure to conventional and organic soils for 5 minutes by iceberg lettuce leaf surface resulted in a percent transfer rate of 4.66% and 1.52% respectively (Tables 1 & 2). After 5 minutes,

conventional soil had *S*. Newport population of 6.26±0.38 and iceberg lettuce after contact had a population of 4.95±0.46 log CFU (Table 1). Organic soil had a *S*. Newport population of 7.98±0.21 log CFU and iceberg lettuce after 5 min exposure had a population of 6.17±0.55 log CFU (Table 2). The highest rate of transfer was observed after 1 hour in conventional soil to iceberg lettuce surfaces resulting in 81.85% rate of transfer (Table 1). The conventional soil had a *S*. Newport population of 2.85±0.32 log CFU and iceberg lettuce leaf had a population of 3.51±1.10 log CFU (Table 1).

The difference in culturability between cells present in the soil and cells present on the leaf surface could have resulted due to difference in moisture content on the two matrices. Testing of soil moisture conditions indicated that organic soil had a moisture content of 21.05% while conventional soil had a moisture content of 7.72%. The loss of culturability of cells has been observed previously in the environment and is termed as the Viable But Non Culturable (VBNC) state. Starvation and desiccation stresses have been known to induce the VBNC cell state in foodborne pathogens. VBNC state may represent a survival response by non-sporulating bacteria exposed to potentially injurious environmental conditions (Pinto et al., 2011). The increased moisture content of the organic soil might have resulted in a lower transfer rate of pathogen from soil to iceberg lettuce surface. The transfer rate of *S*. Newport to iceberg lettuce leaf surface from organic soil after 1 hour exposure was 2.56% (Table 2). The population of *S*. Newport in organic soil after one hour was 8.06±0.23 and the population on leaf surface was 6.48±1.02 log CFU (Table 2). These results indicate the potential risks associated with produce coming into contact with contaminated soil in relation to time and that dry soils could result in easier release of pathogen from contaminated soil to leaf surfaces.

Compost amended soils resulted in percent cross transfer of 3.18%, 0.58% and 1.87% after 5 second, 5 minute and 1 hour durations of exposure (Table 3). Compost amended soils used in this study were rich in nutrients and the population of *S*. Newport did not demonstrate a decrease as in conventional soil, indicating better moisture retention by the soil. Initial population of *S*. Newport in compost amended soil was 7.56±0.25 log CFU and the population of *S*. Newport after 1 hour on iceberg lettuce leaf was 6.1±0.91 log CFU (Table 3).

Potential of water as a vehicle of cross transfer

In comparison to soil, water had a lower and more consistent percent transfer rate. The transfer rates of *S*. Newport to leaf surface 5 seconds, 5 minutes and 1 hour after contaminated irrigation water contact were 0.36, 0.41 and 0.11% respectively (Table 4). The stock solution used to inoculate leaf sample had a *S*. Newport population of 8.52±0.81 log CFU/ml (Table 4). Spraying of 5 ml of contaminated water resulted in retention of 5.31±0.33 log CFU/g of *S*. Newport on the leaf surface after 5 seconds (Table 4). The population of *S*. Newport on the leaf surface after 1 hour was 4.77±0.43 log CFU/g indicating retention of the pathogen population after the water contamination event (Table 4). The dissemination of *Salmonella* into marine environments could aid in rapid dispersal of the pathogen. *Salmonella* has also been known to survive in a septic system for 10 to 15 days, and thus could enter fresh or agricultural water due to storm run-off or accidental leaks of sewage or septic tanks (Parker & Mee, 1982). *Salmonella* serotypes can also establish persistence in marine ecosystems. Greene et al. (2008) conducted traceback studies to investigate two *Salmonella* Newport outbreaks caused in 2002 and 2005, respectively, by isolates having the same PFGE pattern. Environmental sampling revealed that the rare strain responsible for the outbreaks was isolated from irrigation pond water near the farms two years apart (Greene et al., 2008).

