THE EFFECTS OF AGRICULTURAL FACTORS ON FOODBORNE BACTERIAL PATHOGEN ISOLATION, ATTACHMENT, AND SURVIVAL

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

The pathogens enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella enterica*, and *Listeria monocytogenes* are prominent causes of foodborne illness. If they reach the pre- or post-harvest environment of produce, they can survive for extended periods of time and transfer to post-harvest processing to reach consumers. There are a multitude of factors that influence this survival, bacterial attachment, and isolation, which have been identified for pre- and post-harvest conditions. However, how these pathogens respond to the changes in these systems requires more work to inform effective control methods.

To compare the efficacy of an isolation method in different types of matrices, we used immunomagnetic separation (IMS) to selectively isolate strains of EHEC representing non-O157 serogroups from feces, ground beef, and lettuce. The differing matrices and the presence of native microbes seemed to interrupt isolation, with O111 and O145 beads performing the poorest. To understand how EHEC attachment is influenced by food processing-relevant conditions, we investigated the effects of PBS, 4.5% NaCl, and lettuce exudates on attachment to stainless steel (SS). These simulated limited nutrients, osmotic pressure, and alternative carbon sources that EHEC can encounter during food processing. Initial association to SS was reduced under the 4.5% NaCl for all EHEC strains, but this did not translate to reduced attachment. Variation in attachment was observed in lettuce exudates only, and this variation was driven by strains.

Finally, we evaluated pathogen survival in the pre-harvest environment. We simulated an aqueous environment that EHEC, *Salmonella*, and *L. monocytogenes* might encounter during pre-harvest. Soils were collected from distinctly different environments and extracts were created by leeching water-soluble components from soil. Extracts were inoculated with pathogens and they were monitored to see if the varying chemistries or

microbiomes influenced survival. Initially, survival was reduced in the low-nutrient extract, particularly when native microbes were present. Overall, work done here identified factors like matrix or specific environmental conditions and their effects on the isolation, attachment, and survival. These data provide the basis for further work to improve accuracy of IMS-based detection in complex matrices, determine strain-specific mechanisms for EHEC attachment, and assess associations between soil microbiome composition and pathogen survival.

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DEDICATION

This work is dedicated to my other half, Adam Stein. This mountain was mine to climb, but your support and love were indispensable along the way.

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1. LITERATURE REVIEW – PRE-HARVEST AND POST-HARVEST ENVIRONMENTAL FACTORS LEADING TO PHENOTYPIC CHANGES IN FOODBORNE PATHOGENS

Introduction

Consumption of nutrient-rich fruits and vegetables is a cornerstone of a healthy diet, and consumption of these foods has increased within the past few decades (WHO, 2003). These foods are a rich source of key nutrients, such as numerous vitamins and carbohydrates, which help maintain a robust lifestyle (Brookie et al 2018; Dreher 2018; Robberecht et al., 2017; Rooney et al., 2013). Due to this, increases in demand around the world have driven fresh produce production to achieve large amounts of food that is nutrient-dense in order to meet the needs of the planet's growing population (Huang et al., 2004). Vegetables and fruits are available for consumption in many forms: raw or unprocessed, minimally processed, frozen, cooked, or canned. As of late, there is a significant trend of consuming these products raw (USDA ERS, 2020). The combination of these factors makes ready-to-eat (RTE) produce a prominent concern in the realm of food safety.

For fresh produce, bacterial foodborne pathogens are a persistent concern since these products are frequently consumed raw (Brookie et al., 2018; Dreher 2018), which avoids an important control method of cooking to kill pathogens. Of all domestically acquired foodborne illnesses with an identified food vehicle recorded in the US between 1998 and 2008, 46% were attributed to produce, with 2.2 million of those illnesses associated with leafy greens (Painter et al., 2013). The produce plants encounter many potential sources of contamination pre-harvest, such as water, dust, manure, or wildlife (Brandl 2006), but also continues to be vulnerable in the post-harvest environment on the way to the consumer. These environments provide competitive, complex, and dynamic conditions that can pose significant challenges for pathogens to overcome and survive. From

competition and nutrient limitation to physiochemical stressors like osmotic pressure, pH stress, sanitizers, and temperature changes (Rozen and Belkin, 2001; Beuchat 2002; Hutchison et al., 2004), bacteria that end up in the pre- or post-harvest environment must employ ways to evade, counteract, and survive.

The enteric pathogens Enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella* spp., and *Listeria monocytogenes* are three of the most prominent causes of bacterial foodborne illness in the United States (Scallan et al., 2011). Major concerns with these pathogens are a result of their infectivity and severity of illness (Glynn and Bradley, 1992; Kathariou, 2002; Salvadori and Bertoni 2013). *L. monocytogenes,* in particular, has one of the highest mortality rates (20-30%; Swaminathan and Gerner-Smidt, 2007). Commonly, these three pathogens are attributed to outbreaks sourced to foods like ground beef, unpasteurized dairy products, leafy greens, and other fresh produce (CDC, 2020a, b, c). Compounding the problem of easy contamination, the infectious dose for *Salmonella* and EHEC can be quite low (~100 cells). While these pathogens can persist (Welshimer 1968; Locatelli et al., 2013) or survive (Franz et al., 2005; Hutchison et al., 2004) in the extra-host environment, they are commonly found as commensals in animal reservoirs, like cattle and sheep, which allows them to be shed more widely and cycle through the environment (Foltz 1969; Hussein 2007; Stipetic et al., 2016; Winfield and Groisman, 2003). These combined factors lead to the dispersion of these pathogens throughout pre-harvest environments facilitated by mechanisms such as wind/dust, irrigation and flood waters, fecal deposits, and application of untreated manure (Brandl 2006). Moreover, these pathogens can continue to persist and reach the post-harvest processing environments of foods (Pérez-Rodríguez et al., 2008). Many adaptations or mechanisms that are responses to environmental conditions have been investigated. This review serves as an extensive look into the ways that these

pathogens interact with different components within the pre- and post-harvest environment of fresh produce and what is known about isolation of cells and key phenotypes associated with these microbes, such as survival and attachment.

Incidence of EHEC, *Salmonella* spp., and *Listeria monocytogenes* in fresh produce

EHEC O157 and non-O157

Enterohemorrhagic *Escherichia coli* is a persistent foodborne pathogen in the United States. This Gram-negative bacillus is divided into multiple serogroups, with O157 being the most prominent causing an annual 63,000 illnesses, 2,100 hospitalizations, and 20 deaths (Scallan et al., 2011). In general, *E. coli* can be categorized as EHEC when they can produce Shiga toxin and possess virulence factors like the locus of enterocyte effacement (LEE). Leading causes of infection from non-O157 EHEC come from the serogroups O26, O45, O103, O111, O121, and O145 (USDA/FSIS, 2012). As a collective, non-O157 EHEC are responsible for an estimated 112,000 illnesses and nearly 300 hospitalizations (Scallan et al., 2011). O157 and non-O157 EHEC have been implicated in an annual loss of $280 million in cost of illness and 1,700 quality-adjusted life years (QALY) in the US, which puts a significant burden on the public (Hoffmann et al., 2012).

