CENTER for PRODUCE SAFETY

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FINAL PROJECT REPORT

Project Title
Selection of *E. coli* surrogates with attachment and survival patterns similar to those of human pathogens associated with produce

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Objectives
The goals of the project are to (1) provide science based information on survival of pathogens and indicators in soil and on fresh produce when initially present in either applied manure or irrigation water and (2) identify characteristics of indicators that make them better surrogates of produce associated pathogens. The specific objectives include:

(1) Select E. coli surrogates from 1,346 isolates obtained from poultry, dairy and swine manure or from surface water sources (Cook et al., 2011) that have properties similar to those shown to be important for survival/binding/internalization of human pathogens on fresh produce.

(2) Determine physical, chemical and biological properties including cell surface (electrophoretic mobility, hydrophobicity, surface charge density, extracellular polymeric substance (EPS) composition), cell size, adherence to soils, carbon utilization profiles and genetic factors (including those associated with adhesion, transport, capsular formation, iron scavenging and cell aggregation) for pathogens and indicators.

(3) Monitor die-off of pathogens and indicators in soil and on produce following application through contaminated manure or contaminated irrigation water.

(4) Identify E. coli strains that behave similarly to pathogens by statistical analysis of survival and adherence patterns and by correlating to previously defined physical, chemical and biological characteristics.

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FINAL REPORT

Abstract

Despite the best efforts of industry and regulatory agencies to identify and implement good agricultural practices (GAPs) to reduce or eliminate pathogen contamination, significant outbreaks associated with fresh produce continue to occur in the U.S. and around the world. While manures and irrigation waters are crucial to crop production, these are also potential sources of produce contamination by manure-borne bacteria. Identification of non-pathogenic surrogates for common produce-associated human pathogens may aid in development of better GAPs and processing strategies. In previous studies, we isolated over 1,300 *E. coli* from diverse manure and water sources. In this project, we build on those studies by using this collection of environmental isolates of *E. coli* to compare the behavior of strains with diverse physical, chemical and biological properties with that of produce-associated pathogens, *E. coli* O157:H7 and *Salmonella typhimurium*. Selections of *E. coli* surrogates were based on similarity of isolates to pathogens in terms of biofilm and leaf attachment, carbon source utilization and cell surface characteristics. Behavior of a common quality control (QC) *E. coli* strain was found to be significantly different than that of *Salmonella* in both biofilm formation and leaf attachment ($p < 0.05$). Selected environmental surrogates formed greater biofilms when grown in lettuce lysates, and attached to lettuce in a manner similar to *Salmonella*. Persistence of surrogates, pathogens and the QC strain on lettuce plants was assessed in greenhouse-scale experiments, and it was found that all isolates were above detection levels for 22 days. Die-off rates on lettuce show that the QC strain had the greatest rate of die-off while the pathogens and selected surrogates had statistically similar or lower rates of die-off. In soils (sandy loam and silt loam) with or without poultry litter amendments, the survival of environmental isolates was affected by the presence of organic carbon, and silt loam soil supported survival of all isolates at higher rates than the sandy loam. In soils with no amendment, the surrogates and *Salmonella* stayed above the detection limit of 10^3 cells/g for at least 42 days. The QC strain and *E. coli* O157:H7 fell below detection at the 42-day sampling period in the sandy loam. The data from this project provide support for the use of surrogates selected from the environment as improved representatives of produce-associated human pathogens. The commonly used QC strain *E. coli* ATCC 25922 may not be an adequate surrogate for *Salmonella* but may adequately represent *E. coli* O157:H7 in certain situations. These results provide a foundation for future research into the selection and use of pathogen surrogates for varying environments to enhance GAPs, improve food safety, and reduce risk to public health.

Background

Contamination of food and water by pathogens continues to be a public health issue in the United States. It is estimated that 48 million foodborne illnesses occur each year in the U.S., of which 9.4 million are caused by known pathogens (Centers for Disease Control, 2007). According to a Centers for Disease Control and Prevention report, 46% of foodborne illness in the United States is caused by fresh produce (Painter, et al., 2013). Leafy vegetables were categorized as the highest food safety priority by the World Health Organization (WHO) in 2008 (World Health Organization, 2008). A recent workshop of food safety experts from farm, consumer and fresh produce processing organizations listed identification and implementation of good agricultural

practices (GAPs) as the most important control measure to ensure the safety of fresh produce (Van Boxstael, et al., 2013). Although mitigation strategies are often targeted to post-harvest processing, environmental monitoring programs for pre-harvest safety are increasingly important as the FDA Food Safety and Modernization Act (FSMA) has prioritized prevention by strengthening food safety on the pre-harvest side of production. The economic and public health implications of suggested changes make science-based and agriculturally relevant data for GAPs of paramount importance so they can be used as part of consumer and producer oriented solutions to improve produce safety.

Manure and waters used in agricultural practices are sources of manure-borne bacteria. Although the majority of these bacteria are beneficial and/or harmless, the potential for contamination of crops and water sources with pathogens is of concern and requires increased understanding of pathogen behaviors in these agricultural environments. Two pathogens, *Salmonella* spp. and *Escherichia coli* (*E. coli* O157:H7), are of the greatest concern due to their association with significant outbreaks linked to consumption of fresh produce (Crim, et al., 2014; Goodburn, et al., 2013; Olaimat, et al., 2012).

Indicator organisms are often used as a measure of the presence of fecal contamination since they are present in concentrations of 10^5 to 10^7 cells per gram. Pathogens are present in much lower concentrations, they are often difficult to grow, and working with pathogens requires skilled workers, bio-safety level 2 certification and costly supplies. Monitoring contamination using indicators and in particular *Escherichia coli* (*E. coli*) has a long and well-documented history. *E. coli* is a dominant intestinal commensal organism and an important fecal indicator bacterium. However, targeting both pathogens and indicators in the diverse and complex agricultural environment is problematic. Therefore, studies are warranted to continue to explore ways to make this indicator (or similar organisms) better representatives of pathogen occurrence in agricultural applications such as produce safety.

The need lies in uncovering strains/properties of *E. coli* that make it behave in a manner similar to that of the pathogen while ensuring that it is safe and easy to use for research and quality control purposes. This search for a suitable surrogate for pathogens must follow a specific process (Sinclair, et al., 2012). These steps include defining the problem and environmental system in which the bacterium is found, selecting and prioritizing surrogate attributes, selecting candidate surrogates, conducting experiments with the candidates, assessing or evaluating exposure potential and/or dose response, and risk characterization (Sinclair, et al., 2012). The goal of surrogate use is to provide a non-pathogenic organism that will behave as an actual pathogen would in a specific environment, and allow scientific data to be collected to better understand and remediate risk to public health, in particular the consumption of contaminated fresh produce.

Environmental *E. coli* isolates may be a potential source of improved surrogate organisms. Studies conducted in our lab suggest that phenotypic and genotypic variability of environmental isolates of *E. coli* exists not only between different sources of isolation (i.e., swine, poultry, dairy) but also from within a single source (Bolster, et al., 2010, Bolster, et al., 2009, Cook, et al., 2011). Strain-level differences in physical, chemical and biological properties of *E. coli*

isolates likely improve the probability of survival in harsh, biologically competitive environments like those found in farm habitats (in manure, soil and on plant surfaces).

