CENTER for PRODUCE SAFETY

CPS 2020 RFP
FINAL PROJECT REPORT

Project Title
When the *E. coli* hits the fan! Evaluating the risks of dust-associated produce cross-contamination

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Objectives

1. *Evaluate the role of dust in transferring foodborne pathogens to the surfaces of produce commodities specific to the eastern and western agricultural regions of the United States.*
2. *Understand the role of humidity in the deposition of dust on produce and the survival of foodborne pathogens in dust particulates.*
3. *Test dust particulates from animal operations for the presence of biomarkers indicative of fecal contamination and the presence of enteric pathogens.*

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Abstract

Dust represents an understudied vehicle for microbial dispersal in agricultural environments and produce contamination by microorganisms pathogenic to humans. Dust deposition onto crops during field cultivation is inevitable as plant surfaces serve as a major aerosol sink. Studies have indicated that dust can serve as a vehicle for bacteria. Wind-driven distribution of dust in agricultural environments could also impact food safety when the sources of dust include particles from natural and human-related reservoirs of human pathogens. While the populations of enteric pathogens in water are frequently determined through periodic testing as recommended by the Food Safety Modernization Act (FSMA), and the microbiological quality of soils is monitored, the evaluation of dust and soil-borne particulates is rarely carried out. This study proposed to accomplish the following: 1) To evaluate the role of dust in transferring foodborne pathogens to the surfaces of produce commodities specific to the eastern and western agricultural regions of the United States, 2) To determine the role of humidity in the deposition of dust on produce and the survival of foodborne pathogens in dust particulates, and 3) To test dust particulates from animal operations in Georgia and Arizona for the presence of biomarkers indicative of fecal contamination and the presence of enteric pathogens.

Background

Dust, broadly defined as fine particulate matter resulting from wind erosion on land surfaces and suspended in the air, is an inseparable component of the atmosphere. The suspended particles are transported large distances and are deposited over oceans and land surfaces. Dust represents an understudied vehicle for microbial dispersal in agricultural environments and produce contamination by microorganisms pathogenic to humans. Dust not only affects biological processes in plants, such as stomatal gas exchange, but also the plant surface microbiome. Dust deposition onto crops during field cultivation is inevitable as plant surfaces serve as a major aerosol sink. Studies have indicated that dust can serve as a vehicle for bacteria. Wind-driven distribution of dust in agricultural environments could also impact food safety when the sources of dust include particles from natural (soil, decaying vegetation, feral/wild animal droppings) and human-related (manure-amended soils, silage, municipal sewage-based biosolids, composting, and animal production facilities) reservoirs of human pathogens. It is estimated that dust levels in a field during farm operations could reach 35 mg/m3 of air. While the populations of enteric pathogens in water are frequently determined through periodic testing as recommended by the

Food Safety Modernization Act (FSMA), and the microbiological quality of soils is monitored, the evaluation of dust and soil-borne particulates is rarely carried out.

More information is needed to understand the factors that play a role in the contamination of dust by foodborne pathogens, their survival in dust, and their spread and cross-contamination of nearby produce fields. It is likely that several environmental / atmospheric factors play a role in the cross-contamination of produce by dust from animal feeding operations. These include the temperature and relative humidity, the solar radiation, the soil type and moisture content, and the wind speed and direction. Factors such as the pathogen species/strain may also play a large role in whether or not the organism can cross-contaminate produce via dust as well as the type of crops that are being grown in regions near animal feeding operations. It is therefore critically important to examine dust transport and deposition of pathogens in regions with various characteristics and crops to determine which of these factors contribute the most to any risks associated with contaminated dust. It is also important to determine the safety of different distances relative to animal feeding operations to determine optimal buffer zones to reduce the risks of produce contamination from contaminated dust.

The use of host-associated fecal microorganisms to identify dominant sources of contamination is a scientific approach that has proven to be advantageous for remediating fecally polluted water bodies and to elucidate pathogen transport through agricultural environments (Harwood et al. 2014). Microbial source tracking comprises a suite of methods and an investigative strategy for the determination of fecal pollution sources in the environment that rely on the association of certain fecal microorganisms with a particular host and the identified attributes of these host-associated microorganisms can be used as markers for fecal contamination from the host (Harwood et al. 2014, Shanks et a. 2009). Bacterial genetic markers have been found to be a useful tool for identifying animal and human sources of fecal contamination in the environment (Harwood et al. 2014). Quantitative PCR (qPCR) detection and enumeration of fecal genetic markers provide a rapid means of accurate source identification and are being considered for use as an alternative to culture-based methods for microbial water quality assessment (Harwood et al. 2014, Fisher et al. 2015, Stanley et al. 2016).

In addition, assays for the analysis of metabolites in human and animal feces such as bile salts have been developed and applied for identification of human and animal fecal contamination in aqueous and soil matrices (Battistel et al. 2015, Jeanneau et al. 2011). Bile salts are considered as direct markers because they occur naturally in human and animal fecal matter (Jeanneau et al. 2011). In the human and animal gut, cholesterol, the dominant mammalian sterol, is transformed into bile salts and stanols/sterols. Thus, bile salts can also be used as an indicator of fecal contamination. And finally, assays for the detection of endotoxins (found in the cell wall of all Gram-negative bacteria) can also provide information on the levels of bacterial contamination of a sample.

Research Methods and Results

Objective 1 *– Evaluate the role of dust in transferring foodborne pathogens to the surfaces of produce commodities specific to the eastern and western agricultural regions of the United States.*

and

Objective 2 – *Understand the role of humidity in the deposition of dust on produce and the survival of foodborne pathogens in dust particulates.*

Environmental growth chambers were set up in both Arizona and Georgia and fresh produce, to be used under Objectives 1 and 2, was grown during both years of the project in Arizona (spinach

and romaine lettuce) and in Georgia (tomatoes and bell peppers). Soils from conventional and organic farms were collected from each region and assessed for background levels of bacteria, moisture content, and organic content. The moisture content and organic content were both found to be higher in soils from organic farms. Additional drying steps with the organic soils during the preparation of the dust samples for the experiment were therefore performed. The background levels of bacteria were also found to be higher in organic farm soils (up to 10^5 CFU per gram).

Because of the high background levels of bacteria in the soil to be used in the laboratory experiments, various *Escherichia coli* O157:H7 and *Salmonella* strains were constructed that contained antibiotic resistance genes found either on a plasmid or on the chromosome as well as the gene for green fluorescent protein (GFP) on a plasmid. Although the doubling time varied between strains, there were no statistically significant differences ($P \leq 0.05$) found between the average maximum specific growth rates of these strains and the non-resistant strains.

These constructed strains were then used in dust transfer experiments with high background levels to either inhibit the growth of other bacteria via the use of antibiotics in the growth media, or to enable the differentiation of these strains from the background bacteria through the presence of the green fluorescence under UV light. For the initial dust survival and transfer laboratory experiments, three *Salmonella enterica* strains (*Salmonella enterica* serovars Newport, Typhimurium, and Oranienburg) were used to inoculate soil dust with various particle sizes (<150 microns, 150 to 3,360 microns, or >3,360 microns). The dust was inoculated by placing 5 grams onto the surface of a bacterial culture in a petri dish and spreading using a sterile bent glass rod until mixed (dry inoculation). A cell scraper was then used to recover the inoculated dust. This inoculated dust was mixed with 45 additional grams of dust (hand massaged in a Whirl-Pak® bag until homogenous) of the same type. This resulted in an estimated inoculum level of approximately ~8.0–9.0 $\log_{10}$ CFU/gram dust. The bacteria were then recovered from the dust to determine the survival of the bacteria on the dust (see **Table 1**).

The *S.* Oranienburg used is a *Salmonella* strain that was isolated from a low-moisture food (pecans) and is known to survive in dry environments. This strain was used for the purpose of comparison with the survival of *S.* Newport and *S.* Typhimurium, both of which are more commonly associated with produce-related outbreaks. The *S.* Oranienburg and the *S.* Typhimurium populations were stable in dust particles of all size ranges, whereas approximately a 3-$\log_{10}$ drop was observed in the *S.* Newport populations inoculated onto the dust. The dust particle size did not appear to have a significant effect on bacterial survival/recovery. It is unclear whether this drop in the recovery of the *S.* Newport was due to the bacteria entering a viable but non-culturable (VBNC) state.

For the final transfer experiments conducted at various relative humidity levels and with various produce types, constructed strains of *E. coli* O157:H7 (ampicillin/streptomycin resistance, GFP), *Salmonella* Newport (ampicillin resistance, GFP), and *Salmonella* Typhimurium (ampicillin resistance) were used to spike the dust samples with various particle sizes.