Cattle are reservoirs of EHEC and can shed this pathogen into the environment via feces leading to spread into water and soils (Hussein 2007; Jongman and Korsten, 2017; Oliveira et al., 2012). Manure provides some benefit to EHEC as there is evidence of prolonged survival in this medium (Himathongkham et al., 1999; Hutchison et al. 2004). This increase in survival time can facilitate dispersal, which allows for many routes of contamination throughout the environment like water, soils, or dust (Brandl 2006). These factors affecting transmission highlight the food safety problems with fresh produce. In a survey conducted on produce from 2010 through 2012, it was noted that EHEC, both O157

and non-O157, was present in 10 samples of romaine lettuce and spinach out of ~1,500 samples (Zhang et al., 2018). Since the early 1980s, the number of EHEC of outbreaks associated with produce has ranged from 21% (Rangel et al., 2005) to 10% (Heiman et al., 2015) in the US. Additionally, the seasonality of EHEC outbreaks has been a focus as a majority (up to 45%) of outbreaks in leafy greens occur during the fall (Heiman et al., 2015; Marshall et al., 2020). There may be a correlation between this spike of outbreaks in the fall due to increased shedding of EHEC in cattle feces in the summer months with environmental dispersal occurring over the following months (Dunn et al., 2004; Hancock et al., 1997). But with multiple outbreaks still occurring throughout the year (28% in Spring, 18% in Summer, and 10% in Winter), this does not show the entire picture of what happens once EHEC leaves the host (Marshall et al., 2020). Ultimately, these works put a spotlight on the need to understand more concerning EHEC and how the extra-host environment influence its path from farm-to-fork.

Salmonella enterica

Salmonella is closely related to *E. coli* and has distinct similarities in O-antigens, infectious dose (~100 cells), and asymptomatic carriers (Hu et al., 2010; Sharp 1991). However, as the etiologic agent of over 1,000,000 estimated illnesses annually, non-typhoidal *Salmonella* sits as the top bacterial foodborne pathogen in the US (Scallan et al. 2011). It is also responsible for the most hospitalizations (~20,000 annually) and deaths (370 annually) in the US making *Salmonella* a significant concern in food safety (Scallan et al., 2011). This then leads to a higher estimated financial burden of $3.3 million as an annual cost of illness and the loss of 17,00 QALYs from non-typhoidal *Salmonella* (Hoffmann et al., 2012).

Such high levels of infections and outbreaks of *Salmonella* could be due to several factors such as number of infectious serotypes (100 of 2,700 serotypes), the number of sources of *Salmonella*, its ability to contaminate a wide variety of foods, and the fact that it persists for long periods of time in the non-host environment (CDC, 2020c; Franz et al., 2005). While many illnesses are usually attributed to a zoonotic source (e.g. pets, livestock, animal food products), fresh produce is a significant vehicle for transmission of *Salmonella* (CDC, 2020c; Kimura et al., 2004). When looking to the survey report from 2018, seven samples of leafy greens (romaine lettuce, spinach, and iceberg lettuce) were identified across the three-year study period (Zhang et al., 2018). While this prevalence was relatively low, the mere presence of *Salmonella* underlines the risk associated with consuming fresh produce. Over 20% of reported cases of foodborne illness in developed countries between 1980 and 2016 attributed to fresh produce have been linked to *Salmonella* (Machado-Moreira et al., 2019). While food safety practices have improved, reducing this incidence of salmonellosis from contaminated fresh produce consumption continues to be a challenge.

Listeria monocytogenes

The foodborne pathogen, *L. monocytogenes*, is a Gram-positive bacillus that has proven to be a difficult organism to control in food production. Outbreaks of *L. monocytogenes* are rarer than EHEC and *Salmonella* as it only accounts for a little under 1,600 annual illnesses in the US (Scallan et al., 2011). Where *L. monocytogenes* is of greatest concern is in the severity of illness, as it has the highest hospitalization rate (94%) and a mortality rate ranging from 20 to 30% (Scallan et al., 2011; Swaminathan and Gerner-Smidt, 2007). These high rates of hospitalization and death can be attributed to *L. monocytogenes* infections occurring in susceptible populations like neonates, pregnant women, and the otherwise immunocompromised (Swaminathan and Gerner-Smidt, 2007).

This translates to an annual $2.6 billion in cost of illness and a QALY of 9,000 (Hoffmann et al., 2012).

Generally, *L. monocytogenes* exists ubiquitously in nature and is seen as an opportunistic human pathogen. *L. monocytogenes* is a very resilient microorganism, which allows it to be present in a multitude of environments. This is evident in its ability to tolerate acidic (pH 3.0) and alkaline (pH 12.0) conditions, along with high salt concentrations, and grow at refrigeration temperatures (Lunden et al., 2007; Patchett et al., 1992; Zhu et al., 2005). Additionally, resistance to common sanitizing agents, phenotypes attributed in-part to biofilm formation, make removing *L. monocytogenes* from food processing environments particularly tough (da Silva and De Martinis, 2013). Due to these abilities, *L. monocytogenes* is commonly a concern with foods not normally cooked like RTE deli meats, dairy products, and fresh produce. Since 2011, six *Listeria* outbreaks have been attributed to produce products ranging from cantaloupes and caramel apples to packaged salads and enoki mushrooms (CDC, 2020b). When surveying leafy greens, it was noted that 15 of ~1,500 samples were positive for *L. monocytogenes* over three years of sampling (Zhang et al., 2018). The sources of contamination of fresh, leafy produce been attributed to different environments during pre- and post-harvest (Smith et al., 2018). These constantly shifting environmental conditions require a complex set of responses in order for *L. monocytogenes* to persist. Elucidating key phenotypic responses, like attachment, isolation, or survival, to varying environmental conditions can inform control methods to reduce *L. monocytogenes* outbreaks associated to fresh produce.

Environmental factors impacting specific phenotypes

EHEC, *Salmonella*, and *L. monocytogenes* are all foodborne bacterial pathogens that periodically cause outbreaks that have been associated with fresh produce. Periodic

outbreaks of these pathogens present a significant burden to public health while consumption of produce is still generally associated with good health outcomes. Control of foodborne pathogens on produce is paramount to reducing this burden and methods to do so must be well-informed. There has been extensive evidence to show the source of these pathogens (either due to shedding from an animal host or due to ubiquity in the environment), but how they move through and interact with the pre- and post-harvest environment is not as well understood. Pressures influencing these pathogens within these environments can come from conditions like nutrient limitation, ultra-violet radiation, desiccation, predation, sanitizers, unfavorable temperatures, pH shifts, or osmotic stress (Rozen and Belkin, 2001; Lunden et al., 2007; Patchett et al., 1992; Savageau 1983; Zhu et al., 2005). This constantly shifting array of conditions requires a robust set of responses from these bacteria, and here exist gaps in knowledge. Three of the many phenotypes in need of study are survival, attachment, and isolation and they are analyzed here due to the important roles they play in the pre- and post-harvest environment.

Impacts on isolation – challenges with common methods

An important step in preventing bacterial foodborne outbreaks is isolating these pathogens in foods before they reach the consumer. Certain foods, such as ground beef, are required to be monitored and tested for potential contamination (USDA/FSIS, 2012). Many options to detect EHEC, *Salmonella*, and *L. monocytogenes*, with methods based on colorimetric culture assays, real-time PCR (qPCR), and immunology-based assays (Beutin and Fach 2014; DebRoy et al. 2011; Jadhav et al., 2012; Lee et al., 2015; Verstraete et al. 2011). Using these methods to detect foodborne pathogens has its challenges as accuracy can be influenced by factors like low cell numbers, other microbes, detection of dead cells, or interference from the food matrix (Wang and Salazar, 2016). This variation can lead to

challenges in picking the most accurate and sensitive method for detection and, under some circumstances, a combination of methods may be necessary.