The goal of this project was to build on our previous studies of strain level diversity of environmental isolates of *E. coli* (>1,300 isolates from 4 sources) to compare the behavior of strains with diverse physical, chemical and biological properties with the behavior of produce-associated human pathogens. Properties of *E. coli* isolates that make them appropriate surrogates for produce-associated human pathogens were identified. Candidate surrogates were selected and used in studies where irrigation waters or soils were contaminated with both surrogates and pathogens. The behavior of surrogates and pathogens in soils and on fresh leafy greens was then compared to determine the feasibility of using environmental strains of *E. coli* as surrogates for produce pathogens. This work was a collaborative effort between groups at the USDA-ARS in Bowling Green, KY, Western Kentucky University Department of Public Health (including 1 MS student) and the University of California, Riverside College of Engineering (including 1 PhD student).

Hypothesis

E. coli isolates from environmental sources that possess physical, chemical and/or biological properties similar to those of human pathogens associated with produce colonization will survive and adhere to produce and soil in a manner similar to the pathogen.

Research Methods

Environmental *E. coli* isolates: selection of candidate surrogates

The USDA-ARS unit's collection of *E. coli* isolates (n=1,326), collected and isolated from poultry, swine and dairy manures and from surface water sources, was used as a pool for selection of surrogates. The objective was to use this diverse pool of *E. coli* isolates from multiple environmental sources and characterize them for factors that have been identified as important to the survival and persistence of pathogens on plant and soil surfaces. In this characterization process, isolates were eliminated to narrow the pool from which the final surrogates were selected. Figure 1 provides a visual description of the surrogate selection process and the data used as selection criteria at each step.

Using a subset of isolates from the original culture collection (n= 250) that provided equal representation among source types, data from Cook et al. (2011), growth rates and adhesion assays were used as the basis for additional narrowing of the pool (n=63; Fig. 1). These 63 *E. coli* isolates were then characterized based on characteristics (both genotypic and phenotypic) that have been determined in recent studies to affect the ability of pathogens to survive on fresh produce or in soils, leading to their ingestion, foodborne illness, and increased risk to public health (Brandl, 2006; Cook, et al., 2011; Olaimat, et al., 2012; Yaron, et al., 2014). The pool of 63 surrogates was analyzed for these characteristics, and the resulting data were used to select a group of 18 "candidate surrogates" that would be further characterized and used for surrogate selections (Fig. 1 "63 Isolates"). The 18 candidate surrogate isolates included both high and low biofilm formers when grown on nutrient broth and in lettuce lysates. Representatives from each source type (i.e., poultry, swine, dairy, dry event and wet event water source samples) were selected.

Characterization of isolates: candidate surrogates

The 18 candidate surrogates were evaluated for phenotypic properties (i.e., biofilm formation, curli expression, carbon source utilization and cell surface) and genotypic properties (i.e., phylotype, virulence, adhesion and biofilm genes) as described below; the same isolates were sent to our University of California Riverside collaborator for cell surface characterization. Also, the 18 candidates were tested for attachment to lettuce leaf surfaces and were sent to the *E. coli* Reference Center at Pennsylvania State University (University Park, PA) for serotyping and virulence gene testing. These data were compared with produce pathogens *Salmonella typhimurium* (ATCC 13311), *E. coli* O157:H7 (ATCC 43888), and a common FDA quality control strain *E. coli* (ATCC 25922).

Lettuce lysates

The lettuce exudates were prepared as previously described from store bought green leaf lettuce heads (Brandl, et al., 2008; Kyle, et al., 2010). Minimal salts media was added to the exudates as previously described, with the addition of 19 mM of ammonium chloride (Brandl, et al., 2008). The final product of lettuce exudates amended with minimal salts was used as the primary growth media for all isolates in preparation for experiments (and from this point forward will be referred to as LM media). The growth of isolates in LM media replicated the conditions of the environment due to the low level of nutrients available for growth and the potential environment that pathogens would face when on plant surfaces. Behavior of isolates grown on the low nutrient lettuce lysates was compared with that of isolates grown on high-nutrient Luria-Bertani (LB) broth (Difco, Bectin, Dickinson and Co., Sparks, MD).

Biofilm formation

The biofilm formation of isolates for this study was determined as previously described, using a crystal violet stain assay in 96-well microtiter culture plates (Patel, et al., 2010). Biofilm readings were completed using a BioTek ELx808 absorbance microplate reader (BioTek Instruments Inc., Winooski, VT) and the optical density (O.D.) of each well was measured at 546 nm. O.D. values from residual background dye in control wells were subtracted from wells with cultures.

Curli expression

The expression of curli fimbriae was characterized by growing all isolates on agar plates containing dyes and low salts that are taken up by organisms that produce the curli fimbriae (Römling, et al., 2003). Red colonies visible after 48 h of incubation at room temperature indicated the expression of curli. Curli expression for each isolate was categorized as previously described (Reisner, et al., 2006).

Cell surface characterization

To characterize differences in isolate cell surface when grown under high-nutrient (LB) or low-nutrient (LM) conditions, each of the isolates was grown for 18 h and harvested in the stationary phase. Isolates were grown in either LM or LB (see sub-section *Lettuce lysates*). Zeta potential and effective diameter were assessed by measuring electrophoretic mobility with a ZetaPALS analyzer. Relative hydrophobicity was measured using the microbial adhesion to hydrocarbons test and measuring changes in optical density with a UV-Vis spectrophotometer. Surface charge density was assessed through potentiometric titration using a Titrino auto-titrator. Extracellular polymeric substance (EPS) was characterized by freeze drying and colorimetry.

Genotyping and serotyping

All 18 isolates were genetically characterized as previously described, targeting genes for adhesion, capsule synthesis and siderophore formation (Cook, et al., 2011; Frömmel, et al., 2013; Gomi, et al., 2014; Kaufmann, et al., 2006). Serotyping of *E. coli* for somatic (O) and flagellar (H) antigens was performed by the *E. coli* Reference Center at Pennsylvania State University (http://ecoli.cas.psu.edu/services/index.htm#serotyping). O serotyping was conducted using O-antigenic polysaccharides generated against *E. coli* serogroups O1-O187. H typing was performed by PCR-RFLP analysis of the *fliC* gene. Virulence genes were assessed by PCR analysis for LT, STa, STb, stx1, stx2, *eae*, *cnf*1 and *cnf*2.

Lettuce leaf attachment

Attachment of isolates to lettuce leaves was determined using modifications of methods as described previously (Brandl, et al., 2008; Shaw, et al., 2008). Briefly, three-leaf stage green leaf lettuce plants grown in the USDA-ARS unit greenhouse were used for all experiments. All 18 candidate surrogates were grown in LM media to approximately 1 x 10^9 cells ml^{-1} to be used as the inoculate. Leaves from the plants were aseptically removed and dipped into the inoculate for 30 seconds, and then immediately washed in sterile 0.1X PBS buffer for an additional 30 seconds to remove any loosely adhered cells. DNA was extracted from leaf samples, and the attached surrogate or pathogen cells were quantified by quantitative, real-time PCR (qPCR) as described below.

