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Selection of Pathogen Surrogates and Fresh Produce Safety: Implications for Public Health and Irrigation Water Quality Policy

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SELECTION OF PATHOGEN SURROGATES AND FRESH PRODUCE SAFETY: IMPLICATIONS FOR PUBLIC HEALTH AND IRRIGATION WATER QUALITY POLICY

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SELECTION OF PATHOGEN SURROGATES AND FRESH PRODUCE SAFETY: IMPLICATIONS FOR PUBLIC HEALTH AND IRRIGATION WATER QUALITY POLICY

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SELECTION OF PATHOGEN SURROGATES AND FRESH PRODUCE SAFETY: IMPLICATIONS FOR PUBLIC HEALTH AND IRRIGATION WATER QUALITY POLICY

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Foodborne illness continues to be a substantial public health issue in the United States, with fresh produce being one of the leading causes of outbreaks. Understanding routes of contamination of fresh produce and how pathogens survive on plant surfaces is paramount in improving food safety and reducing risk to public health. The objectives of this study were to select environmental *E.coli* isolates as pathogen surrogates of *Salmonella typhimurium* and *E.coli* O157:H7, assess lettuce plant contamination by spray irrigation water, and evaluate a common industry quality control (QC) *E.coli* strain (ATCC 25922). Selections of *E.coli* surrogates were made utilizing biofilm and leaf attachment data from lab scale assays. Five surrogates were found to be similar in biofilm formation and leaf attachment capabilities of the pathogens, while the common QC strain was significantly different than *Salmonella* in both biofilm formation and leaf attachment ($p < 0.05$). Persistence of surrogates, pathogens and the QC strain on lettuce plants was assessed in greenhouse scale experiments, where it was found that all isolates were above detection levels for 22 days. Die-off rates were calculated for all isolates, with the QC strain having the greatest rate of die-off in the first experiment ($k = -4.52$) and the second greatest in the second experiment (-2.82) while the pathogens and selected surrogates had statistically similar and lower rates of die-off. Based on this information, current policies concerning the sampling and management of irrigation waters and crops for microbial safety may be insufficient. It is recommended that sampling methods and frequencies be

adjusted for irrigation waters and fresh produce, and the use of projected die-off rates not be used for the determination of time intervals needed before a crop is safe to harvest.

Chapter I
Introduction

Contamination of food and water by pathogens has been and continues to be a substantial public health issue in the United States. In 2012 alone, 831 foodborne illness outbreaks were reported in the United States (Centers for Disease Control and Prevention [CDC], 2014a). These outbreaks were responsible for causing 14,972 illnesses, 794 hospitalizations, and 23 deaths (CDC, 2014a). These statistics collected by the CDC only include the illnesses resulting from outbreaks, and do not include illnesses dissociated with an outbreak. An outbreak is described as two or more of the same illnesses caused by the same food exposure (CDC, 2014a). 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 (CDC, 2014a).

According to a Centers for Disease Control and Prevention report, 46% of foodborne illnesses in the United States are caused by fresh produce (Painter et al, 2013). Despite efforts to reduce produce contamination, these issues persist. In terms of fresh produce, leafy vegetables were categorized as the highest food safety priority by the World Health Organization (WHO) in 2008 (World Health Organization, 2008). It should be noted that produce consumption and availability of fresh produce has increased in recent years in the U.S. (Hoelzer, Pouillot, Egan, & Dennis, 2012), as fruits and vegetables are recommended to be part of a healthy diet (U.S. Department of Agriculture [USDA], 2015).

There has been amplified regulatory emphasis on food safety, and particularly produce safety, in recent years within the United States. The passage of the Food Safety

Modernization Act (FSMA) in 2011 enabled the U.S. Food and Drug Administration to improve the safety and security of the U.S. food supply. An addition to FSMA was the "Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption" proposed rule, also known as the Produce rule, in 2013 (U.S. Food and Drug Administration [USFDA], 2013). This proposed rule concentrated on the microbial hazards of produce production based on a "qualitative assessment of risk to public health from on-farm contamination of produce" (USFDA, 2013). The proposed rule offers guidelines to be followed in the production of produce, amended Good Agricultural Practices (GAPs) for producers with emphasis on agricultural water quality, soil amendments, worker hygiene, and other components of produce production along the Farm to Fork process (USFDA, 2013).

For this study, emphasis was directed to the California Leafy Green Products Handler Marketing Agreement (LGMA), which enforces mandatory food safety practices that are audited by the California Department of Food and Agriculture (California Leafy Green Products Handler Marketing Agreement [LGMA], 2013). The LGMA's "Commodity Specific Food Safety Guidelines for the Production and Harvest of Lettuce and Leafy Greens" sets forth specific guidelines for the production of lettuce and leafy green products. The LGMA specifically guides sampling frequencies, assessment of production fields, agricultural water use, soil amendments, crop treatments, and harvest equipment (LGMA, 2013). The sampling frequencies are heavily based on an estimated die-off rate of pathogens, which assumes that pathogens die-off at a known linear rate. The sampling and detection of microbial contamination of agricultural irrigation waters

are of particular interest for the purposes of this study and will be discussed later in greater detail.

Many pathogens have been linked with foodborne illness and produce contamination. Some of the most common are serotypes of *Salmonella enterica,* and *Escherichia coli*, which are of the greatest concern due to their association with significant outbreaks linked to consumption of fresh produce (Crim et al., 2014; Goodburn & Wallace, 2013; Olaimat & Holley, 2012). *Salmonella enterica* serotypes are common foodborne pathogens that cause infection with a low dose of viable cells, and cause symptoms such as nausea, vomiting, abdominal cramps, diarrhea, fever and headache (USFDA, 2012). It has been shown that plants contaminated with *Salmonella typhimurium* could have quantifiable amounts of bacteria for up to four weeks after contamination (Kisluk & Yaron, 2012).

E. coli is a common enteric species that is part of the natural flora of the human gastrointestinal tract, but some groups of *E. coli* can cause disease. There are six pathogenic groups of *E. coli*, but specifically the enterohemorrhagic *E.coli* (EHEC) group is often implicated in foodborne outbreaks and includes the infamous O157:H7 serotype (USFDA, 2012). This group of *E. coli* has a low infective dose similar to that of *Salmonella*, with similar symptoms at onset, often progressing to bloody diarrhea (USFDA, 2012). The dangers of this pathogen lie in the possible complications that can occur, which include hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenia, both of which can prove to be fatal (USFDA, 2012).

Contamination of fresh produce with pathogens can occur in multiple ways during production and processing, which can be caused by various environmental and

management factors (Suslow et al., 2003). One of the challenges in protecting the public from foodborne illness is to understand the many routes of contamination of fresh produce and how this contamination occurs.

Irrigation water and its application to fresh produce crops is an acknowledged area of concern for food safety. Several studies have investigated or deliberated the possible impact irrigation water can have as a route of fresh produce contamination with human pathogens, but methods and findings differ (Brandl & Amundson, 2008; Erickson et al., 2010; Gelting et al., 2015; Oliveira, Viñas, Usall, Anguera, & Abadias, 2012; Solomon, Yaron, & Matthews, 2002; Van Der Linden et al., 2013; Wood, Bezanson, Gordon, & Jamieson, 2010) . Additionally, the time at which the application of irrigation water occurs during the growing process of produce could impact likelihood of contamination (Food and Drug Administration, 2013).

The source of irrigation water can be drawn from multiple sources with the most common being surface waters (rivers or ponds), ground water (well water), or reclaimed water. The quality of surface and ground water sources can be impacted by environmental factors upstream, which may include aspects of the watershed's hydrology, land use within the watershed, topography, and climatic events (Gelting et al., 2015). These factors may determine the level of source water contamination with pathogens. Crops are often irrigated from surface or ground water sources via spray or drip applications (Smith et al., 2011). This application of irrigation water can potentially introduce pathogens to fresh produce if the water source is contaminated. The contamination potential is greatly affected by the type of irrigation method used (Solomon et al., 2002). Spray irrigation causes direct contact with the phyllosphere of the

plant, that portion of the lettuce plant or other leafy greens that is ingested. A general practice in food safety is to use specific organisms as an indicator of fecal contamination and the possible presence of pathogens. Fecal indicator bacteria (FIB) such as *Escherichia coli* (*E. coli*) are often used as indicators of pathogen contamination because they usually exist in higher concentrations than pathogens. As already discussed, *E. coli* are naturally found in the gastrointestinal tract of warm blooded animals and are transported outside the body via fecal matter (Ishii & Sadowsky, 2008). Therefore, fecal waste from animals (wild and domestic) and humans leads to *E. coli* being common bacteria in the environment. Once released, E. coli may survive extended periods of time in harsh environments in soil or water where temperature, moisture levels, UV radiation from the sun, salinity, and other environmental conditions can create a stressful environment (Ishii & Sadowsky, 2008).