<u>For experiments conducted in Georgia</u> at a high relative humidity (~85.0 ± 5.0%) with tomatoes, bell peppers, peaches, and apples, dust particles with three size ranges were used: <150 microns, 150 to 3,360 microns, or >3,360 microns. The dust was inoculated by placing 5 grams onto the surface of a bacterial culture in a petri dish and spreading using a sterile bent glass rod until mixed (dry inoculation). A cell scraper was then used to recover the inoculated dust, which was then mixed with 45 additional grams of dust of the same type and hand massaged in a Whirl-Pak bag until homogenous. This resulted in an estimated inoculum level of ~8.0–9.0 $\log_{10}$ CFU/gram dust. **Peaches and apples were not originally included in the grant proposal, but were added to these studies to provide different produce surface types to better understand the transfer and survival of pathogens from dust.**

The dust transfer to produce was facilitated using pressurized air (via a crop duster) from a distance of ~2.5 inches (5 pumps) to whole fruits (tomatoes, bell peppers, peaches, apples).

The dust particles were allowed to attach to the surfaces of the produce for 90 minutes under a biohazard hood at the same relative humidity as the transfer. The dust was then recovered from the produce samples by placing the individual produce in PBS with 1% Tween 80 in a Whirl-Pak bag and manual massaging for 2 minutes in the solution. These sample eluates were serially diluted using PBS and spread plated on appropriate selective agar containing streptomycin and/or ampicillin. The plates were incubated at 37°C for 24 hours and the colonies were enumerated to determine the numbers of bacteria that were transferred to the produce through the dust contamination. The results for the dust transfer studies conducted with tomatoes, bell peppers, peaches, and apples are shown in **Figures 1–3**. Based upon statistical analyses, comparisons between the transfer/recovery rates of the various bacterial strains on various fruit surfaces are shown in **Table 2**.

Effect of particle size on dust contamination: There were some statistically significant differences in the transfer of pathogens to produce in some instances, suggesting that the particle size can affect the ability of the bacteria to be transferred to the surfaces of the various produce types grown in Georgia. With *S.* Newport, the smaller particle sizes seemed to be transferred more readily to tomatoes and bell peppers, whereas larger dust particles were transferred more readily to peaches. For *S.* Typhimurium, larger dust particles (>3,360 microns) were transferred more readily than small dust particles (<150 microns) to the surfaces of bell peppers and peaches. No difference between the transfer of different dust particles was observed for *E. coli* O157:H7. It appears likely that larger particles are trapped more efficiently by peaches than smaller particle sizes. This is most likely due to the presence of trichomes (fine hair-like structures) on the surface of peaches.

Effect of produce type on dust contamination: In general, no real differences were observed between the dust contamination transfer rates to the surfaces of the fruits with smooth surfaces (tomatoes, bell peppers, and apples) for any of the three bacterial strains; however, larger transfer rates were observed on peaches for both *Salmonella* strains. This greater transfer rate was likely due to the presence of trichomes. Such “peach fuzz” likely traps dust particles (especially particles >150 microns), whereas the smooth surfaces of tomatoes, bell peppers, and apples are less efficient at trapping dust particles.

Effect of bacterial strain on dust contamination: The transfer and recovery rates varied greatly by species/strain. The recovery rates for *S.* Newport ranged between approximately 2.61 $\log_{10}$/g and 4.79 $\log_{10}$/g produce. Likewise, the recovery rates for *S.* Typhimurium were similar (ranging between approximately 2.08 $\log_{10}$ and 4.20 $\log_{10}$/g produce) despite the original inoculum levels being approximately 1.0-$\log_{10}$ higher than the inoculum levels for *S*. Newport. In addition, although the inoculum levels for *E. coli* O157:H7 were between the levels used for *S.* Newport and *S.* Typhimurium, the recovery/transfer rates were much lower (ranging between approximately 0.80 $\log_{10}$ and 1.79 $\log_{10}$/g produce).

The dust transfer experiments conducted in Arizona on leafy greens (whole plants of spinach and romaine lettuce) were conducted at both low (27.0 ± 5.0%) and high (73.0 ± 3.0%) relative humidity levels and with inoculated dust particle sizes of <150 microns and <2,000 microns. The change from size ranges of 150 to 3,360 and >3,360 microns to <2,000 microns was based on the recommendation of the CPS technical committee that a mixture of sizes (i.e., 1 to 2,000 microns) would mimic real-world dust more closely than discrete, non-overlapping size ranges. The dust was inoculated by placing 5 grams onto the surface of a bacterial culture in a petri dish and spreading using a sterile bent glass rod until mixed (dry inoculation). A cell scraper was then used to recover the inoculated dust, which was then mixed with 45 additional grams of dust of the

same type and hand massaged in a Whirl-Pak bag until homogenous. This resulted in an estimated inoculum level of ~8.0–9.0 $\log_{10}$ CFU/gram dust.

The dust transfer to produce was facilitated using pressurized air (via a crop duster) from a distance of ~6.0 inches (5 pumps). The dust particles were allowed to attach to the surfaces of the produce for 90 minutes under a biohazard hood at the same relative humidity as the transfer. The dust was then recovered from the produce samples by removing whole leaves (all leaves from spinach plants, 3 outer leaves and 3 inner leaves from romaine lettuce plants) using sterile scissors and placing the leaves in sterile stomacher bags. The samples were then weighed to determine the weight of the inoculated leaves. A volume of either 50 ml (spinach) or 100 ml (romaine lettuce) of Buffered Peptone Water (BPW) was then added to each bag and the samples were pummeled in a stomacher on high speed for 2 minutes. These sample eluates were serially diluted using PBS and spread plated on appropriate selective agar containing streptomycin and/or ampicillin. The plates were incubated at 37°C for 24 hours and the colonies were enumerated to determine the numbers of bacteria that were transferred to the produce through the dust contamination. The results for the dust transfer studies conducted with spinach and romaine lettuce (outer vs. inner leaves) are shown in **Figures 4–9**. Based upon statistical analyses, comparisons between the transfer/recovery rates of the various bacterial strains on various leafy green surfaces are shown in **Table 3**.

Effect of particle size on dust contamination: For the dust transfer studies conducted on produce grown in Arizona, the larger particle size (<2,000 microns) was generally transferred at greater rates than the smaller particle size (<150 microns) for *S.* Newport and *S.* Typhimurium on all leafy green leaf types at both a low (27.0 ± 5.0%) and high (73.0 ± 3.0%) relative humidity (statistics performed using $\log_{10}$ reductions rather than the number of CFU recovered in order to account for various inoculum levels). This trend was also observed for *E. coli* O157:H7 at low relative humidity, but not at high relative humidity; however, at a high relative humidity, many of the samples had fallen below the detection level of the assay and thus there it is possible that differences may have been observed if the *E. coli* O157:H7 levels could have been quantified.

At a low relative humidity, all three bacterial strains survived better on the dust with larger particle sizes (up to 2,000 µm) than on small particle size dust. At a high relative humidity, the opposite was observed, with all three bacterial strains recovered in greater numbers from the <150-µm dust.

Effect of relative humidity on dust contamination: No statistically significant differences were observed between the transfer *S.* Newport or *S.* Typhimurium via dust on spinach or romaine outer and inner leaves at different relative humidity levels. In contrast, a higher relative humidity resulted in lower transfer and/or survival of *E. coli* O157:H7 on both romaine outer and inner leaves. In addition, the transfer of *E. coli* O157:H7 was lower on spinach at a high relative humidity with the larger particle size dust (<2,000 micron).

Effect of produce type on dust contamination: Overall, no significant differences were observed for the transfer and recovery of any of the bacterial strains on different leaf types (i.e., spinach vs. romaine lettuce outer leaves vs. romaine lettuce inner leaves) when comparing $\log_{10}$ reduction rates.

Effect of bacterial strain on dust contamination: Although some statistical differences were observed between the transfer/recovery rates between the *Salmonella* strains and *E. coli* O157:H7 were found (see **Table 3**), these comparisons do not fully describe the survival of the bacteria in dust. For instance, although the transfer rates were similar for *S.* Newport, *S.* Typhimurium, and *E. coli* O157:H7, their survival differed greatly in the dust. Despite all dust samples being inoculated with approximately 8.0 to 9.0 $\log_{10}$ CFU/gram, the dust inoculated with

S. Newport had an average concentration of 7.72 ± 0.39 $\log_{10}$ CFU/gram, the dust inoculated with *S.* Typhimurium had an average concentration of 8.07 ± 0.23 $\log_{10}$ CFU/gram, and the dust inoculated with *E. coli* O157:H7 had an average concentration of 5.19 ± 1.51 $\log_{10}$ CFU/gram. Thus, *E. coli* did not survive nearly as well in the dust as the two *Salmonella* strains. There was also a significant difference between the survival of the *E. coli* at low and high relative humidity levels in the dust (average surviving concentration at low relative humidity = 6.41 $\log_{10}$ CFU/gram; average surviving concentration at high relative humidity = 3.98 $\log_{10}$ CFU/gram).