Characterization of final surrogates: greenhouse-scale soil and lettuce survival studies

Green leaf lettuce (*Lactuca sativa*) seedlings were grown in commercial potting mixture in the USDA-ARS unit's greenhouse. Growth conditions were controlled in a manner to best simulate commercial production. Moisture content of the soil was monitored and adjusted daily as needed to maintain moisture levels (~70% moisture) using a custom built three-zoned automated drip irrigation system (Fig. 2). Seedlings were seeded and germinated in an organic commercial potting mix (SunGro Horticulture, Agawam, MA), and then transplanted at approximately 7 days post-emergence into a 13.75-cm square pot. Plants were fertilized with Jack's 20-20-20 fertilizer, once at the time of transplanting and again 2 weeks after planting.

Lettuce plants were grown inside the biosafety-certified bay of the USDA-ARS greenhouse, where a smaller (12 x 20 feet) microclimate was built. Inside the microclimate, two air conditioners were installed to control temperatures during the hot summer months and to reduce humidity. Greenhouse temperatures varied due to the outside climate, with average high temperatures near 25°C and lows near 15°C. Light was dependent upon natural sunlight but was supplemented with greenhouse lighting on overcast days.

Greenhouse experiments were carried out with two to five of the final selected environmental *E. coli* surrogates, the two pathogens *E. coli* O157:H7 (ATCC 43888) and *Salmonella typhimurium* (ATCC 13311) , and the QC strain (ATCC 25922). Each of the eight isolates was grown from a frozen sample using the method previously described in LM growth media.

Surrogates persistence experiment

To determine the length of time in days that pathogens and surrogates could potentially survive on lettuce plants, a greenhouse experiment was conducted to determine how well the selected

surrogates, pathogens and QC strain would survive over an extended period of time on 1-month-old lettuce plants. In this experiment the surrogates were applied as a group, along with the *E. coli* O157:H7 and the QC strain. *Salmonella* was applied separately.

On each plant, three replicate leaves of similar size and age were inoculated with a single isolate to simulate spray irrigation. For this, 1 ml of isolate inoculate at approximately 10^7 cells ml^{-1} was evenly distributed onto the leaf surface from the point where the leaf joined the plant stem outward to the middle of the leaf (approximately 5–6 cm). The experiment included three control plants that were placed among the experimental plants to determine if any cross contamination occurred. Sampling was done on days 0, 1, 2, 3, 5, 7, 12, 15, and 22. Day 0 samples were taken 90 minutes post inoculation of the plants. All controls sampled were negative for growth, indicating no cross contamination among the plants.

Modified mTEC agar was used to detect *E. coli* O157:H7 (Difco, BD). LB agar plates (Difco, BD) supplemented with ampicillin (50 µg/ml) were used to detect the QC strain, a green fluorescent protein (gfp) labeled 25922 strain (ATCC 25922gfp) strain. Colonies of 25922gfp were enumerated under black light by counting green fluorescent colonies. *Salmonella* was detected using Improved HiChrome *Salmonella* agar (Sigma-Aldrich Co., St. Louis, MO).

Soils and amendments experiment

A laboratory-scale experiment was conducted to evaluate the survival of two of the selected surrogates, the two pathogens, and the QC strain in two soil types with or without amendments (poultry litter). A sandy loam (Hartsells fine sandy loam consisting of 17.5% clay, 63.1% sand, and 19.4% silt) from Northern Alabama, and a silt loam (Crider silt loam, consisting of 19.0% clay, 4.0% sand, and 77.0% silt) from Western Kentucky, were used in all soil studies. Amendments were added at a 10% w/w level to the soils (Franz, et al., 2008). All soils and amendments were mixed in sterile plastic containers (1.89 L) with a total of 600 g of soil/amendment combination (SAM) in each. Sterile water was added to each SAM to achieve 60% WHC or approximately 81% dry weight ± 4%.

Triplicate soil containers for each SAM were inoculated with a mix of the five isolates to achieve a concentration at approximately 1 x 10^7 cells g^{-1}. Additional triplicate containers were mixed with sterile water for controls for each soil and amendment type. All containers were kept at 20°C with a 16-mm aeration hole in the top corner of each container. On days 0, 7, 21, and 42 the containers were sampled by shaking and mixing of the soil, and then aseptically removing approximately 5 g of SAM. Samples were extracted and the inoculated isolates were enumerated by isolate specific qPCR analysis, as described below. Enrichments of soil samples were done to verify that the *E. coli* and *Salmonella* were present after cell numbers reached detection limits.

DNA extractions and quantitative, real-time PCR (qPCR) assays

The concentration of final candidate surrogates, *Salmonella, E. coli* O157:H7 and QC strain *E. coli* ATCC 25922 were quantified using qPCR assays as previously described (Cook, et al., 2011; Frömmel, et al., 2013; Gomi, et al., 2014; Kaufmann, et al., 2006). All qPCR assays were developed and confirmed to detect only the targeted strain. Taqman assays were carried out in Qiagen HotStarTaq Master Mix and SYBR assays were carried out in QuantiTect SYBR Green PCR mix (Qiagen, Valencia, CA) as previously described (Cook, et al., 2014). DNA from soil or lettuce was extracted using the FastDNA® Spin kit for soils (MP Biomedical, Solon, OH) according to manufacturer's specifications. Soil DNA extracts were further purified to remove

PCR inhibiting molecules (e.g., humic material) using GENECLEAN® Kit (MP Biomedical, Solon, OH) according to manufacturer's specifications.

Statistical Analysis

To test the significant difference between two regression coefficients for independent samples, a z-score was determined using a z-test equation (Paternoster, 1998). The two regression coefficients were considered significantly different if $z > 3.182$ (95% confidence interval, 3 degrees of freedom). Significant differences for means of all other data points were determined using two sample *t* tests in Microsoft Excel with differences considered significant if $p < 0.05$.

Research Results and Discussion

Characterization and selection of candidate surrogates

Biofilm formation is as an important characteristic of pathogens, enabling them to colonize and survive on plant surfaces, and attach and persist in harsh environments (Yaron, et al., 2014). In these studies, biofilm formation was found to be higher when isolates were grown in LM than when grown in the high nutrient environment of LB broth (Fig. 3). In contrast, biofilm formation in nutrient rich media was extremely low for most isolates. Isolates from dry event (DE) surface water samples were the highest biofilm formers. *Salmonella* was the highest biofilm former in LM media, with an average O.D. value of 1.22 ± 0.405. Biofilm formation by *Salmonella* was 9.2 times higher when grown in low nutrient LM media than when grown in high nutrient LB broth. The QC strain 25922 was one of the lowest biofilm formers in LM, with an average O.D. of 0.122 ± 0.019, which was approximately 10 times less than that of *Salmonella*.