Selected *E. coli* strains are also used as surrogates for pathogens for quality control and food safety research because they are easy to grow, highly characterized, and detectable (Kim & Harrison, 2009). While similar, it is important to carefully differentiate the uses of an indicator organism and a surrogate. A FIB is used to indicate fecal contamination only, which can lead to the assumption that pathogens may be present. A surrogate is a microorganism that is used to study the fate of a pathogen within a particular environment because it behaves in a same manner and is often used for environmental assessment of risk (Sinclair, Rose, Hashsham, Gerba, & Haas, 2012).

There are several steps to the process of selecting surrogates, as described by Sinclair, et al., 2012. These steps include defining both the problem and the environmental system in which it is found, selecting and prioritizing surrogate attributes,

selecting candidate surrogates, conducting experiments with the candidates, assessing exposure potential, dose response, and risk characterization (Sinclair et al., 2012). The goal of surrogate use is to provide a non-pathogenic organism that will mimic a pathogen in a specific environment and allow scientific data to be collected in order to better understand and remediate risk to public health.

E.coli strains such as the American Type Culture Collection (ATCC) 25922 strain have been used as surrogates for pathogenic *E.coli* strains, such as O157:H7 (Kim & Harrison, 2009), and for other benchmarking or standard uses. The ATCC Food and Water Testing Reference Strains Guide lists the 25922 *E.coli* strain as a reference or surrogate strain for AOAC International, U.S. Food and Drug Administration (FDA), and International Organization for Standardization (ISO) food microbiology safety assays (ATCC, 2014).

A key step in using *E. coli* as a pathogen surrogate is to understand how this organism varies in the environment relative to produce pathogens so that the most representative surrogate can be selected. Because of the stressful situations that environmental E.coli isolates are under as previously described, it is logical to select surrogates from environmental isolates as they may already possess characteristics or factors needed to emulate those of pathogens. Research of relevant literature identifies several factors that aid pathogens in surviving in soil and on plant surfaces (Brandl, 2006; Yaron & Romling, 2014), which include growth rates, biofilm formation, curli expression, and leaf attachment capabilities. Understanding these factors can aid in the characterization of *E.coli* isolates, and then the selection of those isolates that best imitate actual pathogens.

These factors can be extensive, depending on the production process or environment that the surrogates are being selected. For the pre-harvest environment, surrogates must attach, survive, and persist on the surface of the product. Biofilm formation is key to this occurring, as it allows the bacteria to colonize on a less than ideal surface and survive an extended period of time (Brandl, 2006). Growth rates also play a role, simply because a bacterium that can grow more readily in a low nutrient or stressful environment is much more likely to survive on a plant surface than one that is not. Curli expression is another factor associated with a pathogens ability to survive on produce. These long fimbriae have been shown to aid in the attachment to surfaces (Brandl, 2006) and possibly the formation of biofilms (Reisner, Krogfelt, Klein, Zechner, & Molin, 2006).

The detection of pathogens and surrogate organisms for both microbial contamination testing on a produce product and for research can be difficult over time. After leaf surfaces are contaminated, the culturable number of cells on the plant's surface begins to decline dramatically, especially in a moderate temperature and low humidity environment, such as the normal growing conditions of lettuce in California (Moyne, Harris, & Marco, 2013). Initial decline can lead to the assumption that the bacteria are dead and the produce no longer infective after an estimated time frame. This estimation is often used to calculate the period of time to wait before the harvest of a produce product, after a known contamination event has occurred, or when product sampling should occur post irrigation event. However, while pathogens may not produce countable colonies on growth medias in lab conditions, viable cells are still attached to the plant surface and are potentially able to infect the consumer (Dinu & Bach, 2011).

The situation of viable but non-culturable cells has been shown to occur (Dinu & Bach, 2011; Moyne et al., 2013) and could bring into question the methodology of an assumed rate of linear die-off to determine sampling frequencies and the time period before harvest, as it is difficult to determine if cells have altered to a viable but non-culturable state (Wood et al., 2010). This does not include the use of these die-off rates to differentiate between isolates as to which survives and persists in an environment longer.

For this study, a pool of environmental *E.coli* isolates collected from four environmental sources was used to make selections of specific isolates to be tested as potential pathogen surrogates. These environmental sources include both livestock (poultry, dairy, and swine) and surface water sources, all of which could be the origin of pathogen contamination of irrigation water. The isolates were characterized and analyzed for phenotypic and genotypic properties that are determined similar to that of produce associated pathogens *Salmonella typhimurium*, and *E. coli* O157:H7. Using this data, *E.coli* isolates were selected for a final group of surrogates.

These surrogates were then used in greenhouse experiments to determine their effectiveness as pathogen surrogates when applied to green leaf lettuce plants. The potential of produce contamination via spray, aerial application, irrigation water was assessed using both the selected environmental *E.coli* isolates, and the produce associated pathogens *Salmonella typhimurium*, and *E. coli* O157:H7. The surrogates will also be compared to the commonly used *E.coli* ATCC 25922 strain to determine its effectiveness as a surrogate. Because of the already accepted use of ATCC 25922 strain as a surrogate, it is important to determine its efficacy as a surrogate for pathogens in a produce production setting. With the increased food safety regulations, it is important to

understand if these policies and regulations adequately protect public health, to understand the risks of spray irrigation water usage, and to provide new and improved surrogates to further the prevention of produce associated outbreaks of foodborne illness.

Chapter II

Research Objectives

The goals of this study are to: (1) use phenotypic characteristics of *E.coli* strains to select improved surrogates of pathogens; (2) provide scientific data on the survival and persistence of pathogens, selected surrogates, and a common QC strain on fresh produce when applied via spray irrigation water and; (3) evaluate current policies regarding agricultural irrigation water use and microbial hazards, and their potential impacts on public health.

The research questions include:

1. Can *E. coli* strains from the USDA-ARS collection obtained from poultry, dairy and swine manure or from surface water sources (Cook, Bolster, Ayers, & Reynolds, 2011) be selected that have properties similar to those shown to be important for survival of human pathogens on fresh produce?
2. What are the die-off rates of pathogens and surrogates on produce following application through simulated contaminated irrigation water?
3. Is the common FDA *E.coli* quality control strain (ATCC 25922) an appropriate surrogate for produce-associated pathogens?
4. Are current produce safety policies and guidelines sufficient to protect public health from foodborne illness?

Chapter III

Materials and Methods

Selection of Surrogate Isolates

The USDA-ARS unit's collection of *E.coli* isolates (n=250), collected and isolated from poultry, swine, dairy, and surface water sources, were used as a pool for candidate surrogates. The objective was to use this large pool of diverse *E.coli* isolates from multiple environmental sources and characterize them for certain factors that have been identified as important to the survival and persistence of pathogens on plant and soil surfaces. In this process of characterization, isolates would be eliminated to narrow the pool from which the final surrogates would be selected. Described herein is the process that was used to select for the surrogate isolates. Refer to Figure 1 for a visual description of the selection process and the data used for each step.

With the original pool of isolates (n= 250), previous data from the research in which these isolates were studied (Cook et al., 2011), and preliminary assays, such as growth rates, were used to narrow the selection considerably to a convenient and representative number of isolates (n=63). These 63 *E.coli* isolates were then characterized for further selections using factors derived from relevant literature that common causing foodborne illness pathogens possess. Many factors or characteristics (both genotypic and phenotypic) have been determined by recent studies to affect the ability of pathogens to survive on fresh produce or in soils, leading to their ingestion, foodborne illness, and the risk to public health (Brandl, 2006; Cook et al., 2011; Olaimat & Holley, 2012; Yaron & Romling, 2014). These characteristics include biofilm formation, curli expression, and growth rates.