Immunology-based assays, like immunomagnetic separation (IMS), require a readily available antigen on the cell surface to isolate via antibody binding. In the case of Gram-negative bacteria, the lipopolysaccharide (LPS) component of the outer membrane (OM) is an easy molecule to target. When attempting to isolate different pathogens with IMS, differentiation can get as fine as the serogroup level, like in the case of Shiga toxin-producing *E. coli* (or STECs; Boer and Heuvelink, 2000). While IMS can provide benefits for isolating STECs from foods, like avoidance of isolating background microbiota, there could be issues with using these methods due to phenotypic responses and changes in OM structure of Gram-negative bacteria. In fact, this dynamic cellular structure is capable of shifting and changing due to external stimuli (Rojas et al., 2019). Alterations to this outer structure due to environmental stressors osmotic pressure or sanitizers can disrupt the structure as expression of RpoE modulates gene expression (Dartigalongue et al., 2001; Egler et al., 2005). These points highlight limitations of IMS. Reduction in the capture accuracy of IMS in different samples has been shown, and improvement may come from combining isolation and detection methods. Optimizing IMS in combination with molecular techniques like PCR could overcome some challenges for both methods. IMS can selectively isolate serogroups from various samples with PCR used as confirmation, and more work should be done to develop and validate these combinations of methods.

Impacts on attachment – Biofilms

After survival, bacterial attachment to various biotic and abiotic surfaces is a concerning phenotype in food safety. This is because attachment, or lack thereof, is what keeps bacteria in unwanted places like food processing equipment. Attachment begins with

cells becoming reversibly associated to a surface. This initial association to a surface is due to random close contact and the physical structures on the outside of the bacteria, such as curli and flagella, that interact with other bacterial cells and nearby surfaces (Gorski et al., 2009; Pawar et al., 2005, Shaw et al., 2011). Properties of the surface, such as hydrophobicity and van der Waals forces, can influence these interactions (Nesse et al., 2014; Razatos et al., 1998). This initial association can lead to two outcomes: dispersal or irreversible attachment. This long-term form of attachment is characterized by bacterial cells resisting physical removal from the surface. From there, bacteria of all species can aggregate into a biofilm surrounded by extracellular polymeric substances, like cellulose, which help to protect and adhere the microbes in one location (Saldana et al., 2009; Solano et al., 2002). Signals that trigger biofilm formation have been well investigated within single species populations (da Silva and De Martinis, 2013; Fett 2000; Fratamico et al., 1996; Speranza et al., 2011; Zulfakar et al., 2013), yet this problem still plagues food processing facilities and contributes to outbreaks.

These processes of attachment and biofilm formation are of concern since these responses facilitate persistence of foodborne pathogens in an environment (Vestby et al., 2009). Biofilms created by EHEC, *Salmonella,* or *L. monocytogenes* have been shown to form on plastics (Joseph et al., 2001; Stepanović et al., 2004), stainless steel (Joseph et al., 2001; Kusumaningrum et al., 2003; Speranza et al., 2011), and glass (Bonsaglia et al., 2014; Sasahara and Zottola., 1993). Plastic and stainless steel are of greatest concern since these are the most common food contact surfaces. Under these circumstances, pathogens receive external signals that can modulate the attachment phenotype. Temperature can lead to increases attachment and biofilm formation of *Salmonella* (Speranza et al., 2011; Stepanovic et al., 2003), EHEC (Nesse et al., 2014), and *L. monocytogenes* (Bonsaglia et al.,

2014; Gorski et al., 2003; Mai and Conner, 2007). Additionally, pH (Chagnot et al., 2013; Speranza et al., 2011) and limited available nutrients (Mai and Conner, 2007; Speranza et al., 2011) contribute to attachment and biofilm formation by these pathogens. These data have demonstrated the multifactorial nature of biofilm formation, more work is needed to develop applications that effectively disrupt attachment before biofilms can be established.

Once pathogens have the opportunity to attach to these surfaces, removing them can be challenging and the efficacy of common sanitizers is reduced due to the protective nature of biofilms (Frank and Koffi, 1990; Joseph et al., 2001; Lajhar et al., 2018; Wang et al., 2012). Research has improved our understanding of how specific environmental factors influence attachment and biofilm formation and it is possible to apply this information to control pathogens. For instance, it has been observed that attachment of foodborne pathogens can be interrupted by topographical modifications (Feng et al., 2014) and surfactants usage (Hassan and Frank, 2003). Ultimately, interrupting this attachment and biofilm formation is vital to reduce contamination of produce by foodborne pathogens.

Impacts on survival in soils and water

To ultimately reach the consumer, foodborne pathogens must survive unfavorable environments. Without survival in the pre- and post-harvest environment, pathogens like EHEC, *Salmonella*, and *L. monocytogenes* do not make the journey to a human host via fresh produce. It is clear that this initial survival in manure, soils, and irrigation waters is allowing transmission of enteric pathogens into the growing areas of the pre-harvest environment (Franz et al., 2005; Hutchison et al., 2004; Jongman and Korsten, 2017; Oliveira et al., 2012), and eventual cross contamination into the post-harvest processing environment (Erickson et al., 2018; Gaul et al., 2013; Murray et al., 2017; Stephan et al., 2015). Food safety research tend to focus on how specific factors like desiccation (Koseki et

al., 2015), low pH (Mazzotta 2001; Suehr et al., 2020), or livestock waste handling (Himathongkham et al., 1999; Hutchison et al., 2004) lead to changes in survival for prominent pathogens.

A notable trend in recent outbreaks has been the transmission of enteric pathogens to growing produce from contaminated water. Within these aqueous systems, multiple variables, like soil chemistry, the presence of amendments, and native microbiota, could potentially be influencing the pathogens. There is evidence that the presence of biological soil amendments (manure/poultry litter) in soils increases survival of foodborne pathogens such as *Salmonella* (Shah et al., 2019a). In addition, when soils were amended with poultry manure *E. coli* was able to survive longer when compared to soils amended with dairy manure (Neher et al., 2019) Under conditions designed to simulate runoff events or standing water after heavy rain or flooding, biological soil amendments along with soil in the aqueous environment have identified important chemical components that increase pathogen survival (Neher et al., 2019; Shah et al., 2019b). A trend in both of these systems signaled that higher levels of total nitrogen, phosphorous, and organic carbon extracted from amendments and soil correlated with growth of these organisms. On the other hand, low pH and competition from native microbes for nutrients in agricultural soils and associated standing water have shown to negatively impact the survival of EHEC (Xing et al., 2018). But the majority of these observations are from systems that have been sterilized and lack comparisons made amongst different enteric pathogens. When studies use non-sterilized extracts, pathogen growth is reduced or eliminated (Shah et al., 2019b), highlighting the potential role native soil microbes play in controlling growth of pathogens.

With the continuing problems with pathogens surviving prolonged periods of time in standing water systems and the subsequent transfer to fresh produce, more work should be

done to better understand what specific factors are important. Experiments should be designed to include the multiple factors discussed here, particularly with the presence water-soluble components from soil of various sources relevant to agricultural systems of concern. This, with the addition of multiple applicable pathogens, would expose more on how survival varies and could help in improve control methods for these foodborne bacterial pathogens in water.

Overall implications

Prevention of foodborne illness and outbreaks associated with minimally processed foods like fresh produce will require an intensive understanding of how the pathogens interact with the pre- and post-harvest environment. Conditions in these extra-host environments put selective pressure on pathogen populations and has been shown to influence their survival, isolation, and attachment. These phenotypes are significant when considering the implications to food safety. In particular, pathogen survival in water is important to expand on since contaminated water has been a frequent vector for bacteria, like EHEC, to reach growing produce plants pre-harvest.

It was our goal with the following studies to establish a deeper understanding of how specific environmental factors affect specific bacterial pathogens. We began looking at immunomagnetic separation (IMS) as a detection method for multiple STEC serogroups and compared its accuracy in different food matrices. Later, we assessed attachment of EHEC to stainless steel (a common food-contact surface) when those bacteria were subjected to limited nutrients, osmotic pressure, and an alternative carbon source in lettuce exudates. And finally, survival of EHEC, *Salmonella,* and *Listeria monocytogenes* was assessed in the simulated agricultural water environment of soil extracts, in which were distinctly different chemical/nutrient compositions.