The biofilm data from this study suggested that the nutrient-limiting conditions present in the lysate media resulted in physiological changes and concomitant expression of genes by *Salmonella* and several of the candidate surrogates, which resulted in a significantly higher rate of biofilm formation. Regardless of the growth environment, the QC strain 25922 was unable to replicate the same level of biofilm formation as that of *Salmonella*. This was an early indication that the QC strain 25922 may not be an adequate surrogate of *Salmonella*.

Attachment was assessed for the 18 candidate surrogates, the pathogens, and the QC strain by comparing the ability of each isolate to rapidly attach (i.e., within 30 s) to three leaf-stage lettuce plants. The QC strain 25922 averaged the lowest attachment rate of all isolates (mean attachment of 4.11×10^7 cells g^{-1}) and was significantly different from that of *Salmonella*, which had an average attachment of 1.66×10^8 cells g^{-1} ($p < 0.05$). Several surrogates performed much better than the QC strain in the leaf attachment experiments and showed attachment levels that were statistically similar to those of *Salmonella* (Table 1). Surrogates 1, 2, 3 and 12 were especially impressive in leaf attachment rates, and attachment was not significantly different than that of *Salmonella*. It is noteworthy that lettuce leaf attachment for the QC strain, although significantly different than that of *Salmonella*, was comparable to that of *E. coli* O157:H7. Since attachment and biofilm formation are vital in the ability to contaminate and survive on fresh produce, our results suggest that environmental strains of *E. coli* provided a better model for *Salmonella* attachment than did the QC strain commonly used for validation studies.

Cell surface characterization showed that the pathogens and surrogates had cell surface characteristics (size, zeta potential, relative hydrophobicity and EPS) that were similar (within 15% of each other). However, values were often significantly different due to low variability in repetitions (Table 2A). The same values for the QC strain were very different from the pathogens and many of the candidate surrogates. These results suggest that cell surface characteristics may be important for attachment of pathogens and surrogates to surfaces, particularly when isolates are grown under environmentally relevant nutrient levels (i.e., levels found in lettuce exudates).

Genetic analysis of the candidate surrogates indicated that they possess genes conducive to adherence and survival (Table 2C). Candidate surrogates were positive for the siderophore gene *ironEC*, which aids in the capture of iron in low nutrient environments, and capsular synthesis gene *kpsII*, which may aid in nutrient scavenging and/or cell-cell communication. Serotyping and virulence gene (*LT*, *ST*a, *ST*b, *stx*1, *stx*2, *eae*, *cnf*1 and *cnf*2) analysis showed that the final selected candidates were not serotypes associated with pathogenic strains, and were negative for virulence genes. Therefore the final selected surrogates meet the important criteria of being non-pathogenic. Although not necessarily comparable to *Salmonella*, taken together with phenotypic analysis the results suggest that the environmental isolates have genetic properties conducive to survival in low-nutrient environments and attachment to surfaces.

The selection of the final surrogates from the 18 candidates was based on genetic analysis, biofilm formation and lettuce leaf attachment. Most of the candidate surrogates were positive for curli expression (Table 1). The selected surrogates possessed the needed attachment and adherence capabilities, especially when compared with *Salmonella*, which showed an impressive capability to adhere to surfaces when grown in lettuce lysates (Fig. 3). Before bacteria can persist for extended periods of time on a plant surface, the cell must first attach and survive on the plant surface in the initial contamination event. Because *Salmonella* attached to lettuce leaf surfaces at a much higher rate and formed more biofilm than the QC strain, higher levels of *Salmonella* may be found on lettuce plants due to larger numbers of cells that were able to attach and survive immediately following a contamination event. These data show that the QC strain does not behave as *Salmonella* in either attachment capability or biofilm formation. Surrogates selected from the water and agricultural environments, on the other hand, possessed genotypic and phenotypic properties that were more similar to the pathogen and/or were suggestive of enhanced ability to adhere to surfaces and persist for extended periods in the environment.

Candidate surrogate persistence

Data from the isolate persistence experiment was collected over a 22-day period to better understand the ability of produce pathogens to persist on the surface of lettuce plant leaves during normal growth conditions (Fig. 4). The QC strain 25922 and two surrogates were also used to determine if the surrogates or QC strain persisted in the same manner as the pathogens.

The persistence data show that although variable, pathogens and surrogates persisted in the same manner, with culturable colonies being detected for both *E. coli* O157:H7 and *Salmonella*, the surrogates, and for the QC strain 25922 out to the day 22 sampling period (Fig. 4). Concentrations of the QC strain were lower throughout the sampling period while the pathogens and surrogates persisted at a higher and more similar rate (Fig. 4). All isolates exhibited an approximately 5-log reduction over the first 7 days, before a second “persistence period” began;

from days 7 through day 22 the isolates persisted on the plants surface at a consistent rate, between 10^4 and 10^2 cells g^{-1} of lettuce leaf.

The persistence experiment data indicate that bacteria do not die off at a constant linear rate on lettuce plants. While the first several days following the contamination produces a linear die-off, the bacteria did reach a point where the linear die-off ceased and a persistence period began (Fig. 4). This result indicates that pathogens are capable of persisting on a plant surface for an extended period of time at a level capable of human infection. Plants are often irrigated with spray irrigation, with the majority of irrigation water being applied to the crop within the last 30 days prior to harvest (Smith, et al., 2011). Details of this study suggest that some cells of either *E. coli* O157:H7 or *Salmonella* may be capable of surviving long enough to make it through to harvest. The selected surrogates were able to persist on the lettuce plants and stay at a level consistent with the pathogens, unlike the QC strain. This suggests that survival/persistence of the environmental surrogates would more adequately mimic that of produce-associated human pathogens in studies conducted to evaluate treatment technologies and mitigation strategies.

Due to variability between experiments, increased sample numbers and repetition of experiments would be needed to confirm these results. However, inconsistencies and high variability of data, particularly with QC surrogate, reduces data quality, increases the need for expensive sampling to obtain significance, and limits the reliability for use in process design. In contrast, data from the persistence experiment illustrate how well the survival of the selected surrogates mimics that of *Salmonella* and *E. coli* O157:H7 in a pre-harvest lettuce plant environment (Fig. 4). Concentrations of the surrogates were the same or slightly higher than those of the pathogens, providing a more conservative estimate of pathogen levels. In these studies, the persistence, adherence and die-off of selected surrogates from environmental samples were consistently more representative of that of the human pathogens *Salmonella typhimurium* and *E. coli* O157:H7. To reduce risk to public health, the most representative and accurate surrogates should be used to verify pathogen capabilities, produce data for the development of best management practices, and support suggested good agricultural processes to producers.

Soil survival

The survival of two environmental *E.coli* surrogates, pathogens, and the QC strain in soils with or without amendments was similar. Organic amendments, either fresh poultry litter (FPL) or stored poultry litter (SPL), were added to each soil. Selected physio-chemical properties of the soils and amendments are shown in Table 3. Overall, higher survival was seen in the silt loam soil versus the sandy loam, with the QC strain and *E. coli* O157:H7 reaching the detection limit of 1×10^3 cells g^{-1} by day 42. Survival of the two surrogates was greater than that of the two pathogens and the QC strain. *Salmonella* survived longer and at higher concentrations than *E. coli* O157:H7 and the QC strain in both soil types (Fig. 5).