Thus, the number of bacteria recovered varied by species/strain. The recovery rates for *S.* Newport ranged between approximately 2.84 $\log_{10}$ and 5.84 $\log_{10}$ CFU/g produce. Likewise, the recovery rates for *S.* Typhimurium ranged between 3.31 $\log_{10}$ and 5.65 $\log_{10}$ CFU/g produce. The recovery rates for *E. coli* O157:H7 were much lower, ranging between <1.35 $\log_{10}$ and 3.54 $\log_{10}$ CFU/g produce).

Objective 3 – *Test dust particulates from animal operations for the presence of biomarkers indicative of fecal contamination and the presence of enteric pathogens.*

Dust particulates from various distances (e.g., 162 feet, 1,200 feet, 2,300 feet, and 2 miles) away from poultry and beef facilities were collected using impinger-based dust collectors over a period of two years. Background samples from greater than 3 to 5 miles away from the animal operation were also included during each sampling trip to determine background levels for each region. It was not always possible to collect samples from each distance during each sampling trip due to limited access. A total of 153 dust/aerosol samples were collected in impingers containing sterile pyrogen free water, 153 samples were collected in impingers containing 0.1% peptone, and 24 samples were collected on filter membranes using a high-volume air sampler (see details below).

Dust/aerosol samples were collected in impingers using liter per min (Lpm) pumps (Gilian BDX-11 Air Sampling Pumps; Sensidyne, St. Petersburg, FL) to impinge the particulate matter into 90 ml of pyrogen-free, sterile, nanopure deionized water in one impinger, and in 90 ml of sterile 0.1% peptone in a second impinger collected at the same time. Each sample was collected using the pumps on high volume (4 Lpm) for 1 hour, resulting in an aerosol/dust sample volume of 240 L in a final volume of approximately 85 ml.

High-volume dust/aerosol samples were collected in Arizona using an InnovaPrep ACD-200 Bobcat™ Dry Filter Air Sampler on continuous mode for 1 hour at 200 Lpm). These samples resulted in an air sample corresponding to 12,000 L. The dust particles were captured on a filter and then eluted using a canister containing 8.5 ml of an eluting solution (containing 0.075% Tween20 and 25 mM Tris) under pressure. The fluid turns to foam and passes through the interstitial spaces of the filter to flush the filter and extract the captured particles. This resulted in a final sample volume ranging from ~4 to 6 ml. These large volume samples were added late in the project period because of the low numbers of positives using both cultural and molecular methods. **This was additional work that was not included in the original proposal.**

The water impinger samples collected in Georgia were returned to the laboratory at the University of Georgia on ice and then immediately frozen and stored at -20°C until analyzed for endotoxins and bile salts. The water impinger samples collected in Arizona were returned to the laboratory at the University of Arizona on ice and then a 10-ml aliquot was immediately frozen and stored at -20°C. These samples were subsequently shipped frozen to the University of Georgia for the bile salts / endotoxin assays. The quantification of bile salts was accomplished using a kit-based fluorescent probe (Sigma-Aldrich Bile Acid Assay Kit) to measure NADH reduction from NAD upon the interaction of 3-hydroxysteroid dehydrogenase with bile acids. The tests were performed with replicates of 20 µl of the water samples and compared to a sodium cholate (80 µM) standard for fluorescence production using a 96-well plate reader (excitation 530 nm

/ emission 580 nm). Samples were also tested for the presence of endotoxins using a Pierce™ limulus amebocyte lysate (LAL) Chromogenic Endotoxin Quantitation Kit assay in which the presence of endotoxin will cleave the chromogenic substrate, resulting in a change in color that can be measured using absorbance at 405 nm. Total endotoxin concentration was expressed in endotoxin units (EU) per ml of impinger sample. The results for the bile salts and the endotoxins are shown in **Table 4**.

For the impingers containing peptone, samples from both regions were returned to the laboratory on ice and then they were immediately aliquoted into various volumes for subsequent assay:

1) 50-ml volumes of each sample were immediately assayed using Colilert® QuantiTrays with incubation at 37°C for 24 hours to determine the most probable number (MPN) of total coliforms and *E. coli* present per 100 ml. No *E. coli* were detected in any of the samples collected in either region. No coliforms were detected from the samples collected in Arizona; 14.8% of samples collected in Georgia were positive for coliforms (**Table 5**).
2) *Salmonella* strains were assayed using enrichment in tetrathionate broth for 24 hours at 37°C. A subsequent enrichment was performed in Rappaport broth for 24 hours at 37°C and samples were streaked for isolation onto CHROMagar *Salmonella* plates for the selection of *Salmonella* strains. No *Salmonella* were detected using this method from any of the dust/aerosol samples collected in either Arizona or Georgia (see Table 5). Consequently, all of the samples were examined for the presence of *Salmonella* via qPCR (see below). **This was additional work not included in the original grant.**
3) For *E. coli*, the samples were enriched in EC broth for 24 hours at 44.5°C and then streaked for isolation onto eosin methylene blue (EMB) agar plates. No *E. coli* was detected using this method from any of the dust/aerosol samples collected in either Arizona or Georgia (see Table 5). Consequently, all of the samples will be examined for the presence of STEC via qPCR (see below). **This is additional work not included in the original grant.** These assays will be completed over the next few months and the results will be reported at the CPS Annual Research Symposium.
4) Because of the lack of recovery of bacterial pathogens / indicator organisms from the aerosol/dust field samples, duplicate volumes of 0.1, 1.0, and 5.0 ml were also immediately assayed on R2A plates via spread plating (0.1 ml) or membrane filtration (1.0 and 5.0 ml) and incubated for 5 days at 30°C to determine the number of heterotrophic plate count (HPC) bacteria per sample. HPC bacteria are a general indicator that can help to determine the overall microbial quality of a sample (e.g., high HPC levels indicates poor quality). Colonies were counted and the number of recovered bacteria per sample was determined (see Table 5). **This was additional work not included in the original grant.**
5) A 20-ml volume was frozen and stored at -20°C and the frozen samples from Georgia were shipped to The University of Arizona for qPCR to look for bacterial pathogens and genetic fecal markers. Upon thawing, the 20-ml peptone sample was concentrated by passage through a 0.22-µm MCE membrane filter (47-mm diameter) to capture all bacteria on the membrane. These filters were then placed into separate Qiagen DNeasy® PowerWater® Kits and bead-beated to extract the nucleic acids from the samples. This resulted in a 100-µl concentrated sample extract that was then used for all the subsequent qPCR assays.

   Primers, probes, and gblocks were purchased for the quantitative PCR assays, the PCR parameters were optimized, and standard curves were generated for all of the fecal genetic markers and microbial pathogens. This included assays to detect the *Salmonella invA* virulence gene, Shiga-toxin producing *E. coli* (STEC) virulence genes (*stx1*, *stx2*, and

eae), GFD (an avian wildlife marker targeting 16S rRNA sequences of *Helicobacter* spp.), LA35 (a poultry marker targeting 16S rRNA sequences of *Brevibacterium* spp.), and three bovine-associated qPCR assays (Rum2Bac targeting the 16S rRNA gene sequence of *Bacteroidales* present in ruminants, and CowM2 and CowM3 that are more specifically associated with cattle). The results for the qPCR assays are shown in **Table 6**.

Although numerous samples were positive for the fecal genetic markers (particularly for LA35, Rum2Bac, and GFD markers), the vast majority of these were below the limit of quantification (LOQ) for the qPCR assays. The poultry marker was detected in most samples, even when poultry operations were not nearby. This could be due to the use of chicken composts / fertilizers being used in nearby fields. The three cattle/ruminant fecal genetic markers were only detected in Arizona near a very large cattle feedlot (100,000 head). Nevertheless, the samples that were positive above the limit of quantification (LOQ) for cow/ruminant markers tended to be found at more distant locations (≥2,300 feet) from the concentrated animal feeding operation (CAFO) (see Table 6). One sample that was positive (>LOQ) for both Cow2 and Rum2Bac was located approximately 5 miles away from the CAFO, but in the direction of the wind on the date of sample collection. Two other samples that were positive (>LOQ) for two of the three cattle/ruminant markers were both located approximately 2 miles away from the CAFO.