The best path forward to controlling pathogens in these environments will require well-informed methods. This will come from rigorous study identifying environmental factors in pre- and post-harvest that influence isolation and promote or suppress pathogen survival and attachment

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2. COMPARISON OF IMMUNOMAGNETIC SEPARATION BEADS FOR DETECTION OF SIX NON-0157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* SEROGROUPS IN DIFFERENT MATRICIES[1]

Abstract

Immunomagnetic separation used with culture-based methods has been a useful technique in the detection of pathogens. However, previous studies have not answered many of the necessary questions for real world applications. The objective of this study was to assess the efficacy of different IMS bead types in recovery of the correct serogroup from a mixture of big six non-O157 STEC strains. To determine the impact of different matrices on recovery, samples of sterile phosphate buffered saline (PBS), sterile and non-sterile cattle feces, ground beef, and lettuce were inoculated with 10 CFU ml^{-1} mixture of isolates representing the six serogroups. After a 6 h incubation at 37°C, samples were mixed with IMS beads from three different commercial sources and plated on eosin methylene blue agar (EMB). Three suspect *E. coli* colonies were selected from each EMB plate and multiplex PCR was used to determine the serogroup. The rate of correct identification varied with the serogroup, IMS bead manufacturer, and matrix. Overall, recovery of the correct serogroup became less likely with increase in matrix complexity, with enrichments containing lettuce having the greatest number of bead types with significantly lower likelihood of correct recovery compared to recovery in PBS.

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[1] The material in this chapter was co-authored by Autumn L. Kraft, David W. Lacher, Weilin L. Shelver, Julie S. Sherwood, and Teresa M. Bergholz. Autumn L. Kraft had primary responsibility for IMS work, data analysis, and drafted and revised all versions of this chapter. Julie S. Sherwood conducted work with mEC broth growth experiment, and David W. Lacher provided the primers and methods used for mPCR. Weilin L. Shelver provided the strains used. Teresa M. Bergholz revised all versions of this chapter.

Introduction

With an estimated 176,000 illnesses acquired annually in the U.S., Shiga toxin-producing *Escherichia coli* (STEC) continue to be a public health concern (Scallan et al., 2011). Among the ~400 serotypes of STEC, O157:H7, O26, O45, O103, O111, O121, and O145 are of greatest concern (USDA/FSIS, 2012a). The non-O157 STEC are responsible for most STEC illnesses in the US (Scallan et al., 2011). Since STEC can survive in the gastrointestinal tract and manure of cattle (Fremaux et al., 2006; Hussein 2007) and in the non-host environment (Bolton et al., 2011; Moyne et al., 2011), these pathogens can be transmitted to many types of foods via contaminated feces, soil, and water. From 1998 to 2008, leafy vegetables and beef commodities were associated with 22 and 13% of all bacterial foodborne illnesses, respectively (Painter et al., 2013). More specifically, ground beef and leafy greens, such as lettuce, have been implicated in both O157 and non-O157 STEC outbreaks (White et al., 2016).

An important step in preventing STEC-associated foodborne outbreaks is detecting these pathogens in foods before they reach the consumer. Certain foods, such as ground beef, are required to be monitored and tested for potential STEC contamination (USDA/FSIS, 2012b). Many options to detect pathogens exist, including culture-based methods, polymerase chain reaction (PCR), and immunologically-based techniques (Deisingh and Thompson, 2004). For the detection of non-O157 STEC, methods based on colorimetric assays, multiplex PCR (mPCR), and immunomagnetic separation (IMS) have been developed (Jenkens et al., 2003; Posse et al., 2008; DebRoy et al., 2011; Verstraete et al., 2011; Beutin and Fach 2014).

IMS can be combined with culture-based methods and multiplex PCR to confirm the serogroup of viable cells where IMS serves as a selective step during isolation. IMS has

been used to isolate non-O157 STEC from cattle feces and ground beef in conjunction with culture- and PCR-based methods, though some difficulties were observed in isolating all serogroups equally (Fratamico et al., 2011; Kalchayanand et al., 2013; Noll et al., 2015). While colorimetric-based assays for these STEC have been developed (Posse et al., 2008), the subjective nature of isolate identification could lead to less accurate results. Accuracy of this type of detection can be improved with IMS and using a chromogenic agar in combination with IMS has been shown to have a high sensitivity in detecting STEC (Hara-Kudo et al., 2016). Even with these improvements, there is less information available regarding the performance of IMS in various matrices with competing microbes including a mixture of STEC serogroups. The purpose of this study was to assess the ability of commercially-available IMS beads from three different manufacturers (Dynal (DY), Abraxis (ABR), and Romer (RM)) to recover the correct serogroup from a mixture of big-six non-O157 STEC isolates from cattle feces, uncooked ground beef, and romaine lettuce.

Materials and methods

Bacterial isolates and growth conditions

One isolate from each of the six STEC serogroups was used in this study (Table 1). These six representative isolates were stored at –80°C in Brain-Heart infusion (BHI) medium (Becton Dickinson, Sparks, MD) with 10% glycerol. Each isolate was streaked on Tryptic soy agar (TSA, Hardy Diagnostics, Santa Monica, CA) from the frozen stock and incubated for 24 h at 37°C. Thereafter, a single colony was transferred to 5 ml Tryptic soy broth (TSB, Hardy Diagnostics), which was incubated for 20 h at 37°C. From this culture 10 µl was added into 10 ml of TSB and incubated with shaking at 37°C for 20 h. Strains were grown individually for 6 h at 37°C in mEC to determine their final log CFU ml^{-1}

concentration and in a mixture to verify the proportion of the isolates after the incubation (Table 1).

Table 1. Growth of individual STEC strains and proportions recovered from a mixture in mEC broth after 6 h at 37°C

Serogroup	Strain ID	Source	log CFU ml^{-1} after 6h in mEC broth[a]	Proportion of isolates recovered from mixture in mEC
O26	TW00971/DEC10B	STEC Center, MSU	4.17	12
O45	05-6545	Gilmour *et al.* 2007	4.41	26
O103	03-2444	Gilmour *et al.* 2007	4.28	20
O111	TW01387/CL-37	STEC Center, MSU	4.10	11
O121	03-2832	Gilmour *et al.* 2007	4.19	20
O145	75-83	CDC	4.52	11

[a]Mean of two replicates

Preparation of sample matrices

Isolation of STEC strains using IMS was evaluated from enrichments containing the following sample matrices: phosphate-buffered saline (PBS, Amresco, Solon, OH), cattle feces (sterile and non-sterile), uncooked ground beef, and romaine lettuce. The cattle fecal samples were collected from the North Dakota State University Dairy Unit. Half of the fecal samples were sterilized by autoclaving. Ground beef was acquired from a local grocery store and stored at 4°C. Lettuce leaves were collected from plants grown in the North Dakota Agricultural Research Experiment Station greenhouse facility. The leaves from 15 one-month-old plants were collected and liquefied using a blender (Breville Juicer, Breville, Melbourne, Australia). Each sample matrix type was aliquoted into 1 g portions, stored at 4°C, and used with IMS within three days. For each IMS bead type, three replicates of IMS extraction from each matrix were performed.

Inoculation of enrichment and sample matrices

Following 20 h incubation in TSB, each STEC culture was diluted to 10^{-6} in PBS and 1 ml of each diluted culture was combined into 94 ml PBS to create a final dilution of 10 CFU ml^{-1} for each strain. The concentration of this final dilution was verified by enumeration on TSA. This mixture was used to inoculate the mEC broth (Thermo Scientific, Waltham, MA) and matrices. For each matrix, a combination of 1 g of sample matrix, 5 ml of mEC medium with vancomycin, cefsulodin, and cefixime (VCC) and 1 ml of the diluted, mixed inoculum was vortexed and incubated for 6 h at 37°C. A negative control containing 1 g of the sample, 5 ml of mEC-VCC medium, and 1 ml of sterile PBS was incubated at the same conditions. Each inoculated sample matrix mixture was prepared in multiples to provide enough volume for two 1 ml replicates per IMS bead type.