The sandy loam did not support the survival of the pathogens, surrogates, or QC strain as well as the silt loam, perhaps due to greater surface in the silt soils (Fig. 5). Survival of all isolates was greatest in soils amended with SPL. This was particularly observed in the silt loam soil, perhaps due to higher available nitrogen and organic matter (Table 3). Regardless of the soil type, concentrations of QC strain decreased more rapidly than did concentrations of the surrogates, and the decrease did not correlate with that of *Salmonella* ($p > 0.05$) but was more similar to that

of *E. coli* O157:H7 (Fig. 5). However, *Salmonella* once again showed the ability to persist at a higher level. The surrogates survived at an even higher rate than *Salmonella*, showing that they are capable of behaving like the pathogen in diverse environments. Differences in survival based on soil type show the complexity of the agricultural environment and emphasize the difficulty of establishing "one size fits all" requirements for water and soil quality.

Outcomes and Accomplishments

Data generated from this project support and demonstrate the stated hypothesis: *E. coli* isolates from environmental sources that possess similar physical, chemical, and/or biological properties do behave in the same manner as the produce-associated human pathogens *Salmonella typhimurium* and *E. coli* O157:H7 on lettuce surfaces and in soil. Environmental *E. coli* surrogates behave like *Salmonella typhimurium* and *E. coli* O157:H7 in biofilm formation, attachment and persistence on lettuce leaves, and survival in soils with or without organic amendments. The selected surrogates from this project may also provide a superior alternative to laboratory strains of *E. coli* (such as the ATCC 25922 strain) which are currently used by the industry for quality control and/or research purposes. Future research should evaluate the behavior of these isolates under more diverse environmental (wet/drought conditions, temperature extremes) and processing conditions to verify their suitability as surrogates for human pathogens found in association with fresh produce.

Summary of Findings and Recommendations

- Growth media produced from lettuce lysates produce physiological differences in the manner by which pathogens and surrogates adhere to surfaces.
- Growth environment is an important consideration for any experiment that will characterize the way a pathogen or surrogate behaves.
- *E. coli* isolates from similar environmental sources have a high level of strain diversity.
- Improved pathogen surrogates can be selected, and the agricultural environment may be the best source for these isolates.
- Generic lab strain *E. coli* isolates, such as the FDA strain ATCC 25922, may provide a suitable surrogate for *E. coli* O157:H7 in lab-scale experiments but is not otherwise recommended.
- Pathogens and surrogates can persist on lettuce leaf surfaces at low levels and for extended periods of time, emphasizing the need for use of appropriate model organisms in the development of critical control points and best management practices.
- The environmental strains of *E. coli* selected for use as surrogates in these studies exhibited survival and attachment properties on lettuce and in soil that were similar to those of the *E. coli* O157:H7 and *Salmonella typhimurium* strains.
- Ultimately, data from these studies show that the agro-ecosystem environment (manure, soil, water) is likely a better source of surrogates for produce-associated human pathogens than are common lab strains.
- Future research should (1) demonstrate the consistent behavior of these surrogates under relevant environmental conditions, (2) determine the range of conditions in which the surrogates can be used, and (3) evaluate the diversity and behavior of pathogens represented by the selected surrogates.

References cited

Bolster, C.H., K.L. Cook, I.M. Marcus, B.Z. Haznedaroglu and S.L. Walker. 2010. Correlating transport behavior with cell properties for eight porcine *Escherichia coli* isolates. Environ. Sci. Tech. 44: 5008-5014.

Bolster, C.H., B.Z. Haznedaroglu and S.L. Walker. 2009. Diversity in cell properties and transport behavior among 12 different environmental *Escherichia coli* isolates. J. Environ. Qual. 38: 465-472.

Brandl, M.T. 2006. Fitness of human enteric pathogens on plants and implications for food safety. Annual Review of Phytopathology. p. 367-392.

Brandl, M.T. and R. Amundson. 2008. Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*. Applied and Environmental Microbiology 74: 2298-2306.

Centers for Disease Control. 2007. Preliminary FoodNet Data on the Incidence of Infection with Pathogens Transmitted Commonly Through Food – 10 States, 2006. MMWR 56: 336-339.

Cook, K.L., C.H. Bolster, K.A. Ayers and D.N. Reynolds. 2011. *Escherichia coli* diversity in livestock manures and agriculturally impacted stream waters. Curr. Microbiol. 63: 439-449.

Cook, K.L., A.M.P. Netthisinghe and R.A. Gilfillen. 2014. Detection of pathogens, indicators, and antibiotic resistance genes after land application of poultry litter. J. Environ. Qual. 43: 1546-1558.

Crim, S.T., M. Iwamoto, J.Y. Huang, P.M. Griffin, et al. 2014. Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food — Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 2006–2013. MMWR 63: 328-332.

Franz, E., A.V. Semenov and A.H.C. Van Bruggen. 2008. Modelling the contamination of lettuce with *Escherichia coli* O157:H7 from manure-amended soil and the effect of intervention strategies. J App. Microbiol. 105: 1569-1584.

Frömmel, U., W. Lehmann, S. Rodiger, A. Bohm, J. Nitschke, J. Weinreich, et al. 2013. Adhesion of human and animal *Escherichia coli* strains in association with their virulence-associated genes and phylogenetic origins. Applied and Environmental Microbiology 79: 5814-5829.

Gomi, R., T. Matsuda, Y. Matsui and M. Yoneda. 2014. Fecal source tracking in water by next-generation sequencing technologies using host-specific *Escherichia coli* genetic markers.

Goodburn, C. and C.A. Wallace. 2013. The microbiological efficacy of decontamination methodologies for fresh produce: A review. Food Control 32: 418-427.

Kaufmann, M., C. Zweifel, M. Blanco, J.E. Blanco, L. Beutin and R. Stephan. 2006. *Escherichia coli* O157 and non-O157 shiga toxin–producing *Escherichia coli* in fecal samples of finished pigs at slaughter in Switzerland. J. Food Protect. 69: 260-266.

Kyle, J.L., C.T. Parker, D. Goudeau and M.T. Brandl. 2010. Transcriptome analysis of *Escherichia coli* O157:H7 exposed to lysates of lettuce leaves. Applied and Environmental Microbiology 76: 1375-1387.

Olaimat, A.N. and R.A. Holley. 2012. Factors influencing the microbial safety of fresh produce: A review. Food Microbiol. 32: 1-19.

Painter, J.A., R.M. Hoekstra, T. Ayers, R.V. Tauxe, C.R. Braden, F.J. Angulo, et al. 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. Emerg. Infect. Dis. 19: 407-415.

Patel, J. and M. Sharma. 2010. Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. Int. J. Food Microbiol. 139: 41-47.

Paternoster, R., Brame, R., Mazerolle, P. and Piquero, A. 1998. Using the correct statistical test for the equality of regression coefficients. Criminology 36: 859-866.