For the high-volume filter samples (Bobcat™ Dry Filter Air Sampler), 1.0 ml of each sample was assayed using IDEXX Colilert™ MPN assays for the detection of coliforms and *E. coli* (neither of which were detected in any of the samples). Serial dilutions of each sample were assayed via spread plating on R2A plates to determine the number of HPC bacteria per sample. A volume of 1.0 ml of each sample was frozen and shipped to the University of Georgia for bile salts / endotoxin assays. Finally, a 2.0-ml volume was concentrated and the nucleic acid extracted in the manner described above for the impinger samples and examined via qPCR for the presence of *Salmonella* and the fecal genetic markers. **The collection of high-volume filter samples and the subsequent assays is all work not included in the original proposal.**

The results from the high-volume dry filter sampler are shown in **Table 7**. Although numerous samples were positive for each fecal marker (including 10 *rum2Bac* marker and 3 *GFD* avian marker that were not detected in the impinger samples collected at the same time from the same location), none of these were above the limit of quantification (LOQ) of the assays and were lower than the genome copies quantified in the impinger samples.

The primary reason that the low-volume impinging method was originally chosen was because it was predicted that it would be gentler on the bacteria in the samples and thus we would be more likely to recover and detect viable organisms than with the use of a high-volume dry air sampler (which would likely desiccate/kill the bacteria). We included these high-volume samples later during the project once it was determined that very few of the qPCR results were above the limit of quantification (LOQ), assuming that the desiccation would not have as great an effect on the nucleic acids. The results were mixed in this regard. Neither coliforms nor *E. coli* were detected in any of the cultures; however, higher levels of HPC bacteria were found in all of the high-volume samples in comparison to the low-volume impinger samples. These levels were often much higher than the 50 times greater amount one would expect, based upon the air volumes sampled (12,000 L versus 240 L). In general, a higher percentage of samples were positive for the genetic fecal markers with the high-volume impingers; however, the concentrations were always lower than that measured for the impinged samples.

A quantitative microbial risk assessment (QMRA) was proposed for this project to determine the risk of contamination of produce with regards to the proximity of produce fields to large animal feeding operations; however, there was not enough quantitative data accumulated from the field samples to accomplish this analysis. The bacterial pathogens were not detected in any of the

cultural or molecular assays. Even total coliform positives were rare in Georgia and were not detected in Arizona. There was substantial data collected on the presence and levels of HPC bacteria; nevertheless, HPC bacteria do not provide any information on the risk of pathogens in a sample. HPC counts can be used to determine how contaminated or "dirty" a sample might be, but the contamination could very well be coming from soil organisms in the region and could be completely innocuous for food safety concerns.

Despite the lack of sufficient data for the implementation of a QMRA, correlations were performed to determine if any relationships between any of the site, atmospheric, and microbial measurements could be identified. Several correlations were found (see **Table 8**). It appears that wind speed, temperature, and relative humidity may have a direct impact on the amount of particulates and thus bacteria that are present in suspended dust samples in the air. In addition, it appears that HPCs have the potential to be used as an indicator of fecal contamination of dust since there were weak to average correlations observed between HPC levels and indicators of fecal contamination such as bile salts (found in feces) and endotoxins (found in the LPS layer of Gram-negative bacterial cell walls). In addition, HPCs can be found in nearly all samples and thus the concentrations found could provide information on the risk of dust contamination of crops.

No correlations between the location of sample collection and the levels of various indicators / genetic markers were observed. This was not surprising since no obvious differences between the number of positive samples at various distances from the animal feeding operations were observed and several samples collected from miles away had quantifiable levels of animal fecal markers. This suggests that dust can become contaminated by animal feces and carried by wind for significant distances where they could potentially contaminate produce fields. The relevant question should thus likely be what level of such contamination can be considered below an acceptable safety threshold for potential food crop contamination grown at a specific distance away?

Outcomes and Accomplishments

The survival of bacterial pathogens and the cross-contamination / transfer of these pathogens by dust to the surfaces of fresh produce were examined in both laboratory and field studies in two diverse growing regions in the United States. The effect of dust particle size, produce type, relative humidity level, and the bacterial strain/species were determined in laboratory studies conducted at the University of Arizona and the University of Georgia using soil dust particles and fresh produce sourced from these regions. Laboratory dust survival / transfer studies were conducted in Georgia on tomatoes, bell peppers, apples, and peaches at a high relative humidity level comparable to that found in this region during the growing season. Laboratory survival / transfer studies were conducted in Arizona on spinach and romaine lettuce plants under both low and high relative humidity conditions. Although this region is typically quite arid, high humidity levels can be experienced during rainfall events.

In addition, field studies were conducted in both Arizona and Georgia to determine the effect of atmospheric conditions (e.g., solar intensity, wind speed and direction, temperature and relative humidity level) and proximity to animal feeding operations on the contamination of dust in these regions. This was accomplished by collecting dust particulates / aerosol samples at various distances (from 162 feet to 5 miles) from animal feeding operations in both regions to determine the levels of bacterial indicator organisms, foodborne pathogens, and fecal genetic markers present.

Summary of Findings and Recommendations

During this study, the survival and cross-contamination of produce by several foodborne bacterial pathogens were studied on dust from organic soils from two vastly different produce growing regions within the United States – Arizona and Georgia. These regions differ geographically, by growing season (winter in Arizona, summer in Georgia), by soil consistency and characteristics, and by average atmospheric conditions, particularly regarding relative humidity levels. Arizona is a dry / arid region, whereas Georgia is lush and quite humid. The types of produce grown in each region also vary greatly.

The survival and transfer studies conducted with dust in a range of particle sizes on produce grown in Georgia (tomatoes, bell peppers, apples, and peaches) and in Arizona (spinach, romaine lettuce outer leaves, and romaine lettuce inner leaves) provided several insights regarding the potential for contamination of produce by dust. For instance, it was determined that the dust particle size may play a role in certain situations in how the dust is transferred to the surfaces of the produce and in whether it is trapped on produce surfaces. The produce type is also a significant determinant on the amount of dust transfer that occurs. For example, produce with irregular surfaces such as some leafy greens may capture dust more readily under certain conditions and produce with surface structures such as the fine hairs (trichomes) on peaches are quite good at trapping dust particles (particularly larger dust particles). There were also differences identified between different species of bacteria and even between different strains of the same species. For instance, under certain conditions, *Salmonella* Newport survived less well in inoculated dust than *Salmonella* Typhimurium, but was still able to contaminate produce at levels comparable to *S*. Typhimurium. *E. coli* not only survived relatively poorly in inoculated dust, but also was transferred to the surfaces of fresh produce at proportionally lower concentrations than the two *Salmonella* strains. The relative humidity can also play a role in the survival and transfer of bacterial pathogens in dust to the surfaces of fresh produce.

For the field sampling, numerous samples were positive for the fecal genetic markers (particularly for LA35 poultry, Rum2Bac ruminant, and GFD avian markers); however, the vast majority of these were below the limit of quantification (LOQ) for the qPCR assays. The poultry marker was detected in most samples, even when poultry operations were not nearby. This could be due to the use of chicken composts / fertilizers being used in nearby fields. Total coliforms (an indicator of fecal contamination) were detected in only a few samples and neither *Salmonella* nor *E. coli* were detected in any of the samples in either growing region. Heterotrophic plate count (HPC) bacteria were detected in nearly all samples and were found to have some correlations to other measurements of fecal contamination such as the presence of fecal bile salts and endotoxins (from the cell walls of Gram-negative bacteria). Thus, HPC bacteria could possibly be used in the future as an indicator for the potential for fecal contamination of dust from animal feeding operations.

Although we could not determine an effect of proximity to animal feeding operations on the risk of contamination of dust by microbial pathogens, it is likely that a larger project with greater numbers of samples and locations is needed to conduct a quantitative microbial risk assessment that will determine the distance from such feeding operations that might be deemed safe for the production of fresh produce.

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APPENDICES

Publications and Presentations

Two publications are currently in progress.

Budget Summary

This research project was awarded $193,378 in grant funds. The entire operating budget, except for the travel funds (to be used to attend the 2023 CPS Annual Research Symposium), has been expended.

Tables 1–8 and Figures 1–9 (see below)

Table 1. The ability of *Salmonella* serotypes to survive in dust after a dry inoculation technique was used. Inoculum was an estimated 8.0 to 9.0 $\log_{10}$ CFU/gram dust.