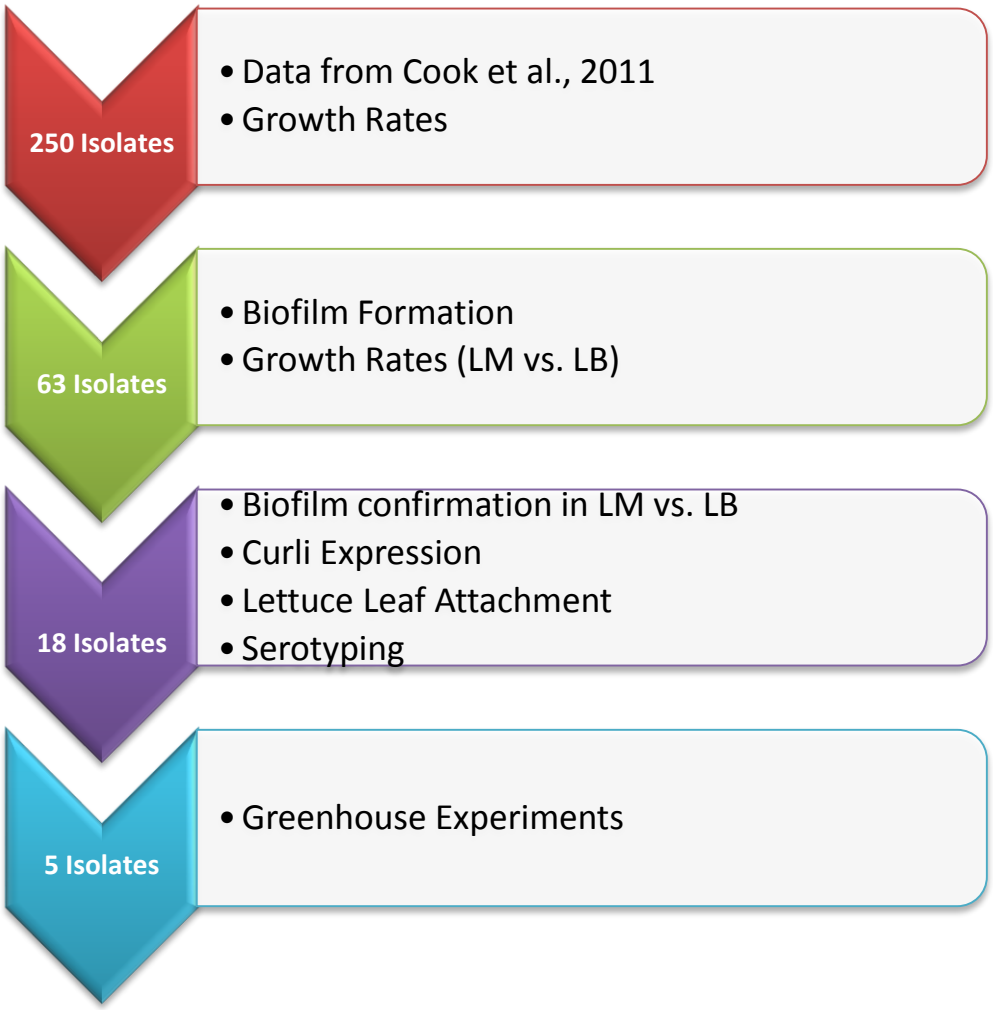

Figure 1. Process of surrogate selection and the corresponding data used for selections

The pool of 63 isolates were analyzed for these characteristics, and the resulting data were used to select a group of 18 isolates that would be further characterized and used for surrogate selections. This group of 18 isolates were selected as a representation of both high and low biofilm formers, growth rates, and multiple curli expressions, and different source types (i.e. poultry, swine, dairy, dry event, and wet event water source samples).

Once the 18 isolates were selected, biofilm data was repeated and confirmed. In addition, the 18 isolates were also tested for adhesion to soil and lettuce leaf surfaces. This data was compared to that of the produce pathogens *Salmonella typhimurium* (ATCC 13311), *E. coli* O157:H7 (ATCC 43888), and a common FDA quality control strain *E. coli* (ATCC 25922). The 18 isolates were also sent to the E. coli Reference Center at Penn State University for serotyping virulence gene testing. Comparing the 18 isolate's characterization data to that of the pathogens and common QC strain, a final selection of five surrogate isolates was made for use in greenhouse studies.

Selections of Pathogens and Control Strain

The pathogens and positive control strain used for the study have been acquired by the USDA-ARS facility in Bowling Green, KY from the American Type Culture Collection (ATCC). The pathogens used included *Salmonella typhimurium* (ATCC 13311) and *E.coli* 0157:H7 (ATCC 43888). The control strain for this study is a common FDA *E.coli* strain (ATCC 25922) that is used for quality control (QC) purposes due to non-pathogenic characteristics and widely accepted use.

Both pathogens were selected because of their links with produce associated outbreaks and known risk to public health. *Salmonella typhimurium* ranks second of all *Salmonella* serotypes in laboratory confirmed human infections, and ranks first in clinical non-human laboratory confirmed infections (CDC, 2014b). *E.coli* 0157:H7 has been the cause of many produce associated outbreaks, and ranks first of all serotypes in laboratory confirmed infections reported to the CDC (CDC, 2014c).

Bacterial Culture Methods

All isolate samples were stored at -80° C until used to start experimental cultures. All bacterial cultures for experimental and characterization purposes were started from the frozen samples. The first passage was started by taking 10µl of frozen sample and streak plating onto Luria-Bertani (LB) agar plate. After incubation overnight at 37° C, a single colony from the plate culture was aseptically selected and used to inoculate either LB broth or lettuce lysate (LM) growth media. For biofilm assays, a second passage from LM to LM was used to ensure that no residual LB broth would impact the physiological and growth state of the cells.

Characterization of Candidate Surrogate Isolates

E.coli strains and pathogens were characterized as described below when grown in lettuce exudates supplemented with minimal salts media. According to Brandl & Amundson, the minimal salts were amended with 19mM of ammonium chloride (Brandl & Amundson, 2008). The lettuce exudates were made using methods derived from Kyle, et al., and Brandl & Amundson from store bought green leaf lettuce heads (Brandl &

Amundson, 2008; Kyle, Parker, Goudeau, & Brandl, 2010) Lettuce leaves were removed from the head by hand, and tore into small pieces less than 2.5cm x 2.5cm in size. De-ionized water was added to the shredded leaves at a rate of 200ml per 50 grams of leaf. The shredded leaf mixture was allowed to set at room temperature for one hour, with mixing and smashing of lettuce material every 15 minutes with a sterile tongue depressor. After one hour, the liquid was strained from the lettuce leaf material, and centrifuged at 10,000 xg for 10 minutes to pellet plant debris. The resulting liquid was then filtered using a 0.8 µm filter, followed by a 0.45 µm filter. The liquid was then filtered through a 0.22 µm filter for sterilization. Minimal salts media was added to the exudates as described by Brandl & Amundson, 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 nutrients available for growth and the potential environment that pathogens would face when on plant surfaces. This low nutrient environment is compared to Luria-Bertani (LB) broth (Difco, Bectin, Dickinson and Co., Sparks, MD) lab growth media, which is high in nutrients.

Biofilm Formation

Biofilm formation has been described as an important ability of pathogens to colonize and survive on plant surfaces, allowing the bacteria to attach and persist in a harsh environment (Yaron & Romling, 2014). The biofilm formation of isolates for this study was determined using derived methods from Patel & Sharma using a crystal violet

stain assay (Patel & Sharma, 2010). Isolates were taken from frozen samples and used to inoculate 150 µl of LM growth media in a 96 well microtiter plate, and then incubated at 37° C for 24 hours. The following day 10 µl of the fresh culture was transferred to 150 µl of fresh LM growth media in a new 96 well plate. This plate was incubated for 48 hours at 37° C. After 48 hours of incubation, the cultures within the plate were removed by aspiration. The wells were then washed once with sterile distilled water. Plates were then air dried for 45 minutes. After drying, 150 µl of Crystal Violet dye (Sigma-Aldrich Co., St. Louis, MO) was added to the plate and incubated for 45 minutes with no shaking. Then the dye was removed by aspiration and the wells in the plate washed an additional three times. The plate was allowed to dry for another 45 minutes and then 150 µl of 95% ethanol was added to the wells to remove the dye from the biofilm formed onto the plastic. The plate was then placed in a BioTek ELx808 absorbance microplate reader (BioTek Instruments Inc, Winooski, VT) and the O.D. of each well was measured at 546nm. Optical Density values from residual background dye in control wells were subtracted out from wells with cultures.

Growth Rates

Growth rates were determined for all isolates by inoculating 100 µl of either LB or LM growth medias with 1µl of fresh culture in a 96 well microtiter plate. Fresh cultures were obtained by inoculating 150 µl of either LB broth or LM growth media the previous day with 10µl of frozen isolate sample. The plate was then placed in a BioTek ELx808 absorbance microplate reader (BioTek Instruments Inc, Winooski, VT) and the growth of the isolate was recorded over a 12-hour period at Optical Density at 546nm.

Growth rates (*K)* were determined using the *GrowthRates* software, © The Bellingham Research Institute, Version 1.8 (Hall, Acar, Nandipati, & Barlow, 2014).

Curli Expression

The expression of Curli fimbriae was identified by growing all isolates on an agar plate prepared according to methods described by Römling et al., which contains dyes that are taken up by the organism if it produces the Curli fimbriae (Römling et al., 2003). Red colonies indicated the expression of Curli (Reisner et al., 2006) after 48 hours of incubation at room temperature, and isolate expressions were categorized by Romling's methods.

Lettuce Leaf Attachment

The attachment capability of isolates to lettuce leaves was determined using methods derived from Brandl & Amundson, and Shaw et al., for the selected isolates (Brandl & Amundson, 2008; Shaw et al., 2008), Three-leaf stage green leaf lettuce plants (approximately 14 days post-emergence) grown in the USDA-ARS unit greenhouse were used. All 18 isolates were grown in LM media to approximately 1 x 10^9 cells ml^{-1} and then 5ml of the inoculate dispensed into sterile 47mm petri dish. 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. The leaves were extracted for DNA and the attached isolate or pathogen cells were quantified using Real Time PCR.