Reisner, A., K.A. Krogfelt, B.M. Klein, E.L. Zechner and S. Molin. 2006. In vitro biofilm formation of commensal and pathogenic *Escherichia coli* strains: Impact of environmental and genetic factors. J. Bacteriol. 188: 3572-3581.

Römling, U., W. Bokranz, W. Rabsch, X. Zogaj, M. Nimtz and H. Tschäpe. 2003. Occurrence and regulation of the multicellular morphotype in *Salmonella* serovars important in human disease. International Journal of Medical Microbiology 293: 273-285.

Shaw, R.K., C.N. Berger, B. Feys, S. Knutton, M.J. Pallen and G. Frankel. 2008. Enterohemorrhagic *Escherichia coli* Exploits EspA filaments for attachment to salad leaves. Applied and Environmental Microbiology 74: 2908-2914.

Sinclair, R.G., J.B. Rose, S.A. Hashsham, C.P. Gerba and C.N. Haas. 2012. Criteria for selection of surrogates used to study the fate and control of pathogens in the environment. Applied and Environmental Microbiology 78: 1969-1977.

Smith, R., M. Cahn, O. Daugovish, S. Koike, E. Natwick, H. Smith, et al. 2011. Leaf lettuce production in california. Univ. CA Veg. Res. Info. Center.

Solomon, E.B., S. Yaron and K.R. Matthews. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Applied and Environmental Microbiology 68: 397-400.

Van Boxstael, S., I. Habib, L. Jacxsens, M. De Vocht, L. Baert, E. Van De Perre, et al. 2013. Food safety issues in fresh produce: Bacterial pathogens, viruses and pesticide residues indicated as major concerns by stakeholders in the fresh produce chain. Food Control 32: 190-197.

World Health Organization. 2008. Microbiological hazards in fresh leafy vegetables and herbs. Microbiological Risk Assessment Series No. 14. World Health Organization, Rome. p. 150.

Yaron, S. and U. Romling. 2014. Biofilm formation by enteric pathogens and its role in plant colonization and persistence. Microbial Biotechnology 7: 496-516.

APPENDICES

Publications and Presentations

Givan, Ethan. 2015. Selection of Pathogen Surrogates and Fresh Produce Safety: Implications for Public Health and Irrigation Water Quality Policy. *Masters Theses & Specialist Projects.* Paper 1545.

Presentations:
Kentucky Water Resources Research Institute Symposium, "Strain diversity in the fecal indicator *Escherichia coli*: implications for its use as a produce pathogen surrogates", Lexington, KY, March 2015.

Kentucky Public Health Association annual conference, "Environmental *E. coli* isolate characterization and selection as improved indicators for pathogens associated with produce contamination", Owensboro, KY, April 2015.

Federation of European Microbiologists, "*Escherichia coli* strain diversity: Selecting isolates for use as pathogen surrogates", Maastricht, The Netherlands, June 2015.

Arkansas Association for Food Protection, "Using the Agricultural Environment to find better surrogates for foodborne pathogens", Fayetteville, AR, September 2015.

UCLA Food-Energy-Water Workshop, "Characterization of Potential Surrogates for Produce Pathogens", Los Angeles, CA, December 2015.

Publications in progress:
Invited book chapter: "Using the environment to select surrogates for produce pathogens"

Paper 1: "Characterization of environmental *E. coli* isolates for use as produce pathogen surrogates"

Paper 2: "Adequacy of environmental *E. coli* strains as surrogates for produce pathogens in soils and on lettuce"

Budget Summary

The funds made available by the Center for Produce Safety were quite sufficient and were applied to accomplish the project's objectives. The funds were supplemented by our USDA-ARS CRIS project base funds that permitted the bulk of the CPS money to be applied to supplies and collaborative work with other universities, which included funding for students. We also attended the CPS symposia and will attend the 2016 CPS Research Symposium in Seattle. We feel that the lack of funding for travel limits the ability to advocate for CPS and the great work done by the association, and minimizes the ability to present work to both national and international groups.

Tables and Figures

Figure 1. Surrogate selection process with experimental stages and corresponding data

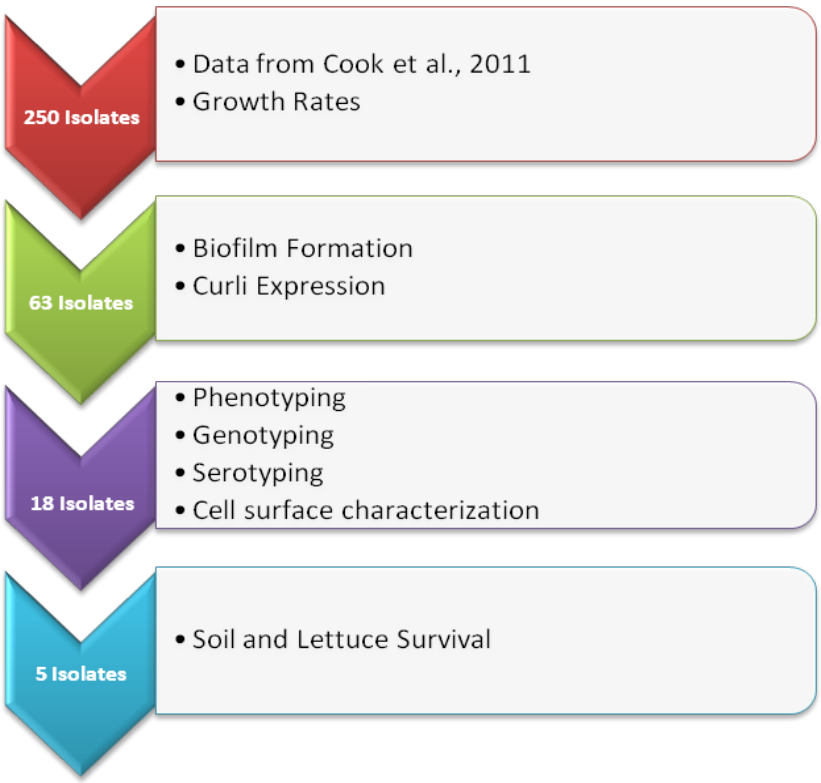

Figure 2. Images, clockwise from top left: Western Kentucky University students Ethan Givan and Aubrey Harston sampling soil; irrigation setup in greenhouse; green leaf lettuce grown for the project; soil collection in Western Kentucky; and lettuce leaf experiments in the laboratory (center image)

Figure 3. Biofilm formation of selected surrogates, pathogens, and the QC strain in either a low-nutrient environment (Lettuce lysates [LM]) or and high-nutrient environment (Luria–Bertani [LB]) media