Bacterial Strain	Dust Particle Size	Survival* on dust (Log_{10} recovered per gram dust)
Salmonella Newport	<150 micron	6.72
	>150 to <3,360 micron	5.52
	>3,360 micron	5.94
Salmonella Typhimurium	<150 micron	8.70
	>150 to <3,360 micron	8.76
	>3,360 micron	8.82
Salmonella Oranienburg	<150 micron	8.24
	>150 to <3,360 micron	8.77
	>3,360 micron	8.93

Table 2. Statistical comparisons of the transfer and recovery of pathogens on the surface of fresh produce at a high relative humidity (85.0 ± 5.0 %).

Bacterial Strain	**Transfer / Recovery**
Salmonella Newport	Peaches > Tomatoes = Bell Peppers
Salmonella Typhimurium	Peaches > Tomatoes = Bell Peppers
Escherichia coli O157:H7	Bell Peppers = Apples = Tomatoes
Produce Type	**Transfer / Recovery**
Tomatoes	*S.* Newport = *S.* Typhimurium > *E. coli* O157:H7
Bell Peppers	*S.* Newport > *S.* Typhimurium > *E. coli* O157:H7
Peaches	*S.* Newport > *S.* Typhimurium

= No statistically significant difference
> Statistically significant greater transfer/survival

Table 3. Statistical comparisons of the transfer and recovery of pathogens on the surfaces of leafy greens at both low (27.0 ± 5.0%) and high (73.0 ± 3.0 %) relative humidity.

Bacterial Strain	Relative Humidity	Transfer / Recovery
Salmonella Newport	Low	Spinach = Rom. Outer Leaves = Rom. Inner Leaves
	High	Spinach = Rom. Outer Leaves = Rom. Inner Leaves
Salmonella Typhimurium	Low	Spinach = Rom. Outer Leaves = Rom. Inner Leaves
	High	Spinach = Rom. Outer Leaves = Rom. Inner Leaves
Escherichia coli O157:H7	Low	Spinach = Rom. Outer Leaves = Rom. Inner Leaves
	High	Spinach = Rom. Outer Leaves = Rom. Inner Leaves
Produce Type	**Relative Humidty**	**Transfer / Recovery**
Spinach	Low	*S.* Newport = *S.* Typhimurium = *E. coli* O157:H7
	High	S. Newport = S. Typhimurium > E. coli O157:H7
Romaine Lettuce (Outer Leaves)	Low	S. Newport = S. Typhimurium > E. coli O157:H7
	High	S. Newport = S. Typhimurium > E. coli O157:H7
Romaine Lettuce (Inner Leaves)	Low	S. Newport = S. Typhimurium > E. coli O157:H7
	High	*S.* Newport = *S.* Typhimurium > *E. coli* O157:H7

= No statistically significant difference
> Statistically significant greater transfer/survival

Table 4. Quantification of bile salts and endotoxins detected in dust/aerosol samples collected near animal operations in Arizona and Georgia.

Distance from Animal Operation (feet)	Growing Region	Bile Salts % positive (µM*)	Endotoxin % positive (EU/sample)*
162	Arizona	-	-
	Georgia	33.3% (0.34)	100% (2.80)
500	Arizona	80.0% (2.60)	70.0% (0.89)
	Georgia	not detected	33.3% (2.10)
1,200	Arizona	90.0% (2.65)	70.0% (0.87)
	Georgia	-	-
2,300	Arizona	60.0% (2.31)	70.0% (1.16)
	Georgia	33.3% (0.32)	100% (6.79)
10,560 (2 miles)	Arizona	50.0% (1.91)	60.0% (0.96)
	Georgia	-	-
>15,840 to 26,400 (> 3 to 5 miles)	Arizona	80.0% (2.35)	100% (0.89)
	Georgia	38.9% (0.30)	83.3% (2.82)

* Geometric mean

Table 5. Recovery of bacterial indicators and pathogens from dust aerosol samples collected in Arizona and Georgia.

Distance from Animal Operation (feet)	Growing Region	HPC Bacteria (Log_{10} MPN per 100 ml ± SD)	Total Coliforms (Log_{10} MPN per 100 ml ± SD)	*Escherichia coli* (Log_{10} MPN per 100 ml ± SD)	*Salmonella* spp. (Log_{10} MPN per 100 ml ± SD)
162	Arizona	-	-	-	-
	Georgia	2.29 ± 1.28	< 1.61 ± 1.05	not detected	not detected
500	Arizona	3.00 ± 0.72	not detected	not detected	not detected
	Georgia	2.31 ± 1.43	< 1.78 ± 1.35	not detected	not detected
1,200	Arizona	3.08 ± 0.74	not detected	not detected	not detected
	Georgia	-	-	-	-
2,300	Arizona	3.29 ± 0.79	not detected	not detected	not detected
	Georgia	2.61 ± 0.78	not detected	not detected	not detected
10,560 (2 miles)	Arizona	2.93 ± 0.78	not detected	not detected	not detected
	Georgia	-	-	-	-
>15,840 to 26,400 (> 3 to 5 miles)	Arizona	2.27 ± 0.50	not detected	not detected	not detected
	Georgia	2.96 ± 1.27	< 1.23 ± 0.74	not detected	not detected

SD = standard deviation

Table 6. Detection of bacterial pathogens and fecal genetic markers from dust aerosol samples collected in Arizona and Georgia using quantitative polymerase chain reaction (qPCR).

Distance from Animal Operation (feet)	Growing Region	qPCR Results for Fecal Genetic Markers Total Percent Positive (% positive above LOQ w/gc per sample*)					
		LA35 (Poultry Marker)	*GFD* (Avian Marker)	*CowM2* (Cow Marker)	*CowM3* (Cow Marker)	*Rum2Bac* (Cow Marker)	*Salmonella* spp. (*invA* gene)
162	Arizona	-	-	-	-	-	-
	Georgia	100% (66.7% - 15,233 gc)	100% (0%)	0.0%	0.0%	0.0%	0.0%
500	Arizona	96.4% (10.7% - 13,140 gc)	64.3% (0%)	0.0%	17.9% (0%)	42.9% (0%)	0.0%
	Georgia	100% (66.7% - 16,009 gc)	100% (0%)	0.0%	0.0%	0.0%	0.0%
1,200	Arizona	100% (7.1% - 15,010 gc)	60.7% (0%)	0.0%	17.9% (0%)	35.7% (0%)	0.0%
	Georgia	-	-	-	-	-	-
2,300	Arizona	100% (7.1% - 10,309 gc)	71.4% (0%)	3.6% (3.6% - 487,050 gc)	14.3% (0%)	35.7% (3.6% - 7,365 gc)	0.0%
	Georgia	100% (66.7% - 24,756 gc)	100% (66.7% - 4,780 gc)	0.0%	0.0%	0.0%	0.0%
10,560 (2 miles)	Arizona	96.4% (10.7% - 10,254 gc)	67.9% (0%)	3.6% (3.6% - 11,560 gc)	28.6% (14.3% - 76,592 gc)	32.1% (3.6% - 9,520 gc)	0.0%
	Georgia	-	-	-	-	-	-
>15,840 to 26,400 (> 3 to 5 miles)	Arizona	100% (14.0% - 19,363 gc)	78.6% (0%)	0.0%	14.3% (7.1% - 8,963 gc)	42.9% (7.1% - 5,589 gc)	0.0%
	Georgia	100.0% (94.4% - 19,761 gc)	94.4% (0%)	0.0%	0.0%	0.0%	0.0%

LOQ Limit of Quantification

Table 7. Detection of bacterial pathogens and fecal genetic markers from dust aerosol samples collected in Arizona using a high-volume dry air sampler.