Greenhouse Experiment Methods

Green leaf lettuce (*Lactuca sativa*) seedlings were grown in a commercial potting mixture in the USDA-ARS unit's greenhouse. The ARS facility houses a state of the art 4,086 ft^2 biosafety level-2 certified greenhouse that was used for all greenhouse experiments. Growth conditions were controlled in a manner to best simulate commercial production and to produce healthy lettuce plants. Moisture content of the soil was monitored and adjusted daily as needed to maintain moisture levels (approximately 70% moisture weight in potting mix) using a custom built three zoned automated drip irrigation system. Seedlings were seeded and germinated in an organic commercial potting mix (SunGro Horticulture, Agawam, MA) , and then transplanted at approximately seven days post-emergence into a 13.75cm square pot. Plants were fertilized with Jack's 20-20-20 fertilizer (Brandl & Amundson, 2008; Solomon et al., 2002) once at the time of transplanting and again two weeks after.

Lettuce plants were grown inside the Biosafety certified bay of the USDA-ARS greenhouse, where a smaller 12' x 20' 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 23° C and lows near 15° C. Light was dependent upon natural sunlight, but was supplemented with greenhouse lighting on overcast days with a 12 hour light period.

Greenhouse experiments were carried out using the five final selected *E.coli* surrogate candidates P1, P2, P3, DE1, WE2, 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 were grown from a frozen sample using the method previously described in LM growth media. On day 0 of the experiment, the 10ml culture was centrifuged for 10 minutes at 10,000xg to pellet the cells. The liquid was poured off, and the cells washed and re-suspended in 10ml of sterile 0.1XPBS buffer. The cultures were centrifuged and washed a second time, and re-suspended in 10ml of 0.1XPBS buffer. From this stock inoculate containing approximately 1 x 10^9 cells ml^{-1}, additional dilutions were made to achieve desired cell concentration for experimental purposes.

Isolate Die-off Experiments

To determine the ability of the five selected surrogates to survive on plant surfaces similar to that of pathogens, greenhouse experiments were conducted to measure the die-off rates (*k*) of the selected surrogates in comparison to the pathogens and the QC strain. For these experiments, one month old green leaf lettuce plants (post-emergence) were used. Three replicate leaves of similar size and age were inoculated per plant with a single isolate to simulate spray irrigation. To do this, 1ml of isolate inoculate at approximately 10^7 ml^{-1} cells was evenly distributed onto the leaf surface from the point of where the leaf joined the plant stem outward to the middle of the leaf (approximately 5-6 centimeters) with a pipette. Each plant was inoculated with one isolate, with a total of six plants being inoculated per isolate (one per sample day), for a total of 48 plants per experiment. An additional three control plants were placed among the experimental plants to determine if any cross contamination occurred. All plants were randomly distributed within the greenhouse and sampling was done in a randomized order to reduce

the variability that different environmental conditions within the greenhouse could cause. Sampling was done on days 0, 1, 2, 3, 4, and 7 with control samples being taken on days 0, 2 and 7. Samples taken on Day 0 were taken 90 minutes post inoculation of the plants.

Prior to sampling, sterile 50mL tubes were prepared by adding 0.5ml 1.0mm Zirconia/silica beads and 12-15 2.0mm garnet beads (Biospec Products, Bartlesville, OK), then weighed and recorded. For sampling, each leaf was removed from the plant using a sterile scalpel. The leaf was cut where it joined the core stem of the plant, and then again 4-5 centimeters from the end of the leaf. The 5 centimeter section of the leaf sample was placed in the correct sterile pre-weighed 50mL tube, and the rest of the leaf discarded into the Biohazard waste bin. Once all leaf samples were collected, the sampled plants were discarded as well.

The samples were then brought back to the lab where they were re-weighed with the leaf sample and recorded, so that the weight of the leaf could be determined. Leaf samples averaged 1.2 grams. Then 5mL of sterile 0.1X PBS was added to each 50ml tube containing the samples. The sample tubes were placed into a GenoGrinder homogenizer, (SPEX Sample Prep, Metuchen, NJ) at 1500 rpm for 1 minute. The samples were then removed, vortexed for approximately 15 seconds, and the liquid from the sample poured off into prepared 15ml tube. This liquid was used for serial dilutions, plating and quantification of the surviving isolate cells. For *E.coli* surrogate candidates, *E.coli* O157:H7, and the QC strain, modified mTEC agar plates were used for serial dilution plating (Difco, Bectin, Dickinson and Co., Sparks, MD). For the quantification of *Salmonella typhimurium,* Improved HiChrome Salmonella agar was used (Sigma-Aldrich Co., St. Louis, MO).

Die-off rates (*k*) were calculated using the methods described by Cook et al., 2014 where $N = N_0 \times e^{-k_1 t}$ is used as the decay model (Cook, Netthisinghe, & Gilfillen, 2014). The decay rate was obtained from the slope of the plot of $\ln N_t/N_0$ (Cook et al., 2014). To calculate the time required to achieve a 1 log reduction (90% reduction) in cell concentration (T^{90}) the *k* rate was used, where $T^{90} = -\ln(0.1)/k$ where 0.1 is proportion of cells left in the population. All data was plotted using SigmaPlot 12.5 (Systat Software, 2013) and linear regression test was used to generate regression parameters and statistics which included k^{-1} day, standard error, and r^2 values.

Isolate 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 one month old lettuce plants (post-emergence). In this experiment the surrogates were applied as a mix, along with the *E.coli* O157:H7 and the QC strain. *Salmonella* was applied separately.

Three replicate leaves of similar size and age were inoculated per plant with a single isolate to simulate spray irrigation. For this, 1 ml of isolate inoculate at approximately 10^7 cells/ml was evenly distributed onto the leaf surface from the point of where the leaf joined the plant stem outward to the middle of the leaf (approximately 5-6 centimeters) with a pipette. A total of 30 plants were used for the experiment, which 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 any growth, indicating no cross contamination among the plants.

To detect *E.coli* O157:H7 from the surrogates, modified mTEC agar media was used (Difco, Bectin, Dickinson and Co., Sparks, MD) where *E.coli* O157:H7 colonies are a tan color, and most other *E.coli* strains are purple. To detect the QC strain, a green fluorescent protein (gfp) labeled 25922 strain was acquired from ATCC. This 25922gfp strain was detected by plating samples onto LB agar plates (Difco, Bectin, Dickinson and Co., Sparks, MD) supplemented with Ampicillin (50µg/ml). Colonies of 25922gfp were enumerated under black light by counting green fluorescent colonies.

Statistical Analysis

For isolate die-off experiments, $N_t = N_0 \times e^{-k_1 t}$ was used as the linear decay model. The plot of $\ln(N_t/N_0)$ was used to determine the linear regression coefficient of the best fit line. To determine if there were significant differences between the regression coefficients of isolate die-off rates, normal comparison of means tests could not be used. To test the significant difference between two regression coefficients for independent samples, a z score is determined using the z-test equation:

$$z = \frac{b1 - b2}{\sqrt{SEb1^2 + SEb2^2}}$$

Where b_1 and b_2 are the regression coefficients of samples being compared, respectively, and SEb_1 and SEb_2 are the standard errors for both samples one and two (Paternoster, Brame, Mazerolle, & Piquero, 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 $p < 0.05$.

Chapter IV

Results

Characterization of Candidate Isolates

Results for the 18 candidate surrogates and the final 5 surrogates will only be displayed and discussed for this study due to earlier selections being made primarily on representation of the group and not for specific pathogen characteristics. Results of experiments and testing of the group of 63 and 250 are not shown.

As Figure 1 illustrates, 18 candidate surrogates were tested for curli expression, biofilm formation in LM growth media, lettuce leaf attachment and were also serotyped. The results of the serotyping and curli expression characterization for the 18 candidate surrogates, the two pathogens, and the QC strain can be found in Table 1. Average values were computed for the data points of all 21 isolates. Of the 21 isolates tested for Curli expression, 16 were positive for the expression and only one swine isolate could be confirmed as negative for the expression. The remaining four isolates were unable to be categorized due to unusual growth expressions, as noted in Table 1.