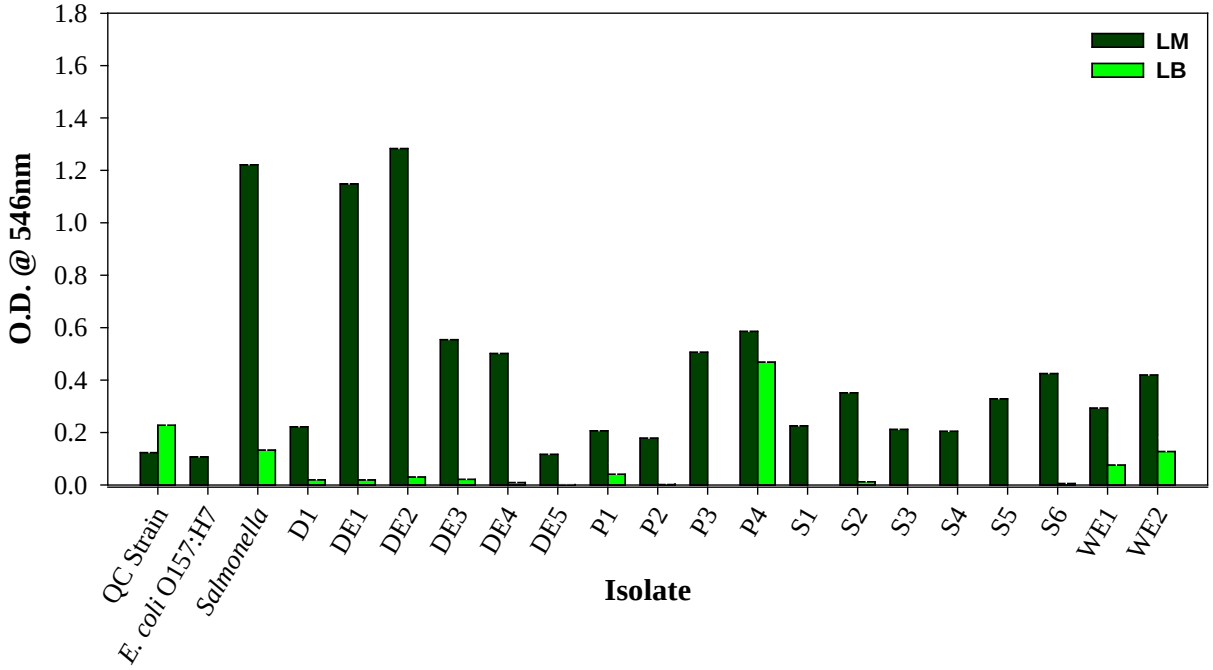

Table 1. Source, serotype, curli expression, and leaf attachment of *E. coli* O157:H7, *Salmonella*, QC strain and surrogates

Source	Isolate	Serotypes[α]		Curli[β]	Leaf Attachment[δ]
		O type	H type		
Poulty	Surrogate 1	100	34	Other1	2.24 ± 0.82
	Surrogate 2	43	2	Rdar	1.45 ± 0.63
	Surrogate 3	1	+	Rdar	1.92 ± 0.95
	Surrogate 4	1	+	Rdar	0.66 ± 0.26
Dairy	Surrogate 5	88	+	Bdar	1.30 ± 0.33
Swine	Surrogate 6	NEG	+	Bdar	1.53 ± 0.21
	Surrogate 7	NEG	NEG	SAW	1.15 ± 0.47
	Surrogate 8	111	5	Bdar	0.80 ± 0.23
	Surrogate 9	109	45	Rdar	0.66 ± 0.10
	Surrogate 10	86	10	Other2	0.33 ± 0.14
	Surrogate 11	128	NEG	Bdar	0.26 ± 0.06
Surface water	Surrogate 12	21	NEG	Bdar	1.38 ± 0.52
	Surrogate 13	21	NEG	Bdar	0.64 ± 0.15
	Surrogate 14	110	+	Rdar	0.55 ± 0.31
	Surrogate 15	110	+	Rdar	0.59 ± 0.47
	Surrogate 16	13	4	bdar	0.39 ± 0.20
	Surrogate 17	R	18	Rdar	0.66 ± 0.23
	Surrogate 18	19	4	Bdar	1.24 ± 0.33
Controls	QC Strain	6	1	other	0.54 ± 0.16
	E. coli O157:H7	157	7	Other3	0.46 ± 0.66
	Salmonella	176	NEG	Bdar	2.19 ± 0.47

[α]Serotyping conducted by *E. coli* reference center at Pennsylvania State University

[β]Rdar indicates Curli/Cellulose postive; Bdar indicates Curli positive/Cellulose negative; SAW indicates Curli/Cellulose negative

[γ]No entry indicates below mean growth rates; + indicates mean growth rates; ++ indicates above mean growth rates

[δ]Ratio to mean lettuce leaf attachment when isolates grown in low nutrient lettuce lysate (LM)

Table 2.

A. Statistical comparison of characteristics of surrogates and QC strain to pathogens when grown in LM

Isolate	Serotype[α]	*Salmonella*				*E. coli* O157:H7			
		Size	Zeta Potential	Relative Hydrophobicity	EPS Composition	Size	Zeta Potential	Relative Hydrophobicity	EPS Composition
		t-score[β]							
Surrogate 1	O100:H34	6.70*	-19.40*	1.23	6.00*	7.78*	-18.10*	3.18*	7.31*
Surrogate 2	O43:H2	-1.25	8.97*	-1.51	5.20*	1.99	-1.58	1.14	10.46*
Surrogate 3	O1:H+	1.94	1.55	0.15	1.57	3.99*	-5.60*	1.54	6.78*
Surrogate 4	O21:H+	6.12*	4.20*	7.26*	2.75	7.34*	-4.16*	11.49*	4.98*
Surrogate 5	O19:H4	2.49*	-30.27*	-7.48*	4.88*	4.40*	-25.73*	-6.27*	9.90*
QC Strain	O6:H1	8.31*	-48.30*	-7.31*	-0.77	8.87*	-31.90*	-6.06*	2.48

[α]Serotyping conducted by E. coli reference center at Pennsylvania State University

[β]Calculated t-score using independent samples t-test comparing each surrogate and QC strains to both pathogen strains

*p<0.05

B. Carbon source utilization

Isolate	4%NaCl	Fructose	Glucose	Lactose	Maltose	Mannose	Sucrose	Aspartic Acid	Malic Acid	Mannosamine	Minocycline	Serine	Glycerol	Acetic Acid	Citric Acid	Propionic Acid
Surrogate 1	+	+	+	+	+	+	+		+	+			+	+		+
Surrogate 2		+	B	+	+	+		+	+	+	+	+	+	+		+
Surrogate 3		+	B	B	+	+		+	+	+	+	+	+	+		+
Surrogate 4		+	+	+	+	+	+		+				+	+		+
Surrogate 5		+	B	+	+	+	B			+		+	+	+		+
QC Strain		+			+	+				+		+	+	+		
E. coli O157:H7		+	B	B	+	+				+		+	+	+		
Salmonella	+	+	+		+	+				+			+		+	

No entry indicates no amplification for the gene; (+) positive result, (B) borderline positive result