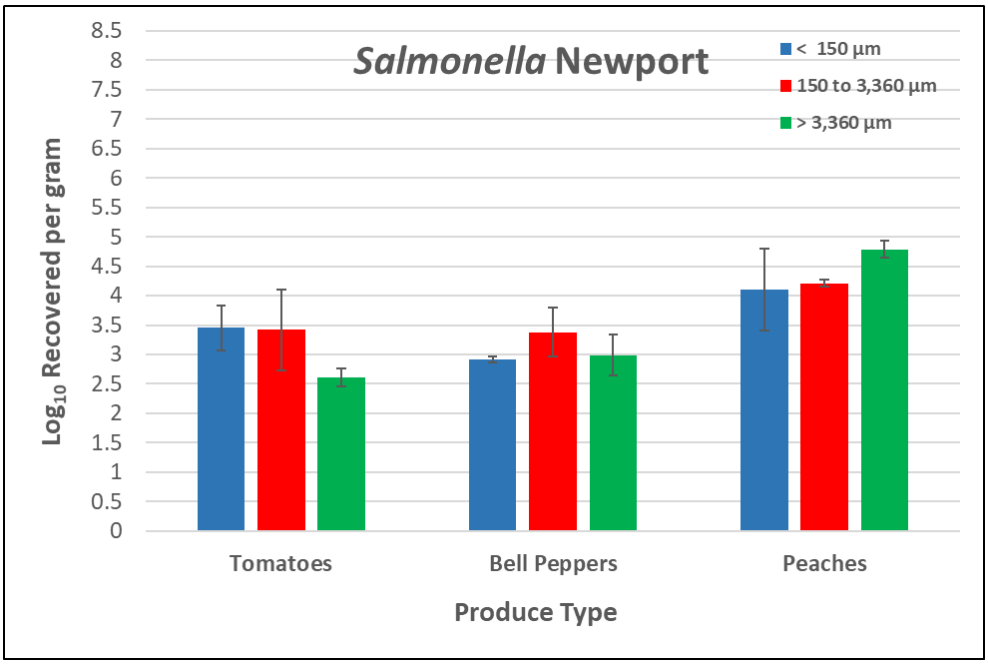
Distance from Animal Operation (feet)	qPCR Results for Fecal Genetic Markers Total Percent Positive (% positive above LOQ w/gc per sample*)						HPC Bacteria (Log_{10} MPN per 100 ml ± SD)
	LA35 (Poultry Marker)	*GFD* (Avian Marker)	*CowM2* (Cow Marker)	*CowM3* (Cow Marker)	*Rum2Bac* (Cow Marker)	*Salmonella* spp. (*invA* gene)	
162	-	-	-	-	-	-	-
500	100% (0%)	60.0% (0%)	0.0%	20.0% (20.0% - 78.6 gc)	100% (0%)	0.0%	6.58 ± 0.72
1,200	100% (0%)	80.0% (0%)	0.0%	0.0%	80.0% (0%)	0.0%	5.85 ± 0.81
2,300	100% (0%)	60.0% (0%)	0.0%	0.0%	80.0% (0%)	0.0%	6.23 ± 0.58
10,560 (2 miles)	100% (0%)	60.0% (0%)	0.0%	0.0%	100% (0%)	0.0%	5.94 ± 0.78
>15,840 to 26,400 (> 3 to 5 miles)	100% (0%)	60.0% (0%)	0.0%	0.0%	100% (0%)	0.0%	5.05 ± 0.41

LOQ Limit of Quantification
SD Standard Deviation

Table 8. Correlations observed between atmospheric and microbial measurements in dust / aerosol samples collected near animal feeding operations.

Measurement # 1	Measurement # 2	Correlation	Strength	Relationship
Temperature	Humidity	-0.48	Weak	As temp. increases, humidity decreases (as expected)
Temperature	LA35 marker	0.51	Weak	As temp. increases, poultry marker increases
Humidity	LA35 marker	0.49	Weak	As humidity increases, LA35 poultry marker increases
Humidity	Endotoxins	0.51	Weak	As humidity increases, Gram-negative bacteria increase
Humidity	HPCs (high vol. filter)	0.60	Average	As humidity increases, HPCs increase
Wind Speed	HPCs	0.42	Weak	As wind increases, bacterial levels in air increase
Wind Speed	Small particulates	0.47	Weak	As wind increases, small particulates in air increase
Wind Speed	Medium particulates	0.44	Weak	As wind increases, medium particulates in air increase
Wind Speed	Large particulates	0.46	Weak	As wind increases, large particulates in air increase
Small particulates	HPCs	0.37	Weak	As small particulates in air increase, HPCs increase
Small particulates	HPCs (high vol. filter)	0.72	Average	As small particulates in air increase, HPCs increase
Small particulates	Endotoxins (high vol. filter)	0.78	Average	As small particulates in air increase, Gram-negative bacteria increase
Medium particulates	HPCs	0.74	Average	As medium particulates in air increase, HPCs increase
Medium particulates	HPCs (high vol. filter)	0.81	Average	As medium particulates in air increase, HPCs increase
Medium particulates	Endotoxins (high vol. filter)	0.78	Average	As medium particulates in air increase, Gram-negative bacteria increase
Large particulates	HPCs	0.75	Average	As large particulates in air increase, HPCs increase
Large particulates	HPCs (high vol. filter)	0.83	Average	As large particulates in air increase, HPCs increase
Large particulates	Endotoxins (high vol. filter)	0.73	Average	As large particulates in air increase, Gram-negative bacteria increase
HPCs	HPCs (high vol. filter)	0.42	Weak	Correlation between small & high volume HPC samples
HPCs	Bile Salts	0.38	Weak	As HPCs increase, bile salts (fecal indicator) increase
HPCs (high vol. filter)	Bile Salts	0.73	Average	As HPCs increase, bile salts (fecal indicator) increase
HPCs	Endotoxins	0.36	Weak	As HPCs increase, Gram-negatvie bacteria increase
HPCs (high vol. filter)	Endotoxins	0.47	Weak	As HPCs increase, Gram-negatvie bacteria increase
Cow3	Bac2Rum	0.66	Average	As cattle marker increases, ruminant marker increases
Total coliforms	Bile Salts	0.52	Weak	As total coliforms increase, bile salts (fecal indicator) increase

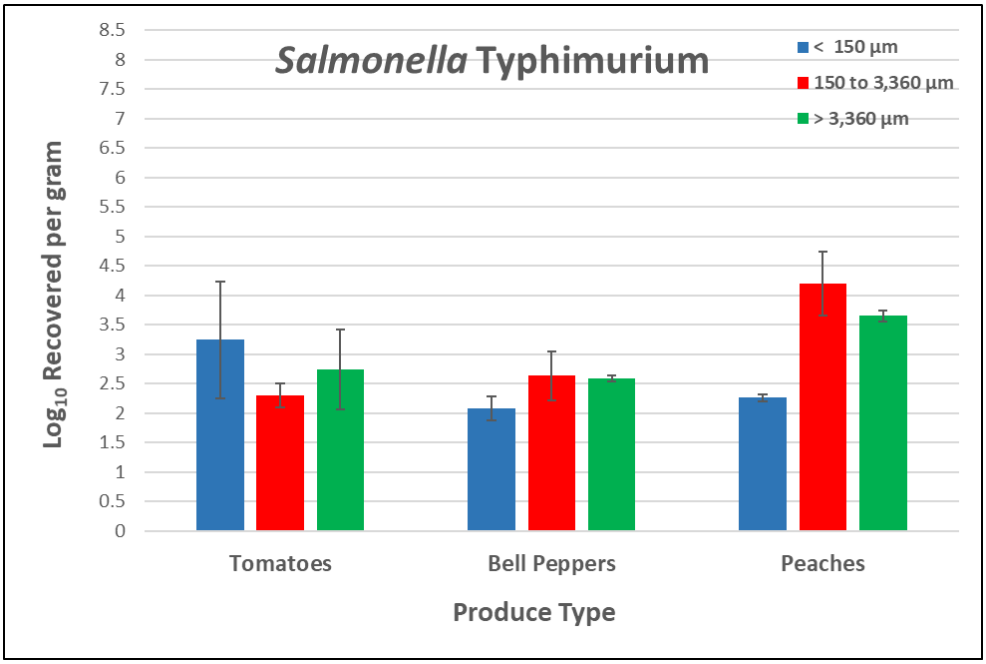
Figure 1. Cross transfer* of *Salmonella* Newport** from dust of varying particle sizes (<150 µm to >3,360 µm) to the surfaces of fresh produce at a relative humidity of approximately 85%.



* The recovery/transfer was significantly greater for the <150 µm particle size dust than for the >3,360 µm particle size dust on tomatoes and for the >3,360 µm dust particle size than for the 150 to 3,360 µm dust particle size on peaches. No significant differences between the dusts with different particle sizes were observed with bell peppers.

** Dust average inoculum was 7.93 ± 0.10 $\log_{10}$/g dust for tomatoes, 8.02 ± 0.40 $\log_{10}$/g dust for bell peppers, and 7.95 ± 0.19 $\log_{10}$/g dust for peaches.