Serotyping data from Penn State University confirmed that all chosen isolates were *Escherichia coli*. Typing also provided the O and H types, which allowed the confirmation that the selected candidates were not of the same serotypes commonly associated with being pathogenic or causing foodborne illness. The serotyping also determined that the selected candidates were negative for virulence genes *LT*, *ST*a, *ST*b,

Table 1.
Serotype, growth rate, and curli expression of candidate surrogates and pathogens

Isolate	O type[1]	H type[2]	Curli[3]	Growth Rate[4]
P1	100	34	Other1	
P2	43	2	Bdar	++
P3	1	+	Rdar	
P4	1	+	Rdar	
D1	88	+	Bdar	++
S1	NEG	+	Bdar	
S2	NEG	NEG	SAW	+
S3	111	5	Bdar	++
S4	109	45	Rdar	++
S5	86	10	Other2	+
DE1	21	NEG	Bdar	
DE2	21	NEG	Bdar	
DE3	110	+	Rdar	
DE4	110	+	Rdar	+
DE5	13	4	bdar	
S6	128	NEG	Bdar	
WE1	R	18	Rdar	+
WE2	19	4	Bdar	++
QC strain 25922	6	1	Other3	++
E.coli O157:H7	157	7	Other4	++
Salmonella	NA	NA	Bdar	

[1] Performed using antisera generated against E. coli serogroups; NA indicates not applicable

[2] Performed by PCR-RFLP analysis of fliC gene; NA indicates not applicable

[3] Rdar indicates Curli/Cellulose postive; Bdar indicates Curli positive/Cellulose negative; SAW indicates Curli/Cellulose negative; Other indicates could not be categorized

[4] No entry indicates below average value; + indicates average value; ++ indicates above average value;

*stx*1, *stx*2, *eae*, *cnf*1 and *cnf*2 and therefore can be considered non-pathogenic. This was significant data, as any surrogate selected must be a non-pathogenic *E.coli*.

Biofilm formation was found to be higher when isolates were grown in LM than when grown in the high nutrient environment of LB broth, which can be seen in Figure 2. Biofilm formation in LB broth was almost non-existent for most isolates. Isolates from Dry Event (DE) water samples overall were the highest biofilm formers, specifically for DE1 and DE2 at an Optical Density (O.D.) of 1.148 ± 0.194 and 1.283 ±0.182 respectively, with two poultry isolates (P3 and P4) having noteworthy biofilm formation as well. *Salmonella* was the highest biofilm former in LM media with an average O.D. value of 1.22 ± 0.405. The QC strain 25922 was one of the lowest biofilm formers on LM with an average O.D. of 0.122 ± 0.019, which was approximately 10 times less than that of *Salmonella*. The biofilm formation of the QC strain 25922 and *Salmonella* were significantly different with a p-value of 0.043. The biofilm formation of *E. coli* O157:H7 was similar to the QC strain 25922 at 0.107 ± 0.007 with a no significant difference p-value of 0.247.

Swine isolates overall had highest growth rates compared to the rest of the isolates tested (Table 1.), and it was observed that isolates with higher growth rates did not form biofilms. Growth rates in LB were approximately double the growth rates in LM, which demonstrated the effect of the low nutrient levels of the LM growth media.

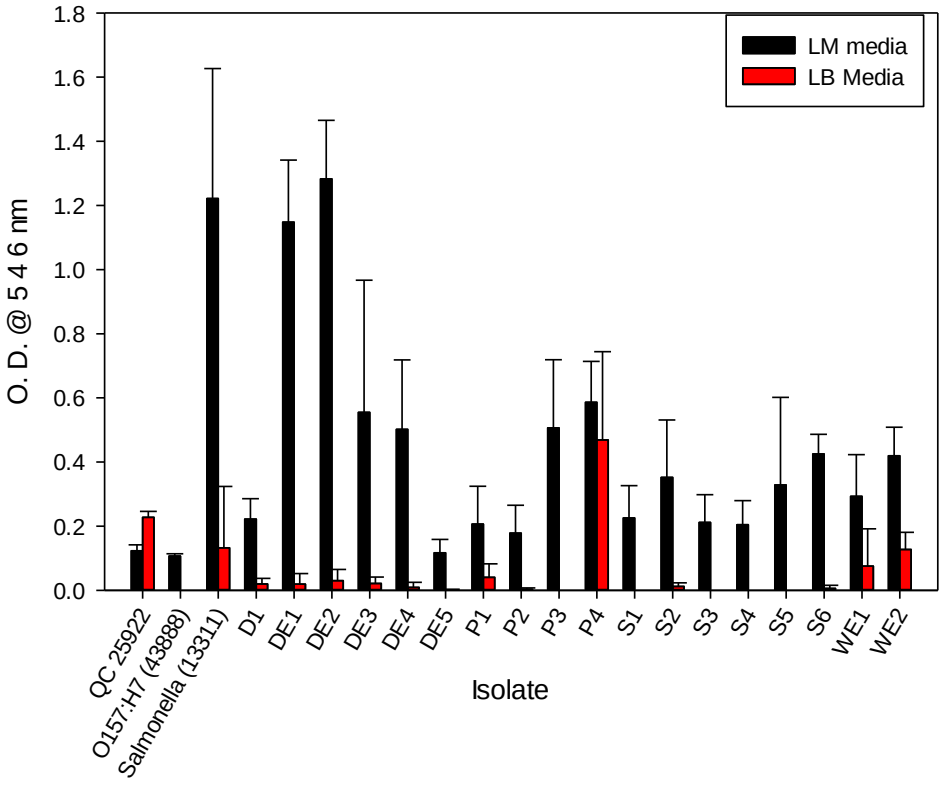

Figure 2. Biofilm formation of candidate isolates, QC strain 25922, and pathogens in both LM and LB growth media

Leaf attachment of the 18 candidate surrogates, the pathogens, and the QC strain were assessed by comparing cells attached to a 3 leaf lettuce plant. The QC strain 25922 averaged the lowest attachment rate of all isolates tested with an average attachment of 4.11×10^7 cells g^{-1} which was statistically different than that of *Salmonella* ($p = 0.034$) which had an average attachment of 1.66×10^8 cells g^{-1}. Several surrogates performed much better than the QC strain in the leaf attachment experiments, while being similar to that of *Salmonella* (Figure 3). Isolates P1, P2, P3 and DE1 were especially impressive in leaf attachment rates, and compared to *Salmonella* very well. P1, P2, P3, and DE1 were all not significantly different than *Salmonella* with p-values of 0.835, 0.396, 0.794 and 0.504, respectively. Leaf attachment data for the isolates can be found in Figure 3.

After the characterization of the isolates, the data resulted in five candidates being selected as surrogates for the selected pathogens. The selected surrogates (n = 5) and the characteristics that primarily led to its selection can be found in Table 2, and the statistical comparison of those isolates and the QC strain to the pathogens for biofilm and leaf attachment can be found in Table 3.

Candidate Isolate Die-off Experiments

The candidate isolates were assessed for individual die-off rates in these two replicate experiments, so that die-off rate calculations could be determined for each candidate surrogate isolate (n =5), the two selected pathogens *E. coli* O157:H7 and *Salmonella* and the QC strain 25922. The die-off rates were calculated using the first four days following the simulated contamination event to utilize the most linear die-off range.

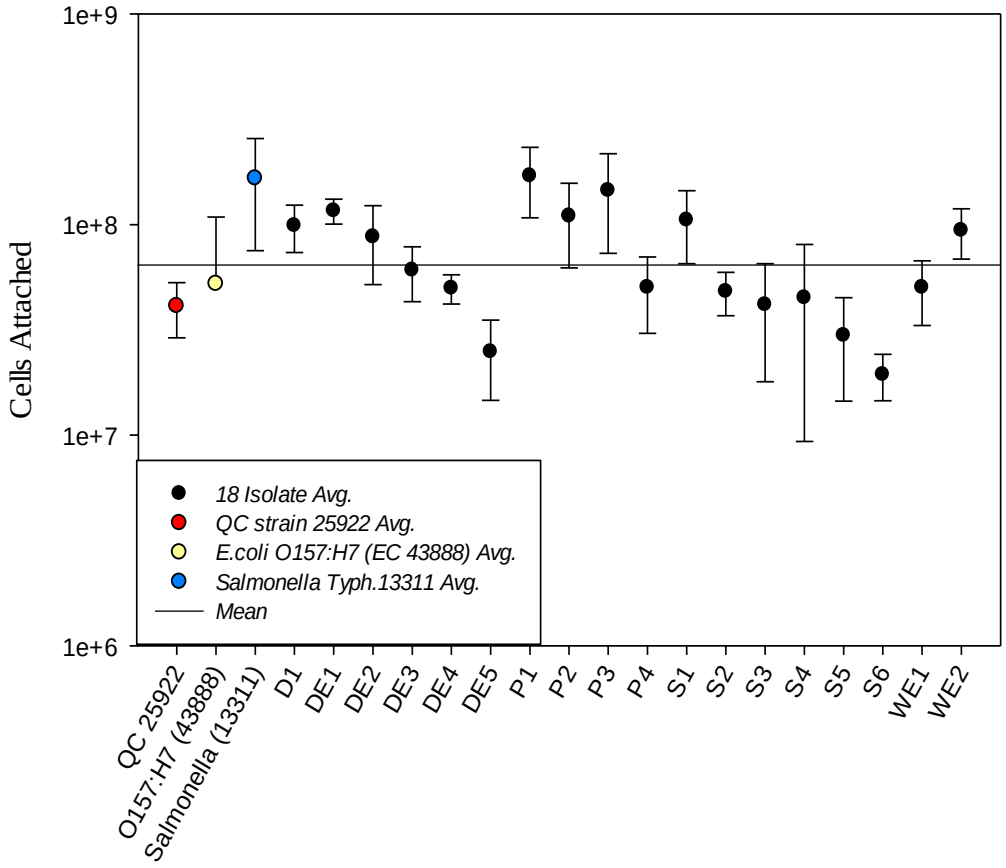

Figure 3. Attachment of selected isolates, pathogens, and QC strain 25922 to lettuce leaves