Potential of dust as a vehicle of cross transfer

 The conventional soil was filterable through the sieve and dust was made using this type of soil without any problems. However, the high moisture content and size particle of the organic soil was not filterable through the sieve and hence, dry portions of the organic soil were used to produce dust. Dust contact was facilitated using pressurized air from a distance of 6 inches from leaf. Conventional soil dust particulate had a *S*. Newport population of 9.43±0.04 CFU/g (Table 5). Contact with leaf surface by dust particulate resulted in a population of 3.61±0.23 log CFU/g retention of *S*. Newport on leaf (Table 5). The transfer rate percent was 0.002% (Table 5). The immediate cross transfer of pathogen from dust to leaf surface indicated the risks associated with dust based dispersion of pathogenic bacteria to produce surfaces. Soil particulates can be dispersed in the field due to winds (Ravi et al., 2011) and aerosolized dust particulates have been associated with pathogen transfer in poultry facilities. An assessment of the presence of *Salmonella* and *Campylobacter* in aerosols within and outside poultry sheds revealed that bacterial levels in air are correlated to their levels in poultry litter (Chinivasagam, Tran, Maddock, Gale, & Blackall, 2009).

A study on the microbial composition of a high-throughput chicken slaughtering facility over a four-month period indicated that the highest microbial counts were found in the areas that had the highest amounts of airborne particulates. A strong correlation was observed between the presence of *Salmonella* and airborne particulates that could have been introduced into the facility by birds. Dust was the only environmental factor in the study that had a significant influence on the dispersal of *Salmonella* spp. (Lues, Theron, Venter, & Rasephei, 2007). Our results indicate that *Salmonella* serotypes can associate themselves with particulate matter and can be dispersed from their source. Soil, composts and manure are suspected as sources of foodborne pathogens and studies have shown that these can be aerosolized and lead to pathogen spread (Brandl, 2006; Millner, 2009).

Agricultural fields are constantly subject to wind based transport of sediments, dusts and aerosols (Ravi et al., 2011). Soil particulates dispersed by wind can range up to 1 mm in diameter (Zobeck & Van Pelt, 2006). The use of moisture depleted organic soil resulted in a loss of culturability of *S.* Newport. No bacteria was enumerated on both soil and leaf.

Bioluminescent imaging

Biophotonic imaging of leaf after contact indicated that post soil, water and dust contact, transfer occurred to lettuce leaf surface (Figures 1, 2 & 3). Surface roughness might play an important role for soil and dust contact based transfer as *Salmonella* was located near the grooves of the veins on the leaf surface (Figures 1 & 3). The contact with agricultural water resulted in a more even distribution of pathogen on leaf surface (Figure 2).

Conclusion

The results from this study indicate that soils, water and dust can serve as potential vehicles of *Salmonella* contamination. The contact time of leaf surfaces with these vehicles and the environmental conditions could play an important role in the amount of cross contamination and the detection of pathogen on iceberg lettuce leaf surfaces.

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Tables

Table 1. Transfer of *S*. Newport from conventional soil to iceberg lettuce leaves

Contact time	*S*. Newport population in soil (Log CFU)	*S*. Newport population on leaves (Log CFU)	% transfer rate
Initial	6.49±0.40		
5 sec		5.09±0.49	3.84
5 min	6.26±0.38	4.95±0.46	4.66

1 hr	2.85±0.32	3.51±1.10	81.85

Table 2. Transfer of *S*. Newport from organic soil to iceberg lettuce leaves

Contact time	*S*. Newport population in soil (Log CFU)	*S*. Newport population on leaves (Log CFU)	% transfer rate
Initial	8.40±0.18		
5 sec		6.33±0.28	0.85
5 min	7.98±0.21	6.17±0.55	1.52
1 hr	8.06±0.23	6.48±1.02	2.56

Table 3. Transfer of *S*. Newport from compost soil to iceberg lettuce leaves

Contact time	*S*. Newport population in soil (Log CFU)	*S*. Newport population on leaves (Log CFU)	% transfer rate
Initial	7.56±0.25		
5 sec		6.07±0.37	3.18
5 min	7.59±0.32	5.36±0.29	0.58
1 hr	7.82±0.27	6.10±0.91	1.87