Recovery of isolates via IMS

The IMS beads were obtained from three manufacturers: Dynabeads® (Dynal Biotech inc., Lake Success, NY), Abraxis (Abraxis, Warminster, PA), and Romer (Romer Labs® inc., Union, MO). The ABR and RM beads were available for each of the six serogroups. However, the DY had only four bead types available, targeting serogroups O26, O103, O111, and O145. Each bead type was distributed into micro-centrifuge tubes in the volumes of 50 µl for RM, and 20 µl for ABR and DY as directed by the manufacturers. After 6 h incubation, inoculated sample matrices and negative controls were vortexed and two 1 ml aliquots were transferred to separate micro-centrifuge tubes containing a single bead type. Tubes were rotated on a sample mixer (Dynal Biotech) for 15 minutes at 42 rpm at room temperature. Tubes were then transferred to the Magnetic Particle Concentrator (MPC®-S, Dynal Biotech). After shaking for 5 min, the supernatant was decanted and discarded. The magnets were removed and beads were washed with 1 ml sterile PBS + 0.05% Tween 20 (EM Science, Gibbstown, NJ) and shaken manually for 1 min. Washing steps were repeated 3 times. After the final wash, beads were suspended in 100 µl of PBS+0.05% Tween 20, spread-plated on EMB (Becton Dickinson) with VCC, and incubated for 16 h at 37°C.

Multiplex PCR (mPCR) assay

From the EMB-VCC plates, three colonies were selected for serogroup confirmation by multiplex PCR (mPCR). Prior to mPCR, DNA was extracted from each of the three colonies where the selected colonies were added to a 40 µl mixture of tris EDTA buffer (TE buffer, Amresco) and proteinase K (5 mg ml^{-1}, VWR, Radnor, PA) and incubated at 55°C for 10 min followed by 80°C for 10 min (Isiko et al., 2015). Lysates were stored at -20°C and used in subsequent mPCR as a DNA template.

Primers were designed to target the O antigen-specific polymerase gene *wzy* from six common non-O157 STEC serogroups (O26, O45, O103, O111, O121, and O145). Primers for the conserved gene *mdh* were included as a positive control. To facilitate easier distinction of the amplicons under standard electrophoretic conditions, two separate primers mixes were created. Both primer mixes contained 10 µM of each primer. Primer mix #1 contained the primers for *mdh*, O45, O103, and O121, while primer mix #2 contained the primers for *mdh*, O26, O111, and O145 (Table 2). Each 25-µl PCR sample contained 2.5 µl of 10× Ex Taq buffer (TaKaRa Bio Inc., Otsu, Japan), 2.0 µl of a mix containing a 2.5 mM concentration of each dNTP, 0.5 µl of either primer mix #1 or #2, 0.5 U Ex Taq HS (TaKaRa Bio Inc.), 1 µl of DNA template, and 18.9 µl of distilled water. Amplification utilized 35 cycles of 94°C for 30 s, 51°C for 30 s, and 72°C for 30 s. The resulting fragments were mixed with Envision dye (Amresco, Solon, OH) and loaded into a 2% agarose gel with a 100 bp ladder and run for 45 min at 100 volts. Gels were visualized on a gel imager (Alpha Innotech, San Leandro, CA) using FluorChem software (Alpha Innotech). Performance of the mPCR was verified using the 24-isolate non-O157 set from the STEC Center (http://shigatox.net/new/reference-strains/non-o157-group.html).

Data analysis

Enrichments with the 5 different matrices were inoculated with the 6-strain mixture 3 independent times. From each replicate of an enrichment, 2 subsamples (technical replicates) were used with each of the 16 bead types. Each technical replicate for each bead type was plated onto EMB agar and 3 colonies were classified with the mPCR. A total of 18 colonies were assessed with mPCR for each bead type in each matrix. The percent correct identification was calculated as the number of colonies with the serogroup matching the target serogroup for the bead divided by 18 and then multiplied by 100. The total number of

colonies tested with the mPCR after IMS extraction from each matrix for each bead and serogroup was 1,440 (16 total bead types x 3 biological replicates x 2 technical replicates x 3 colony picks x 5 matrices). To compare recovery of the correct serogroup from a particular bead type across matrices, odds ratios (OR) and 95% confidence intervals were calculated using the ratios of correctly recovered colonies in sterile feces, non-sterile feces, ground beef, and lettuce compared to the ratio of correctly recovered colonies in PBS (Table 3).

Table 2. PCR primers used for molecular O-typing

Primer mix	Primer name	Primer sequence	Amplicon
1 & 2	mdh_F41	AGGCGCTTGCACTACTGTTA	835 bp
	mdh_R875	AGCGCGTTCTGTTCAAATG	
1	wzy45_F188	ATCGCGTTCGTCTGGATGAAAT	443 bp
	wzy45_R630	AGCGCCCCTGATATCTCCTACAG	
	wzy103_F929	CCCCGCGGGTATTTGCTAT	184 bp
	wzy103_R1112	TCGTATGCGTTCGTTCTAAGATAA	
	wzy121_F306	TACAGCCGGTAGTGTTGAAAGGAT	626 bp
	wzy121_R931	CGCCCGTGTTAATATTCCAAGTC	
2	wzy26_F223	TTAGGCGGTACCCATGAAGTCA	242 bp
	wzy26_R464	GGTGCCATAAAGACAAAACAAAGA	
	wzy111_F495	TTCCGTAATTTGCATCCTGATAC	549 bp
	wzy111_R1043	TTTGCAAATCCATAAACAACTCC	
	wzy145_F754	ATGGGCAGTATCTCTGGTATTGAA	334 bp
	wzy145_R1087	TTGAAAGCCCGGATATTAGGAA	

Results and discussion

STEC growth in enrichment broth

Prior to evaluation of the IMS beads, growth of each strain individually in the modified EC (mEC) enrichment broth was quantified, verifying that each strain could grow to similar levels over the 6 h period (Table 1). While TSB and modified TSB are common enrichment broths for STEC, mEC was selected for use as the lactose and bile salts present in EC broth were found to be more suitable for enrichment of strains representing 8 serogroups of STEC from cattle feces compared to enrichment in TSB (Stromberg, Z.R. et al., 2015). To determine if strains could out-compete each other when grown together in mEC, the proportion of each strain present after 6 h enrichment was determined using a serogrouping mPCR. In the mixture, serogroups O45, O121, and O103 were present in slightly higher proportions that serogroups O26, O111, and O145 (Table 1). Isolates being unable to grow at equal rates and out-competed can be a concern when using IMS for detection. In the future, it may be necessary to use a specific selective enrichment media for the isolation of some serogroups.

IMS recovery from mEC enrichments with PBS

The ability of each type of IMS bead to recover the targeted serogroup was first evaluated for the mixture of 6 strains enriched in mEC in the presence of PBS as the sample matrix. The percent correct identification was calculated for each of the 16 bead types (Fig 1a). For some serogroups, percent correct identification was low, such as 5.6% for ABR O45, 27.8% for ABR O111, 11.1% for DY O111, 5.6% for ABR O145, and 33.3% for DY O145. The lower percentages for O111 and O145 could have been due to the lower proportions of these serogroups seen when enriched in the mixture (Table 1), though RM O111 and O145 had 100% correct identification. Cross-reactivity amongst the strains did

occur, with O103 and O121 commonly returned for ABR O45, and O111 commonly returned for ABR O145. With the absence of a complex matrix and competing native microbiota, recovery from PBS was used to determine the baseline for evaluation of IMS detecting these six serogroups in other matrices.