Table 2.
Final surrogates selected from the 18 candidates to be used in greenhouse studies

Isolate	Source	Serotype [φ]	Primary [χ] Characteristic
P1	Poultry	O100:H34	Leaf Attachment
P2	Poultry	O43:H2	Leaf Attachment
P3	Poultry	O1:H+	Leaf Attachment/ Biofilm
DE1	Dry Event Water	O21:H-	Biofilm
WE2	Wet Event Water	O19:H4	Leaf Attachment

φ Serotype as determined by Penn State Univerisity *E.coli* Reference Center

χ Characteristic responsible for the final selection of that isolate based on characterization data described in this study

Table 3.
Statistical comparison of selected surrogates and QC strain to Salmonella and E.coli O157:H7 in biofilm formation and lettuce leaf attachment

Isolate	*Salmonella*		*E. coli* O157:H7	
	Biofilm t	Attachment t	Biofilm t	Attachment t
P1	-4.169	0.230	1.47	1.796
P2	-4.36**	-0.950	1.42	1.55
P3	-2.710	-0.278	3.25	1.67
DE1	-0.284	-0.810	9.32**	1.65
WE2	-3.350	0.297	6.07**	1.76
QC strain 25922	-4.692**	-3.69**	1.43	1.03

Note. **$p < 0.05$

The temperatures were consistent for both experiments. However, natural sunlight was less in the second of the two experiments, which could not be controlled for due to overcast days. This might have affected the variability between the two experiments. The resulting die-off rates (*k*) and T90 values are shown for experiment one and experiment two in Table 4 and Table 5, respectively.

The QC strain 25922 had the lowest calculated die-off rate and T^{90} for the first experiment and the second lowest in the second experiment with rates of -4.52 and -2.82 respectively. However, when statistical comparisons were made, it was found that there was no statistical difference between the die-off rates of the QC strain and *Salmonella.* Z-scores for a Salmonella and QC strain comparison for experiment one and two were 1.95 and 0.93 which are both less than the critical value of 3.182. Comparing the QC strain to *E.coli* O157:H7 for experiment one and two produced non-significant z scores as well, which were 1.86 and 1.69 respectively. This is partially due to the high standard error for the QC strain data. There was also no significant difference between the surrogates and the pathogens, indicating that all five selected surrogates did die-off in a same manner as the pathogens. The z-scores calculated for the comparisons of the selected surrogates and the QC strain to the pathogens can be seen in Table 6. Tables 4 and 5 show the die-off rates (k) and T^{90} values, both of which indicate that the pathogens and surrogates are very similar, more so than the QC strain.

The T^{90} values for the surrogates and pathogens provided the time in days to achieve a one log reduction in cell numbers. The QC strain had the lowest T90 values in both experiments, with a T^{90} value of 0.51 for experiment one and 0.82 for experiment two. Both the pathogens had a T^{90} value of 0.96 for experiment one which was consistent.

Table 4.
Experiment 1 die-off rates (k) of selected surrogates, pathogens, and QC strain

Isolate	k^{β}	$r^{2\,\gamma}$	$T^{90\,\delta}$
P1	-2.66	0.94	0.87
P2	-1.74	0.81	1.32
P3	-2.54	0.95	0.91
DE1	-2.52	0.95	0.91
WE2	-2.77	0.96	0.83
QC strain 25922	-4.52	0.91	0.51
E.coli O157:H7	-2.41	0.91	0.96
Salmonella	-2.41	0.95	0.96

$^{\beta}$ Decay constant (k) determined from the the linear regression plot of $\ln(N_t/N_0)$ where k is the slope of the regression line.

$^{\gamma}$ Coefficient of determination of the regression of the plot of $\ln(N_t/N_0)$

$^{\delta}$ Time in days to achieve a 90% reduction in cell numbers

Table 5.
Experiment 2 die-off rates (k) of selected surrogates, pathogens, and QC strain

Isolate	k^{β}	$r^{2\,\gamma}$	$T^{90\,\delta}$
P1	-1.91	0.94	1.21
P2	-2.17	0.81	1.06
P3	-3.17	0.95	0.73
DE1	-1.72	0.95	1.34
WE2	-2.34	0.96	0.98
QC strain 25922	-2.82	0.91	0.82
E.coli O157:H7	-2.1	0.91	1.10
Salmonella	-2.27	0.95	1.01

$^{\beta}$ Decay constant (k) determined from the the linear regression plot of $\ln(N_t/N_0)$ where k is the slope of the regression line.

$^{\gamma}$ Coefficient of determination of the regression of the plot of $\ln(N_t/N_0)$

$^{\delta}$ Time in days to achieve a 90% reduction in cell numbers

Table 6.
Results of z-tests comparing die-off (k) rates of selected surrogates and QC strain to the rates of *Salmonella* and *E.coli* O157:H7

		Salmonella	*E.coli* O157:H7		*Salmonella*	*E.coli* O157:H7
	Exp. 1	Z	Z	Exp. 2	Z	Z
QC Strain 25922		1.95	1.86		0.93	1.69
P1		0.503	0.421		0.684	0.571
P2		1.134	1.005		0.189	0.206
P3		0.28	0.229		1.408	2.17
DE1		0.238	0.195		0.903	0.839
WE2		0.787	0.617		0.11	0.489

Note. Z-scores were considered significant if z > 3.182 (3df) using a 95% confidence interval.

with the T^{90}s of the selected surrogates, with P2 having the highest at 1.32 days. In experiment two, die-off for all isolates used was lower overall, with *Salmonella* and *E.coli* O157:H7 having T^{90} values of 1.01 and 1.1 days, respectively. Again in experiment two, the selected surrogates were similar to the pathogens, with P1, P2, and DE1 all having T^{90} values over 1 day.

Isolate Persistence Experiment

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. The QC strain 25922 and a mix of two surrogates were also used to determine if the surrogates or QC strain persisted in the same manner. Die-off rates were not calculated for these data, as the data collected went beyond the linear die-off period.

The persistence data of the isolates can be viewed in Figure 4. The resulting data showed that although variable, pathogens and surrogates persisted in the same manner out to 22 days, with culturable colonies being detected for both *E.coli* O157:H7 and *Salmonella*, the surrogate mix, and for the QC strain 25922 out to the day 22 sampling period. Sampling could not continue out past day 22 due to the condition of the lettuce plants. However, as seen in Figure 4, the QC strain numbers were lower throughout the sampling period while the pathogens and surrogate mix persisted at a higher and more similar rate. All isolates saw approximately a 5 log reduction over the first 7 days, before

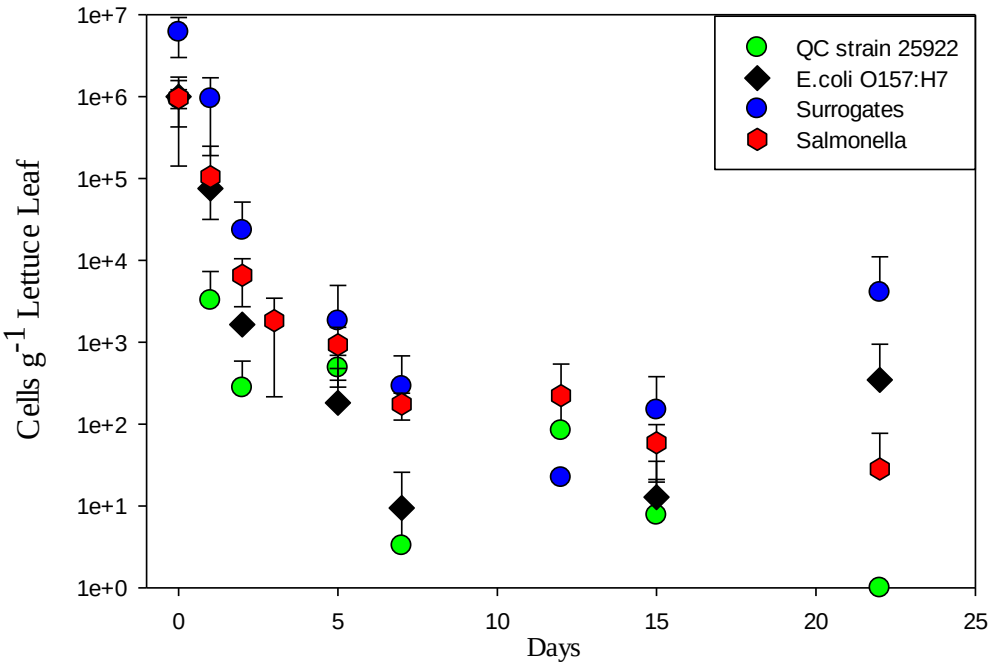

Figure 4. Persistence of pathogens, QC strain 25922, and selected surrogates on lettuce plants

the persisting period began. From days 7 through day 22 the isolates persisted on the plants surface at a consistent rate, hovering between 10^4 and 10^2 cells g^{-1} of lettuce leaf. In a separate repetitive experiment, the QC strain 25922 was compared to *Salmonella* and *E.coli* O157:H7 strains. Figure 5 shows the comparison of die-off for QC strain 25922 and both pathogens. Here it can be observed that the QC strain does die-off at a visibly faster rate, and as a result, was at a considerably lower cell concentration by day four. A similar observation can be made in Figure 6, where the selected surrogates have been added for comparison.