C. Genotypic characterization of surrogates and control strains

Isolate	Phylogrouping			Adhesion						Capsule Synthesis	Siderophore		Gene Fragment								
	chuA	*yjaA*	*TspE4C2*	*agn*	*eae*	*ihA*	*paa*	*sfa-foc*	*tsh*	*kpsII*	*ironEC*	*irp2*	Ch5	Co3	H4	H6	H7	H9	H14	H24	P1	P4
Surrogate 1			+	+					+		+		+		+	+	+	+	+			
Surrogate 2	+		+	+					+		+		+		+	+			+			
Surrogate 3	+			+		+						+					+					
Surrogate 4			+	+					+					+			+					
Surrogate 5	+	+	+	+					+	+							+					
QC Strain	+	+	+	+		+		+	+		+	+								+	+	+
E. coli O157:H7	+			+	+	+	+		+			+		+					+	+	+	
Salmonella																						

No entry indicates no amplification for the gene; + indicates positive amplification for the gene

Figure 4. Persistence of pathogens, selected surrogates, and QC strain on lettuce plants

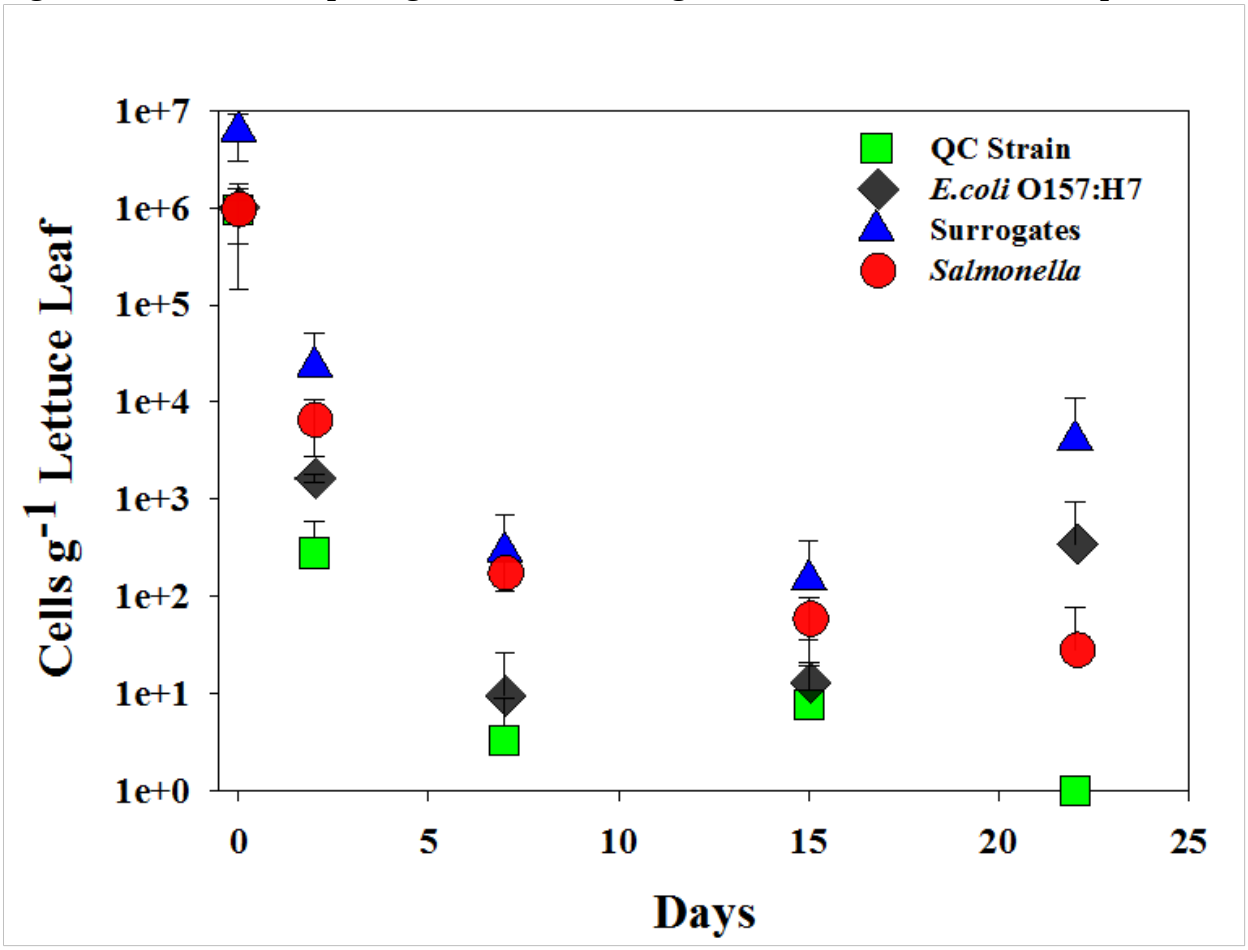

Table 3. Initial soil and poultry litter treatments physicochemical and chemical parameters

Source	pH	OM[α]	Total C	NH4-N	NO3-N	P	K	Na	Al	Mg	Mn
		------%	----	----------	----------	----------	----------	----------	----------	----------	----------
							mgkg^{-1}				
Silt loam											
NPL	5.83 ± 0.27	3.69 ± 0.31	21440.8 ± 1789.6	0.09 ± 0.02	3.53 ± 0.38	67.35 ± 3.51	216.66 ± 2.80	161.96 ± 2.17	1122.93 ± 8.28	166.93 ± 3.05	297.41 ± 3.99
FPL	7.32 ± 0.30	8.75 ± 2.29	50896.5 ± 13338.0	51.55 ± 6.42	0.40 ± 0.08	829.48 ± 95.43	2597.58 ± 135.75	808.90 ± 50.14	937.27 ± 5.79	608.16 ± 70.17	349.85 ± 2.04
SPL	7.62 ± 0.14	6.51 ± 0.58	37863.7 ± 3363.0	113.59± 2.95	0.21 ± 0.05	583.67 ± 131.96	2141.30 ± 167.40	679.40 ± 61.83	972.66 ± 23.97	480.99 ± 85.83	461.84 ± 8.12
Sandy loam											
NPL	6.42 ± 0.24	1.76 ± 0.05	10236.50 ± 294.10	0.10 ± 0.01	3.03 ± 0.32	218.74 ± 9.46	70.93 ± 4.21	58.16 ± 1.43	682.55 ± 6.38	91.89 ± 7.01	48.60 ± 1.69
FPL	6.93 ± 0.80	5.73 ± 0.34	33309.2 ± 1972.3	73.2 ± 2.6	0.34 ± 0.05	1450.97 ± 758.9	3423.4± 1427.4	1110.7 ± 460.0	534.62 ± 32.39	732.6 ± 445.3	89.78 ± 21.43
SPL	8.24 ± 0.91	5.36 ± 2.88	31176.1 ± 16764.7	160.28 ± 0.40	0.26 ± 0.04	729.87 ± 84.96	1752.87 ± 65.48	592.50 ± 25.92	616.47 ± 3.60	336.13 ± 42.75	72.85 ± 1.75

[α]OM = Organic Matter; total organic carbon (%) x 1.72; conversion factor assumes organic matter contains 58 % organic carbon

Figure 5. Concentration of *Salmonella*, quality control (QC) strain, and surrogates in silt loam or sandy loam soil with fresh poultry litter (FPL), stored poultry litter (SPL) or no poultry litter (NPL) on final day of sampling