Percent correct recovery from enrichment with matrices

With the addition of cattle feces, both sterile and non-sterile, beads targeting serogroups O26 and O45 had the highest rates of correct identification for both matrices, while O111, O121, and O145 were less accurately recovered (Fig 1b). For serogroup O103, the percent correct identification was higher in sterile feces compared to the non-sterile feces (Fig 1b). In particular, O145 had the lowest rates of recovery in these two matrices, with serogroups O121 and O45 commonly returned for the O145 beads in sterile feces. RM beads had the highest rates of correct identification for both matrices compared to DY and ABR beads (Fig 1b). With the addition of food matrices, lettuce or ground beef, the percent correct identification decreased across bead types, with RM having the highest percent correct identifications with >80% for all serogroups from ground beef (Fig 1c). Most of the 0% correct identifications were seen in the lettuce and ground beef matrices with O111, O121, and O145 having the lowest rates of recovery for ABR and DY beads. Serogroups O45, O103, and O121 were most common wrongly identified by ABR and DY O111 beads.

Table 3. Odds ratios and 95% confidence intervals for recovery of each serogroup and IMS bead in each matrix compared to recovery from PBS.

		Sterile Feces		Non-Sterile Feces		Ground Beef		Lettuce	
Serogroup	**Bead**	**OR[a]**	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**
O26	ABR	5.6	(0.25, 126)	0.33	(0.054, 1.96)	**0.06**	(0.011, 0.37)	**0.036**	(0.0057, 0.23)
	RM	8.4	(0.4, 175)	0.4	(0.082, 1.94)	1.6	(0.23, 10.9)	**0.2**	(0.043, 0.94)
	DY	12	(0.57, 231)	4.9	(0.49, 48.6)	**0.057**	(0.011, 0.302)	**0.18**	(0.042, 0.78)
O45	ABR	**290**	(17, 5000)	**432**	(16, 11320)	0.32	(0.012, 8.27)	3.4	(0.32, 36.3)
	RM	0.18	(0.008, 3.9)	1	(0.019, 53)	1	(0.019, 53.1)	**0.027**	(0.014, 0.52)
O103	ABR	0.32	(0.012, 8.3)	**0.014**	(0.0007, 0.27)	0.12	(0.0057, 2.5)	0.12	(0.0057, 2.5)
	RM	0.18	(0.008, 3.9)	0.066	(0.0034, 1.3)	1	(0.019, 53.1)	0.32	(0.012, 8.3)
	DY	2.3	(0.36, 14)	0.29	(0.067, 1.2)	0.45	(0.1042, 1.9)	**0.18**	(0.042, 0.78)
O111	ABR	0.36	(0.084, 1.5)	**0.14**	(0.033, 0.63)	**0.017**	(0.0017, 0.17)	**0.0084**	(0.0004, 0.17)
	RM	0.087	(0.0043, 1.8)	0.32	(0.012, 8.3)	0.12	(0.0057, 2.5)	**0.011**	(0.0006, 0.22)
	DY	0.18	(0.008, 3.9)	0.18	(0.008, 3.9)	0.47	(0.039, 57)	0.18	(0.008, 3.9)
O121	ABR	**0.23**	(0.054, 0.97)	**0.18**	(0.042, 0.783)	**0.082**	(0.017, 0.39)	**0.0084**	(0.0004, 0.17)
	RM	2.1	(0.18, 26)	0.44	(0.069, 2.8)	0.19	(0.034, 1.1)	0.44	(0.069, 2.8)

[a] bolded values denote that odds of recovery are significantly different ($p < 0.05$) from recovery in PBS.

Table 3. Odds ratios and 95% confidence intervals for recovery of each serogroup and IMS bead in each matrix compared to recovery from PBS (continued).

		Sterile Feces		Non-Sterile Feces		Ground Beef		Lettuce	
Serogroup	**Bead**	**OR[a]**	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**
O145	ABR	0.32	(0.012, 8.3)	3.4	(0.32, 36)	0.32	(0.012, 8.3)	0.32	(0.012, 8.3)
	RM	**0.0007**	(0, 0.04)	0.052	(0.0027, 1)	1	(0.019, 53)	**0.033**	(0.0017, 0.64)
	DY	0.57	(0.13, 2.5)	1	(0.25, 3.9)	0.052	(0.0027, 1)	0.52	(0.0027, 1)

[a] bolded values denote that odds of recovery are significantly different ($p < 0.05$) from recovery in PBS.

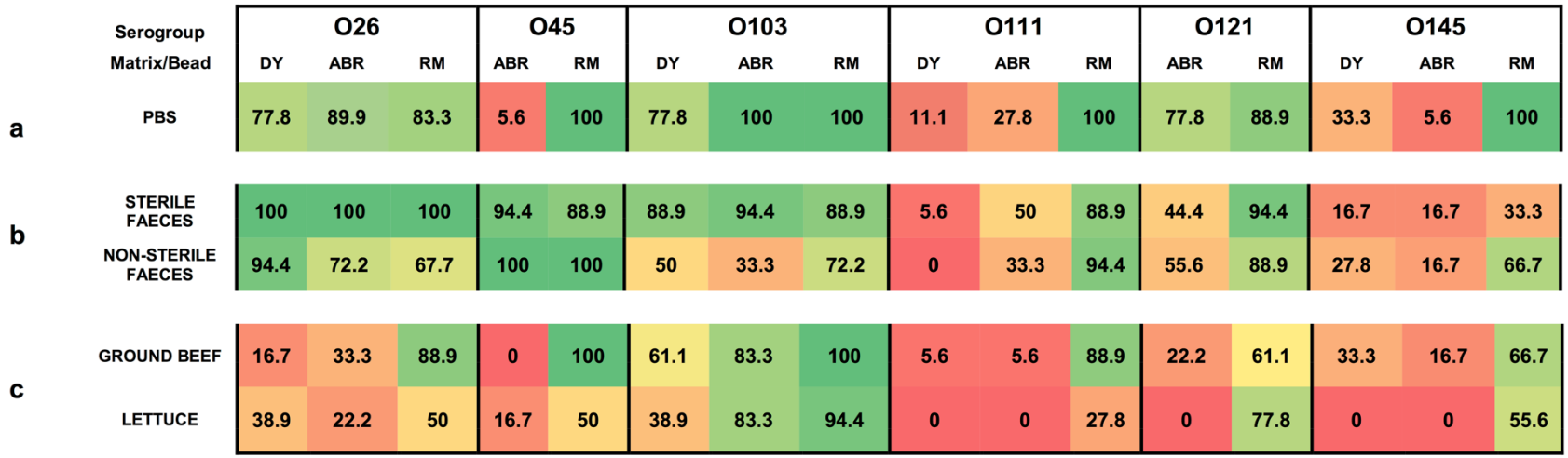

Figure 1. Heat map containing the percent correct identification for each matrix, serogroup, and bead manufacturer. Percentages are calculated from the PCR results from *E. coli* colonies selected for confirmation. The manufacturers are labeled as such: Dynal: DY, Abraxis: ABR, and Romer: RM. Sample matrices tested include: (a) PBS, (b) sterile and non-sterile cattle fecal samples, (c) uncooked ground beef, and lettuce.