It can be seen that the surrogates die-off much more consistently with the pathogens at the same or slightly less rate which led to similar cell concentrations for the surrogates and pathogens, while the QC strain was noticeably lower. The QC strain 25922 fell a full log below that of *Salmonella* in the first 24 hours following the contamination event, but was consistent with the *E.coli* O157:H7 numbers. After the second day, a separation began to occur as the QC strain 25922 cell numbers fell below that of both pathogens, and continued to die-off at a faster rate through the next three days. The QC strain 25922 had fallen below 1×10^2 cells g^{-1} of lettuce leaf while both pathogens were still slightly above 1×10^3 cells g^{-1} of lettuce leaf.

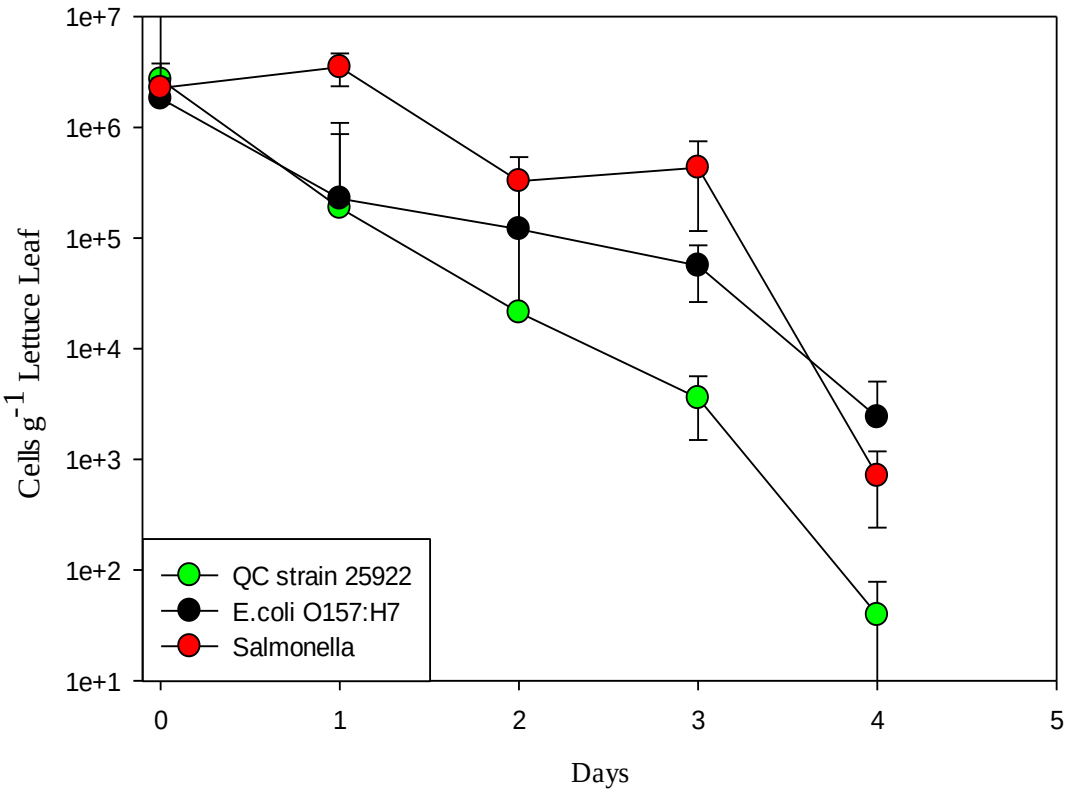

Figure 5. Comparison of survival on lettuce leaf surfaces for QC strain 25922, *E.coli* O157:H7 and *Salmonella*

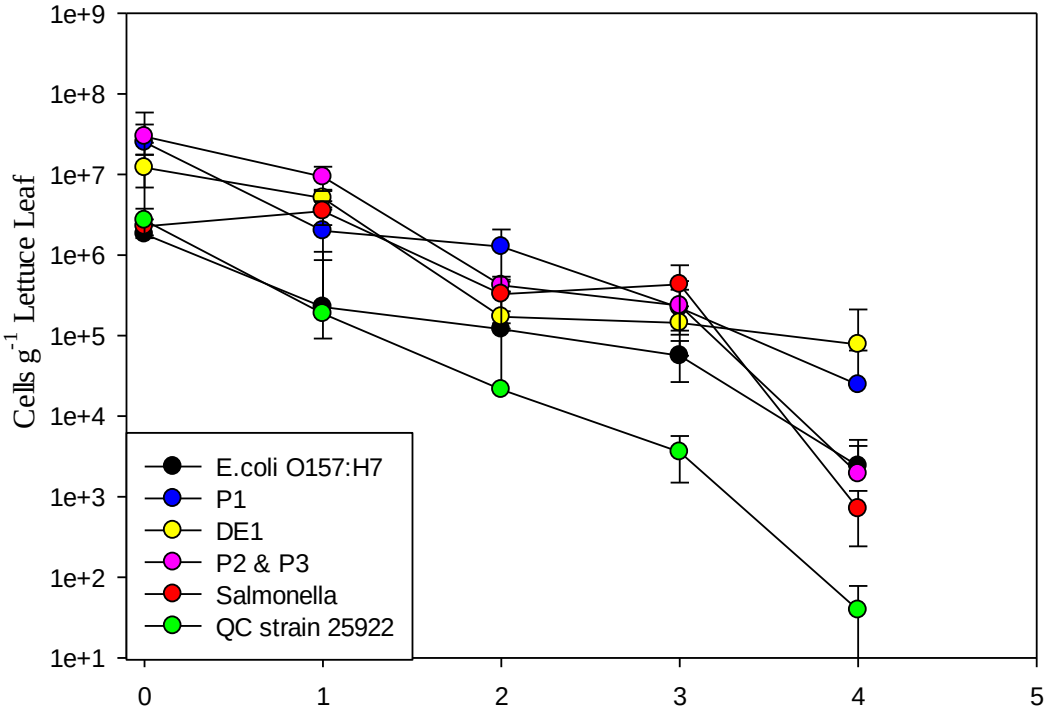

Figure 6. Comparison of survival on lettuce leaves for four selected surrogates, pathogens, and QC strain

Chapter V
Discussion

Characterization and Selection of Candidate Surrogates

The selection of the surrogates from the 18 candidates was based on biofilm formation and lettuce leaf attachment capabilities of the isolates, as most of the isolates were already positive for curli expression. The selected surrogates possessed the needed attachment and adherence capabilities, especially when compared to that of *Salmonella*, which showed an impressive capability to adhere to surfaces. This was especially true when *Salmonella* had been grown in a low nutrient environment of the LM media. Growth environment is an important consideration for any lab scale experiment that will characterize the way a pathogen or surrogate behaves in an environmental setting. Because of the initial observation of Salmonella to adhere and form biofilms, these two characteristics became the primary focus of isolate selection.

The biofilm data from this study suggested that the higher stress levels of the low nutrient environment caused *Salmonella* and several of the candidate isolates to create a higher rate of biofilm. This is an important characteristic, as biofilm formation is a characteristic that allows pathogens to survive on surfaces (Yaron & Romling, 2014). 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*. A similar trend is noticeable with the leaf attachment data, as the QC strain did not show attachment capabilities similar to that of *Salmonella*, although it was comparable to that of *E.coli* O157:H7. Because both attachment and biofilm formation are vital in the ability to

contaminate and survive on fresh produce, the QC strain is already at a disadvantage as a surrogate of *Salmonella*.