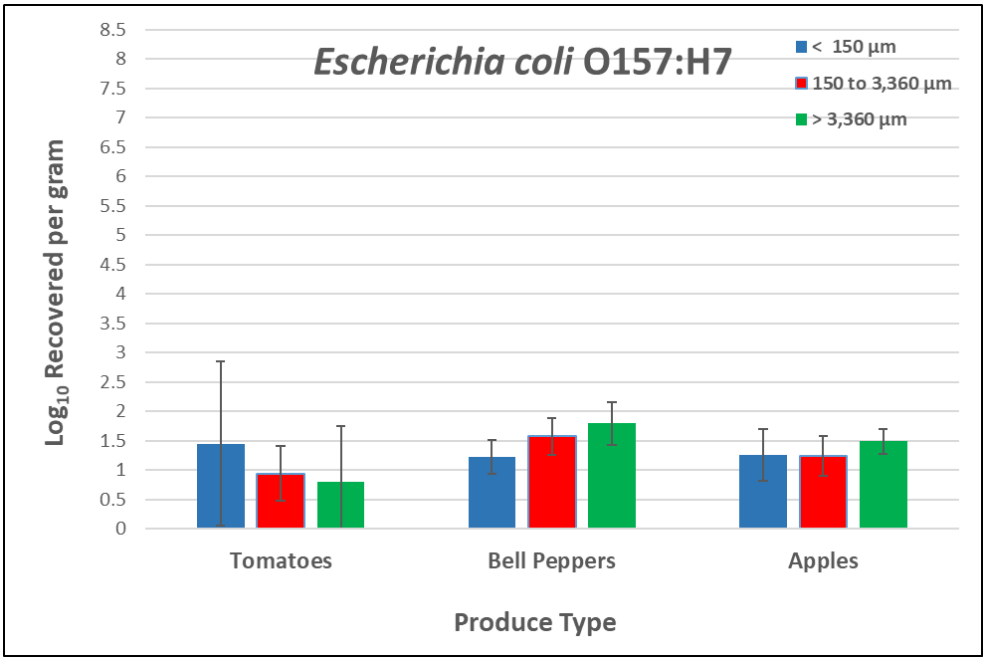
Figure 2. Cross transfer* of *Salmonella* Typhimurium** from dust of varying particle sizes (<150 µm to >3,360 µm) to the surfaces of fresh produce at a relative humidity of approximately 85%.



* No significant differences were observed between the transfer/recovery of dust of varying particle sizes on tomatoes; significantly less transfer/recovery was observed for the <150 µm particle size dust in comparison to the >3,360 µm particle size dust on bell peppers and peaches.

** Dust average inoculum was 8.89 ± 0.20 $\log_{10}$/g dust for tomatoes, 9.08 ± 0.10 $\log_{10}$/g dust for bell peppers, and 9.02 ± 0.16 $\log_{10}$/g dust for peaches.

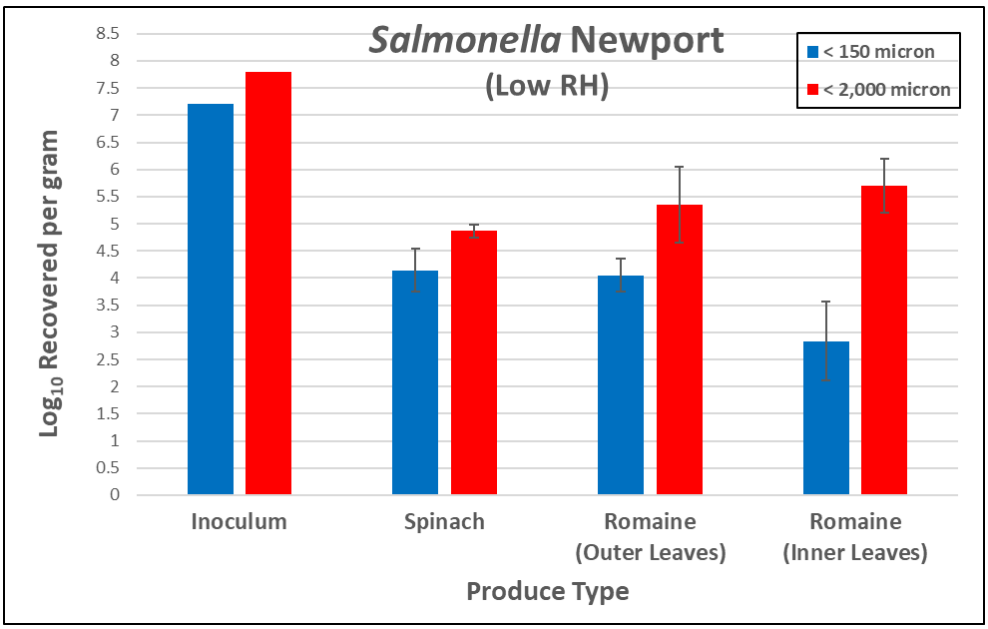
Figure 3. Cross transfer* of *Escherichia coli* O157:H7** from dust of varying particle sizes (<150 µm to >3,360 µm) to the surfaces of fresh produce at a relative humidity of approximately 85%.



* No statistically significant differences were observed between transfer/recovery rates for any of the dust particle sizes on any of the produce types.

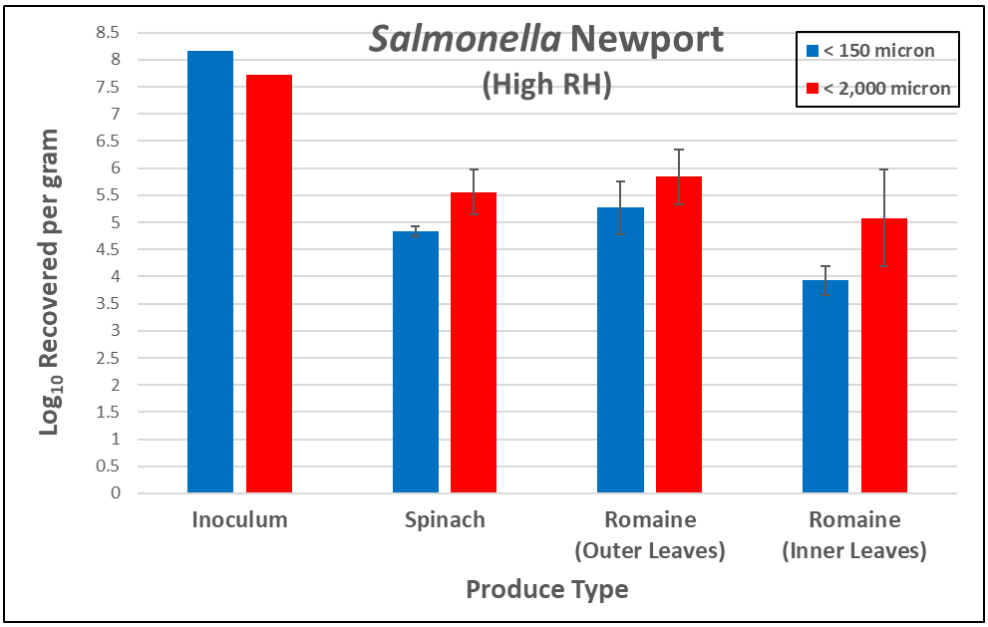
** Dust average inoculum was 8.48 ± 0.15 $\log_{10}$/g dust for tomatoes, 8.70 ± 0.06 $\log_{10}$/g dust for bell peppers, and 8.72 ± 0.13 $\log_{10}$/g dust for apples.

Figure 4. Cross transfer* of *Salmonella* Newport from dust of varying particle sizes (<150 µm to <2,000 µm) to the surfaces of leafy greens at a relative humidity of 25.9 ± 3.5%.



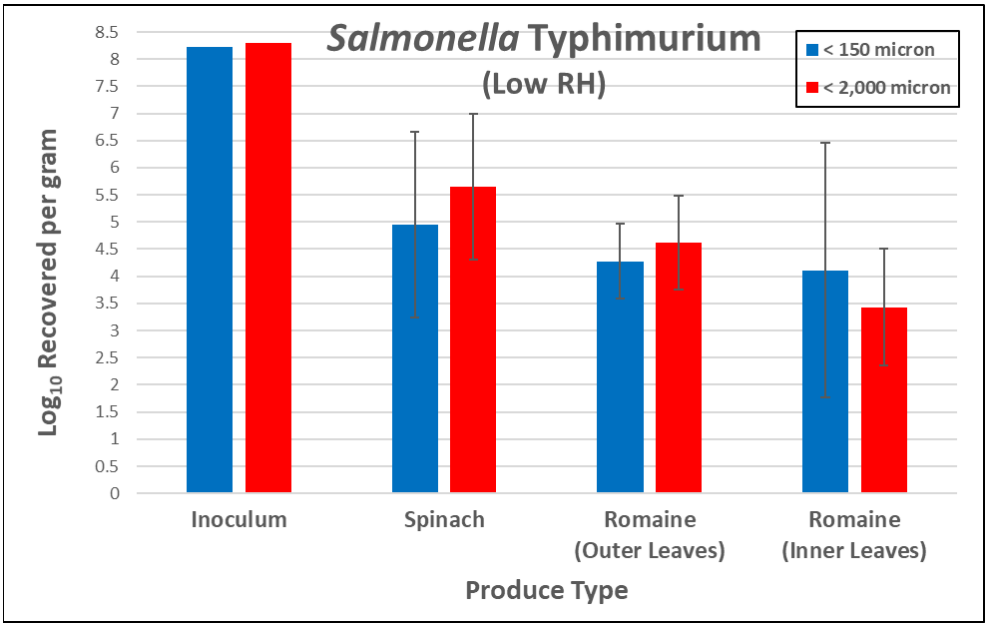
* The recovery/transfer was significantly greater for the <2,000 µm particle size dust than for the <150 µm particle size dust on spinach and romaine lettuce outer and inner leaves.