Comparison of recovery within a bead type across matrices

To compare recovery of the correct serogroup for a given bead type across the diversity of matrices, odds ratios (OR) were calculated, providing a likelihood that recovery would be the same, enhanced, or diminished in each complex matrix compared to recovery from PBS (Table 3). Enrichment in the presence of sterile cattle feces, representing a complex matrix without native microbiota, did not significantly affect recovery for the majority of the bead types. ABR O121 and RM O145 were significantly less likely to recover the correct serogroup compared to their performance in PBS. ABR O45 was significantly more likely to recover the correct serogroup compared to its performance in PBS. Enrichment in the presence of non-sterile cattle feces significantly impacted recovery for 4 of the bead types. ABR O103, O111, and O121 were significantly less likely to recover the correct serogroup from enrichment with cattle feces. Like sterile feces, ABR O45 was significantly more likely to recover the correct serogroup in non-sterile feces compared to its performance in PBS. For O45 ABR, the percent correct identification from the fecal samples was greater than from PBS. In PBS for ABR O45, serogroups O103 and O121 were commonly returned. With the addition of the fecal matter, O103 and O121 recovery was lower, which may indicate that ABR O45 was more effective in sterile feces as O121 and O103 may have been negatively influenced by components of the fecal material.

For bead types ABR O26, DY O26, ABR O111, and ABR O121, enrichment in the presence of ground beef led to significantly lower likelihood of correct recovery. Enrichment with lettuce led to the highest number of bead types with significantly reduced recovery, with only 7 bead types having similar levels of recovery in this matrix compared to recovery from PBS (Table 3). These results suggest that the nature of the sample matrix may be impacting the efficacy of IMS-based recovery. Matrices have added complexity that range

from varied physical structures to antimicrobial properties, as well as native microbes, all of which could limit the efficacy of IMS. Interference amongst native bacteria and the IMS beads may be a factor limiting the success of IMS in these matrices. Many sample types will have competing microbiota that are similar enough to the target STEC that the selectivity of the enrichment media is less impactful and could lead to competing interactions amongst the IMS bead antibodies and the non-target bacteria. Alternatively, it has also been observed in another study that antibodies targeting the O-antigen of STEC can cross-react with other serogroups leading to non-specificity (Stromberg, L.R. et al., 2015).

Overall, the RM beads were more likely return the correct serogroup for the antibody-labeled bead, with similar recovery from PBS across feces and ground beef for 5 of the 6 serogroups. DY beads appeared to be the least reliable, having 0% correct identifications, including O111 for non-sterile feces and lettuce and O145 for lettuce. Poor recovery of O111 and O145 with DY beads was observed by Verstraete et al. where they reported that even in pure cultures, strains of serogroup O111 and O145 were recovered with very low efficiency by DY beads (Verstraete et al., 2010). Specifically, for serogroup O111 it has been observed in other studies that recovery was poor and that this may be due to low-affinity between the antibodies on the IMS bead and the O-antigen (Noll et al., 2015). Examining a larger sample of isolates from each serogroup may uncover if the low correct identification is due to a specific isolate or is a common trend across multiple strains of the same serotype.

In conclusion, IMS-based recovery of the 6 serogroups was variable, and dependent on the sample matrix. RM beads were the most effective in detecting the six STEC in various matrices used in this study. Serogroups O26, O45, and O103 had the highest number of 100% correct identification across all matrices. Of all the matrices, ground beef

and lettuce proved to be the most difficult to use IMS as an isolation method since these two contained the lowest percent correct identification for all the O-types. Interferences in the feces, ground beef, and lettuce may be impacting the efficacy of IMS in those matrices. These results indicate that procedural modifications are needed to broadly use IMS as a reliable method of detecting non-O157 STEC in complex matrices.

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3. IMPACTS OF OSMOTIC STRESS, LIMITED NUTRIENTS, AND LETTUCE EXUDATES ON EHEC ATTACHMENT TO A STAINLESS-STEEL SURFACE

Abstract

Foodborne pathogens, like Enterohemorrhagic *Escherichia coli* (EHEC), are a concern during food processing. Under these circumstances, bacterial cells will readily become attached to food processing surfaces like stainless steel (SS). This is a complex response that can lead to reoccurring contamination and makes these pathogens difficult to eliminate. What environmental conditions influence or trigger initial attachment processes requires more elucidation. The goal of this work was to determine if limited nutrients (PBS), osmotic stress (4.5% NaCl), or lettuce exudates would lead to changes in EHEC attachment on a SS surface. Reversibly (associated) and irreversibly attached cells were recovered over a two-hour period from SS coupons and comparisons were made between serogroups O26 and O157, across time, and among different environmental conditions. We discovered that the 4.5% NaCl treatment led to significantly less association of EHEC to SS than the three other conditions after 30 min ($p<0.01$). In the lettuce exudates, what appeared to be a significant difference in attachment between serogroups ($p<0.01$) was revealed to be significant variation among strains within serogroups. With these results, the differences amongst strains indicates that attachment responses to the tested conditions are strain-specific and this should be considered in future studies on EHEC attachment.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is a bacterial pathogen responsible for a significant number of foodborne illnesses (Scallan et al., 2011). While EHEC typically resides in the gastrointestinal tract of warm-blooded animal reservoirs, such as cattle

(Naylor et al., 2003), it is capable of surviving outside of the host once shed into the environment (Wang et al., 1996). Through many vehicles, such as from contaminated water or manure, EHEC is able to make contact with produce in the pre-harvest environment. This has led to a notable number of outbreaks linked to fresh produce, especially leafy greens (Painter et al. 2013, Scallan et al., 2011). Once present on produce, these pathogens are capable of surviving prolonged periods on and inside the plants (Islam et al., 2004, Solomon et al., 2003, Solomon et al., 2002), on machinery used to process produce (Kusumanigrum et al., 2003), and eventually reach a consumer who may become infected after ingesting contaminated foods. Throughout this process, EHEC will encounter and must overcome many obstacles, or environmental factors, that can involve desiccation, nutrient limitation, sanitizer stress, or osmotic pressure stress. When possible, EHEC will employ sets of responses to cellular damage and metabolic disruption caused by changing environmental conditions (Lajhar et al., 2017; Storz and Hengge 2011).

Survival of microbes in new environments is ensured by phenotypic responses that provide some advantage in that environment. One phenotype of interest is bacterial attachment. This is because attachment, or lack thereof, dictates if bacteria will remain in the environment. Attachment ranges from cells being reversibly associated to a surface to irreversible adherence. Initially, bacterial cells may come in contact with a surface and become reversibly attached, or associated. This process is facilitated by physical structures on the outside of the bacteria, such as curli and flagella, that interact with other bacterial cells and the surfaces on which they may land (Pawar et al., 2005, Shaw et al., 2011). Properties of the surface, such as hydrophobicity and van der Waals forces, also influence these interactions (Nesse et al., 2014; Razatos et al., 1998). As this is a transient state, bacteria may disassociate and disperse, or they may transition into irreversible attachment.

This form of attachment is characterized by bacterial cells resisting physical removal from the surface. Once irreversible attachment occurs, bacteria can aggregate into a biofilm surrounded by extracellular polymeric substances, like cellulose, which help to protect and adhere the microbes in one location (Saldana et al., 2009). As more cells aggregate, they ultimately form a mature biofilm. These processes of attachment and biofilm formation are of concern since these responses facilitate persistence in an environment and resistance to commonly used sanitizers (Vestby et al., 2009; Wang et al., 2012). Within the food processing environment, this is an issue since biofilms are difficult to remove and cells can sporadically break away from a biofilm and spread, potentially contaminating more food and food-contact surfaces over time. Ultimately, well-informed methods of interrupting attachment could be important in controlling EHEC.