Although the QC strain was not found to die-off at a statistically different rate than the pathogens or selected surrogates, it does attach to lettuce leaves and form biofilm at a statistically different rate than *Salmonella*. Before bacteria can be in a situation to persist for extended periods of time on a plant surface, the bacteria 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 a lettuce plant simply because a larger number of cells were able to attach and survive immediately following a contamination event. These data show that the QC strain does not behave as *Salmonella* in both attachment capability and biofilm formation, unlike several of the candidate surrogates selected in this study.

Isolate Persistence and Die-Off

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 event does see a linear die-off, the bacteria did reach a point where the linear die-off ceased, and a persistence period began (Figure 4). This 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). This indicates that there will be multiple irrigation events occurring on a crop

up until the harvest, increasing the risk of a contamination occurring. The capability of pathogens to survive 20 or more days on the crop is a serious concern if produce is contaminated within a few days of harvest. This data suggests that both *E.coli* O157:H7 and *Salmonella* are capable of surviving long enough to make it through the harvest, processing, and distribution of the product and potentially infect the consumer.

The current requirements of the LGMA require that agricultural water being used to irrigate crops must be sampled at the point closest to use no less than 18 hours apart and at least monthly (LGMA, 2013). The farmer is required to either treat or not use water that exceeds the EPA recreational surface water standard of 126 CFU/100 ml of water sample, and if water exceeding this level is used on a crop, then crop sampling must be done for both *E.coli* and *Salmonella* (LGMA, 2013). As already discussed, a crop will be irrigated multiple times within the last 30 days before harvest, and yet the farmer is only required to sample once in that time frame. Multiple irrigation events could occur after the final water sample is taken but before the crop will be harvested, creating an unknown situation of possible contamination. If contamination of the crop with pathogens did occur inside the last few weeks before harvest, and after the last water-sampling period, those pathogens could be expected to persist on the plant surface until after harvest.

FSMA allows the use of an estimated die-off of a 0.5 log reduction per day to calculate the time period needed to wait before harvest if a crop has been sprayed with contaminated irrigation water (USFDA, 2014). This 0.5 log per day reduction is an assumed rate of die-off to a point where pathogen cell numbers drop below what should be a health risk. Data from this study indicates that die-off is linear only for the first 4-7

days, which is followed by a persisting period where cell numbers stay consistent and detectable by standard lab culture methods. Using the 0.5 log per day reduction for *Salmonella* in the Isolate Persistence Experiment in this study, with a starting population level of approximately 1 x 10^6 cells, by day 12 the estimated number of surviving cells would be approximately zero. However, looking at the data in Figure 4, it can be seen that cell numbers for *Salmonella*, *E.coli* O157:H7, and the mix of surrogates are all still at or above this estimated number at day 22. These data brings into question the projection of die-off rates to the point of assumption that pathogen populations have fallen to a safe level. Considering the data from other studies concerning viable but not culturable cells and their potential to be infective (Dinu & Bach, 2011) complicates the methodology even more.

Crop sampling is required by the LGMA if it is known that contaminated irrigation water has been used on a crop. The LGMA specifies that the crop field should be sampled by taking nine 100 gram samples in a Z pattern to test for the presence of *E.coli* O157:H7 or *Salmonella* within 10 days or less of harvest (LGMA, 2013). This sampling technique may prove to be hit or miss on detecting pathogens in an entire field of lettuce. It is known that temperature, moisture levels, UV radiation from the sun, salinity, and other environmental conditions can create a stressful environment and potential decline of bacterial population (Ishii & Sadowsky, 2008), and these conditions may not impact an entire crop field equally. A quick observation of a single lettuce plant will indicate to the observer that environmental conditions that bacteria will experience on a micro scale may differ on a single plant. This fact alone makes it hard not to question the representation that nine 100 gram samples give to an entire lettuce crop

field. Viable but non culturable cells come into play here as well, where cells may no longer be quantifiable with normal lab assays. This situation could potentially lead to faulty sampling results from crops that were known to be contaminated.

Data from the die-off experiments show that the QC strain did not die-off at a rate that was statistically significantly different from those of the pathogens, and that the selected surrogates had similar die-off rates as well. However, statistics do not always tell the whole story. The QC strain had a high amount of variability, especially in experiment one. This high variability led to a high standard error, which affected the statistical test. Inconsistencies and high variability is not a preferred quality for a surrogate, as it makes the results from any experiment using the surrogate questionable. In Figure 6, the plotted data illustrates how well the selected surrogates from this study mimic *Salmonella* and *E.coli* O157:H7 in a pre-harvest lettuce plant environment. The surrogates are the same or slightly higher, which would give a more conservative estimation of pathogen levels. It is important to remember that many food safety research labs cannot use pathogens, and will be using a surrogate to collect data from. This fact alone makes the selected surrogates in this study more representative surrogates of *Salmonella typhimurium* and *E.coli* O157:H7 strains used. 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 policies and regulations, and support suggested good agricultural processes to producers.

Recommendations

The data collected from this study builds upon the research and expertise of many

other studies and researchers. This allows for the formulation of recommendations for the produce industry with the objective of shifting the paradigm of policy and regulation to help producers and reduce public health risk.

Based on the data collected in this study, the first recommendation would be to use proven pathogen surrogates similar to the ones selected in this study in the place of the QC strain 25922. The selected surrogates outperformed the QC strain in biofilm formation, leaf attachment, die-off, and persistence while meeting or exceeding the values produced by *Salmonella* and *E.coli* O157:H7. The use of the QC strain may be more appropriate for *E.coli* O157:H7 in lab scale assays and experiments, but not in greenhouse studies with realistic environmental conditions.

As previously described, the LGMA requires at least monthly agricultural irrigation water sampling that will be used for foliar application to pre-harvest plants. Source waters can change dramatically within a short time period due to various factors effecting the watershed and levels of pathogens (Gelting et al., 2015). Considering the fact that crops are irrigated numerous times within the last month before harvest (Smith et al. 2011), it is a logical assumption that multiple irrigation events could occur close to harvest, but after the last irrigation water samples were taken for that month. Unfortunately, this could leave a time frame where contaminated water could be used on the crop prior to harvest, and not be detected by a sampling that met the requirements. The data from this study indicates that pathogens have the ability to persist on that crop until harvest, and potentially infect the consumer. Because of this, the recommendation is to change water sampling frequency to include a requirement for producers to sample irrigation water for every irrigation event within the last 21 days before harvest. This will

ensure that no contaminated water is used to irrigate the crop within a reasonable timeframe of harvest that could lead to surviving pathogens at infective levels reaching the consumer.

The use of an estimated die-off rate by FSMA has been described, where a 0.5 log reduction estimation can be used to determine the interval of time that a producer must wait before harvesting a crop if the water used did not meet microbial standards. Persistence data collected in this study show that this estimation of 0.5 log daily reduction may not be accurate in the projection of when pathogens reduce to a safe level. The persistence ability of *Salmonella* and *E.coli* O157:H7 presented in this study exceeded the estimated levels computed using the 0.5 log per day reduction methodology. The recommendation for this issue is to not use a projected die-off rate without continued research on the die-off tendencies of pathogens that provide a more accurate understanding of the time periods needed to reach safe consumption levels. Furthermore, it is recommended that until this data is available, water and crop testing be the sole method of determination of microbial hazards, and a no harvest of crops found to be contaminated.

The use of crop sampling is described by the LGMA as a requirement for producers who know that a crop has been irrigated with water that is known to exceed the microbial water quality standard of 126CFU/100ml. This testing must be completed within 10 days of the crops harvest to test for *Salmonella* and *E.coli* O157:H7. The recommendation for this is require the crop sampling to be within three days of the irrigation event that caused the potential contamination, and to increase the required number of samples from 9 to 15. This increase of samples increases the likelihood of the

detection of pathogens if they do exist on the plants, and the faster sampling time post-irrigation increases the chance that the pathogens will be in a culturable state and well above the detection limit of normal lab culture methods. This helps to eliminate the issue of non-culturable but viable cells going undetected, making sure that the crop really is safe for harvest and sale to the public.

Chapter VI

Conclusion

The contamination of fresh produce by pathogens is a substantial public health concern, evident by the number of foodborne illnesses reported in the U.S. every year. Policies and regulations have moved in the direction of prevention by increasing the demands on producers to ensure their products are safe and secure along the Farm to Fork process. Surrogates can provide essential data to both industry and government entities if they are selected carefully and used correctly. These surrogates provide a better understanding of the die-off, persistence, and unique characteristics of the pathogens that are the origin of outbreaks. It can be concluded that the surrogates selected in this study do in fact provide a better representation of two problematic produce pathogens than the currently used strain in a pre-harvest foliar environment. In addition, the data gained from the use of those surrogates and the pathogens together illuminates gaps in policy and regulations that could have negative impacts on human health. The need for continued research in the persistence, survival, and cell characteristics of these pathogens is apparent. The use of surrogates in continued research will provide scientific data that is invaluable to the protection of public health. Further understanding gained from future research can be used to improve policies and regulations regarding microbial food safety practices, and reduce the risk to public health.

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