Figure 5. Cross transfer* of *Salmonella* Newport from dust of varying particle sizes (<150 µm to <2,000 µm) to the surfaces of leafy greens at a relative humidity of 72.9 ± 1.4%.



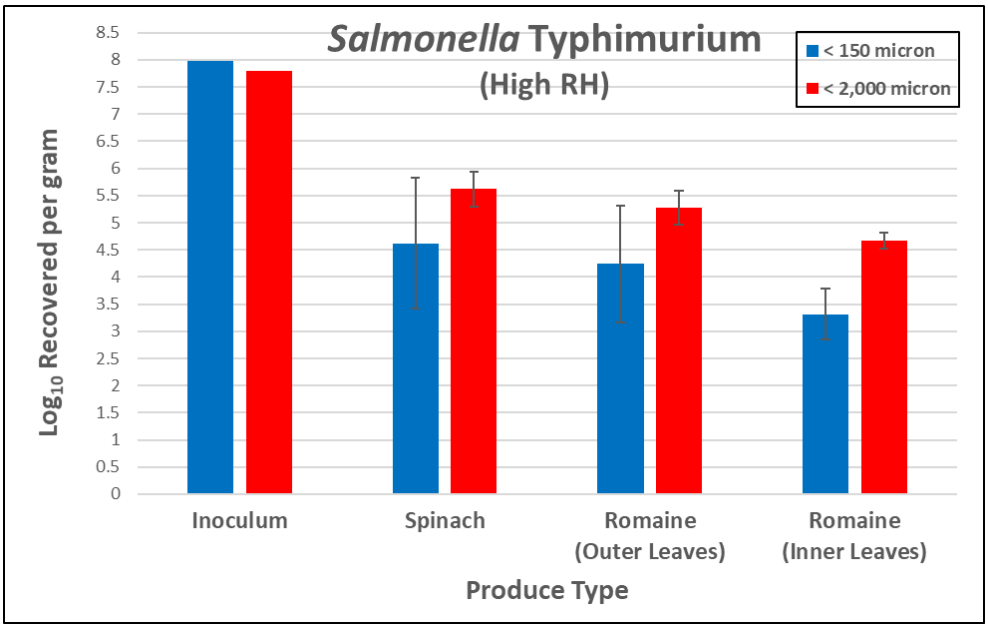
* The recovery/transfer was significantly greater for the <2,000 µm particle size dust than for the <150 µm particle size dust on spinach. No significant differences in recovery/transfer were observed between the two particle sizes for romaine outer or inner leaves.

Figure 6. Cross transfer* of *Salmonella* Typhimurium from dust of varying particle sizes (<150 µm to <2,000 µm) to the surfaces of leafy greens at a relative humidity of 25.5 ± 3.2%.



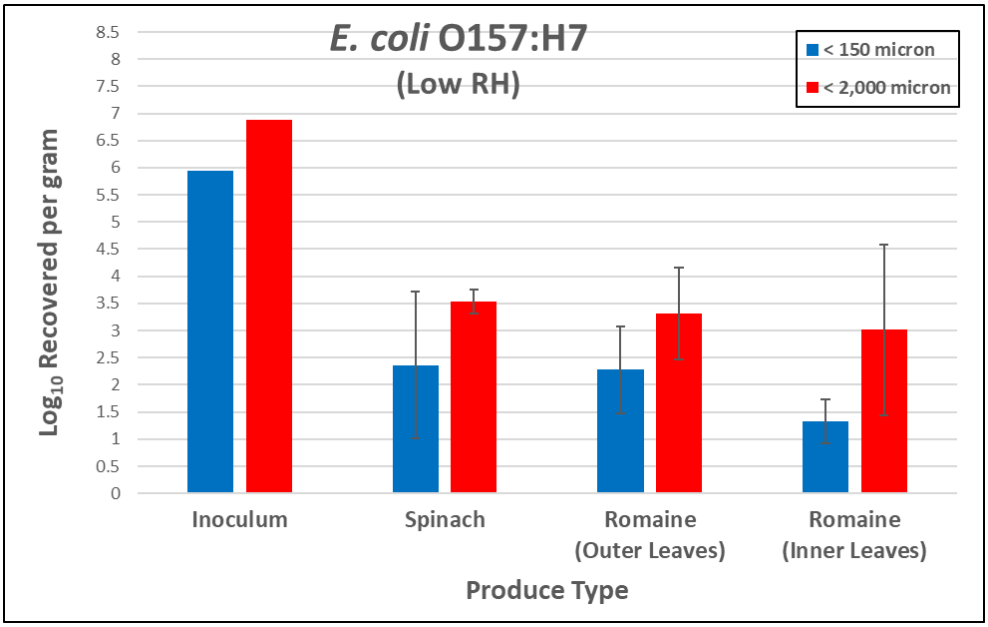
* No significant differences were observed between the recovery/transfer for dust of varying particle sizes on spinach or romaine lettuce outer and inner leaves.

Figure 7. Cross transfer* of *Salmonella* Typhimurium from dust of varying particle sizes (<150 µm to <2,000 µm) to the surfaces of leafy greens at a relative humidity of 72.7 ± 2.0%.



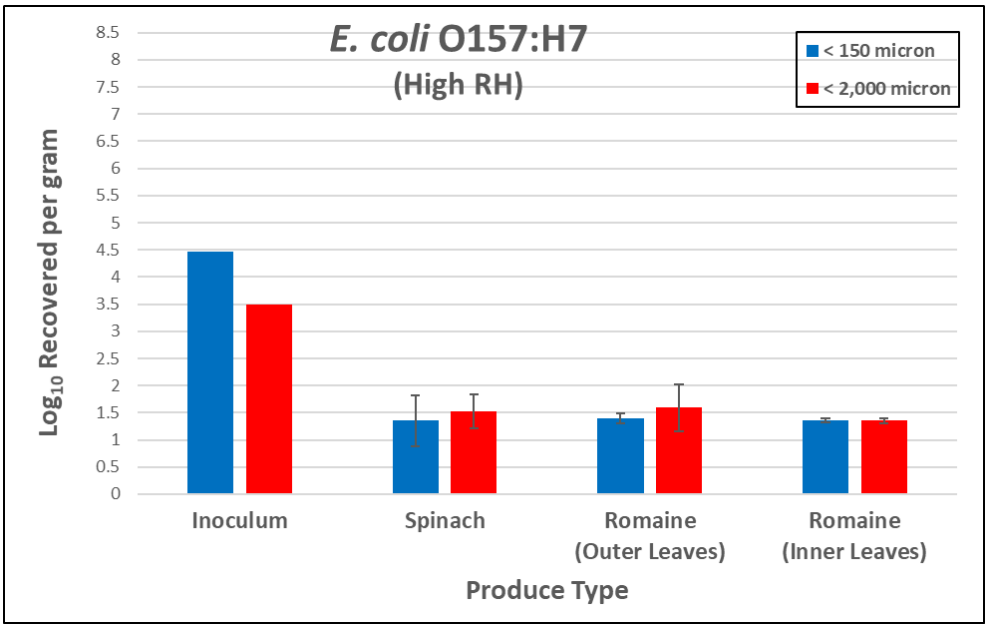
* The recovery/transfer was significantly greater for the <2,000 µm particle size dust than for the <150 µm particle size dust on romaine lettuce inner leaves. No significant differences in recovery/transfer were observed between the two particle sizes for spinach or romaine outer leaves.

Figure 8. Cross transfer* of *Escherichia coli* O157:H7 from dust of varying particle sizes (<150 µm to <2,000 µm) to the surfaces of leafy greens at a relative humidity of 28.5 ± 3.5%.



* No significant differences were observed between the recovery/transfer for dust of varying particle sizes on spinach or romaine lettuce outer and inner leaves.

Figure 9. Cross transfer* of *Escherichia coli* O157:H7 from dust of varying particle sizes (<150 µm to <2,000 µm) to the surfaces of leafy greens at a relative humidity of 74.7 ± 1.1%.



* No significant differences were observed between the recovery/transfer for dust of varying particle sizes on spinach or romaine lettuce outer and inner leaves.