Table 4. Transfer of *S*. Newport from irrigation water to iceberg lettuce leaves

Contact time	*S*. Newport population in water (Log CFU/mL)	*S*. Newport population on leaves (Log CFU/g)	% transfer rate
Initial	8.52±0.81		
5 sec		5.31±0.33	0.36
5 min		5.36±0.51	0.41
1 hr		4.77±0.43	0.11

Table 5. Transfer of *S*. Newport from dust to iceberg lettuce leaves

S. Newport population in dust (Log CFU/g)	*S*. Newport population on leaves (Log CFU/g)	% transfer rate
9.43±0.04	3.61±0.23	0.002

Figures

Figure 1. Bioluminescent image showing *S*. Newport transferred from soil to Iceberg lettuce
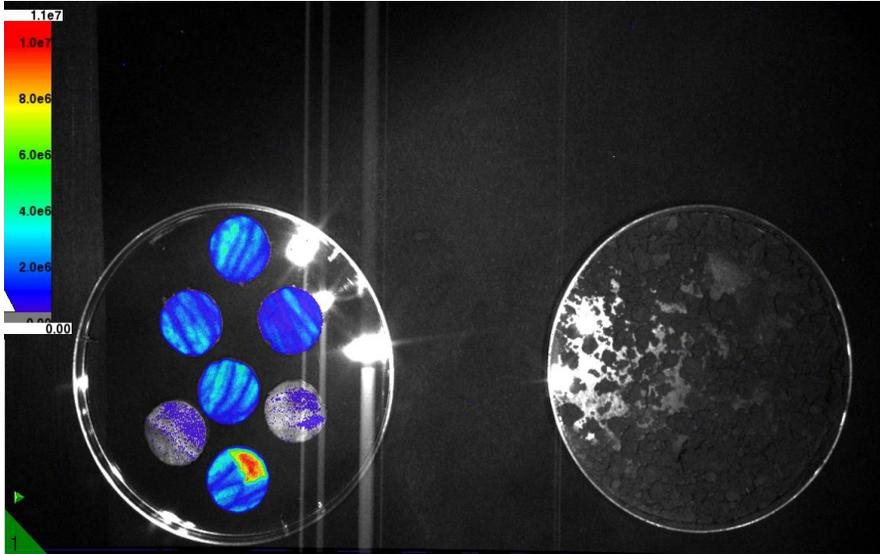
discs

Figure 2. Iceberg lettuce leaf surface after spray contact with *S*. Newport containing irrigation water
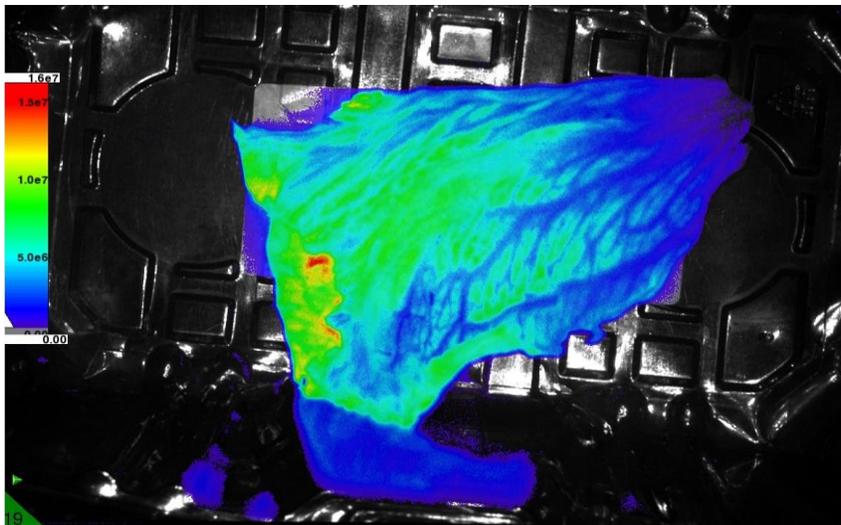

Figure 3. Iceberg lettuce leaf surface after spray contact with *S*. Newport containing dust