In previous studies, the roles of flagella and curli during attachment have been explored (Pawar et al., 2005, Shaw et al., 2011). Specifically, when key genes associated with these extra-cellular structures, like *csgD*, *csgA,* or *fliC*, are knocked out, attachment is significantly reduced (Saldana et al., 2009; Sharma et al., 2016). However, attachment is not eliminated, meaning additional factors are influencing this phenotype. There is also evidence that extrinsic factors like temperature and osmolarity impact expression of curli and flagella (Brombacher et al., 2006; Kamp and Higgins 2011; Shin and Park 1995). While these studies have provided insights into molecular structures involved in attachment and environmental conditions that influence their expression, there is little information on produce processing related environmental stresses that affect the initial stages of attachment. Additionally, there is a gap in making comparisons amongst EHEC serogroups. In this study, our aim was to investigate the effects of nutrient limitation (PBS), osmotic stress (4.5% NaCl), and an alternative carbon source (lettuce exudates) on

the association (reversible) and attachment (irreversible) over a two-hour period of eight strains of EHEC. We sought to compare serogroups of EHEC so four of these strains belonged to O26, and the other four to O157.

Materials and methods

Culture preparation

The EHEC strains used in this study are listed in table 4. These strains were prepared from -80°C freezer stocks using Luria-Bertani agar (LBA). After a 24 h incubation at 37°C, single colonies were picked and used to inoculate 5 ml of 0.1% glucose defined minimal media (GDMM, Neidhardt et al. 1974). This broth was incubated with shaking for 20-24 h at 37°C. Finally, 0.5 ml of the overnight culture was transferred to 50 ml of GDMM and was incubated at 37° with shaking until the culture had reached an OD of 0.6-0.7. This took approximately 3 h post-transfer in the GDMM. This culture was centrifuged at 6,000xg for 10 min, the media was removed, and the pellet was suspended in phosphate buffered saline (PBS) to achieve a concentration of 10^4 CFU/ml for use in attachment assay. Every time cultures were prepped for the assay the concentration was confirmed via plate count.

Table 4. Strains used in this study.

Strain	Serotype	Source
EDL-933	O157:H7	Hamburger
93111	O157:H7	Human
DA-10	O26:H11	Human
DA-22	O26:H11	Human
Sakai	O157:H7	Human
MI03-4	O26:H11	Human
MI06-63	O157:H7	Human
Jimmy John's	O26:H11	Human

Environmental condition and attachment surface preparation

The environmental conditions used in the attachment assay were NaCl, lettuce exudates, PBS (Amresco, Solon, OH), and GDMM. Lettuce exudate was collected from locally bought romaine lettuce plants that were juiced using a Breville Juicer (Breville, Melbourne, Australia). The exudate was centrifuged multiple times to eliminate all visible plant material and aliquots of liquid lettuce exudates were stored in the -20°C freezer until needed. A stock of exudate solution at 2% was prepared using PBS. NaCl was suspended in PBS to a working concentration of 4.5%. The NaCl and exudate solutions were filter sterilized before use in the assay.

A stainless-steel (SS) surface in the form of 2 cm^2 coupons was used to assess EHEC attachment in the tested environmental conditions. These coupons were initially washed with agitation in acetone for 10 minutes, followed by rinsing in distilled water. Next, a laboratory detergent (Liqui-Nox, Alconox Inc.) was used to further wash the coupons for another 10 minutes with agitation. Coupons were rinsed thoroughly in distilled water to remove all detergent and a final rinse in RO-distilled water was used before sterilization via autoclaving.

Attachment assay

The attachment assay was conducted in 6-well culture plates (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, CHN). In each well, a sterile SS coupon was placed using sterilized forceps and 3.2 ml of the environmental condition was added. Before addition of inoculum, concentrations of the test conditions were 1x PBS, 4.5% NaCl, and 2% lettuce exudate. Inoculation involved adding 0.8 ml of the prepared culture, which initiated the timing of the assay. The inoculated 6-well plates were placed on a belly dancer (The Belly Dancer®, Stovall Life Sciences Inc.) for gentle rotation on speed control setting 4 while at

room temperature (~25°C). The assay was prepared so that samples could be taken at 0, 5, 15, 30, 60, 90, and 120 min post-inoculation. Sampling began by removing 100 ul of the solution suspension from the well for dilution and plating. This was necessary to confirm differences in association or attachment were not due to significant differences in bacterial concentrations. Next, the SS coupons were removed using sterile forceps. A brief rinse (2-3 sec) in sterile PBS was used to remove any residual liquid from the environmental condition. Coupons were then placed in 50 ml centrifuge tubes containing 10 ml PBS + 0.5% Tween and an initial 20 sec wash was done to remove loosely attached, or associated, bacteria. Coupons were transferred again to another 50 ml centrifuge tube which contained PBS and acid-washed glass beads. These were vortexed for 2 min to remove attached bacteria from the coupons. From these two 50 ml tubes, 100 ul samples were taken for diluting and plating on LBA. Plates were incubated overnight at 37°C and enumerated using QCount (Color QCount, Spiral Biotech Inc., Norwood, MA).

Statistical analysis

In this study, each strain used was grown in triplicate (biological replicates) with two technical replicates done for every biological replicate. For each of the eight EHEC strains, three different samples were taken (suspension, associated, attached) for each time point in every test condition (GDMM, PBS, NaCl, lettuce exudate). This allows for comparison amongst strains, across the time period of the assay, and with each test condition.

Plate counts collected from the suspension were log transformed and averaged for each strain, test condition, and time point. The bacterial counts from the associated and attached samples were converted from CFU/ml to CFU/cm^2 and the values were log transformed. The goal was to then determine how the environmental conditions impacted

the association or attachment for each strain over time. This was done by calculating the proportion of associated/attached cells of the total observed suspension of cells ({Associated or Attached Count/Media Count}*100). Using Prism 8 (GraphPad, San Diego, CA), these proportions were compared using a two-way ANOVA and any significantly different proportions amongst treatments were identified (Figure 2, 3). Further, a nested t-test was used to observe any nested effects occurring within the data for serogroup attachment in lettuce exudates (Figure 4). A simple linear regression was used to determine if there were any correlations between attachment and association in each condition for all eight strains (Figure 5).

Results

Limited variation between proportions of association and attachment of serogroups

During the assay, a representative sample from the cells suspended in the media was collected for enumeration, which served as a reference for future comparison. There was no significant change in cell numbers in these media counts over time (Table A1). Therefore, the number of cells in the media was used to calculate the proportion of cells in each state over time. The cells associated with and attached to the stainless-steel surface were enumerated in parallel. Proportions of the associated and attached cells were calculated for each strain in each condition across all time points and are graphed in figure 2.

Initially, the proportions of associated cells were compared among the test conditions to determine if there were any significant differences between serogroups. For GDMM, PBS, and lettuce exudates, proportions of associated cells increased by approximately 5% over the 120 min incubation period (Figure 2a). Among these three conditions, there was no point where one serogroup consistently had significantly higher

proportions than the other. At a few time points, like 15, 30, and 60 min, a two-way ANOVA identified some O26 strains had slightly yet significantly higher proportions than O157 strains in lettuce exudates ($p<0.001$ for 15 and 60 min, $p<0.01$ for 30 min). But overall, these differences in association between serogroups was limited to these points.

4.5% NaCl influences association to stainless steel for both O157 and O26

In the 4.5% NaCl condition, a distinct pattern emerged for both serogroups after 30 min (Figure 2a). While the proportion of associated cells in other conditions continue to rise, the proportions in the NaCl condition do not change over the entire incubation period with the O157 strains averaging 41.8% ± 3.9 and the O26 strains averaging 43.5% ± 3.2. While the proportion of associated cells are similar between the serogroups, they are significantly lower than the proportions of associated bacteria recovered from the other three conditions. This was clear from O157 association proportions at 59.5% ± 3.67 in GDMM, 54.8% ± 4.19 in PBS, and 59.7% ± 2.09 in lettuce exudates and O26 association proportions at 61.2% ± 2.53 in GDMM, 58.9% ± 3.73 in PBS, and 62.2% ± 2.59 in lettuce exudates ($p<0.0001$). This signals that NaCl is in some way negatively affecting the initial association of EHEC to this SS surface.

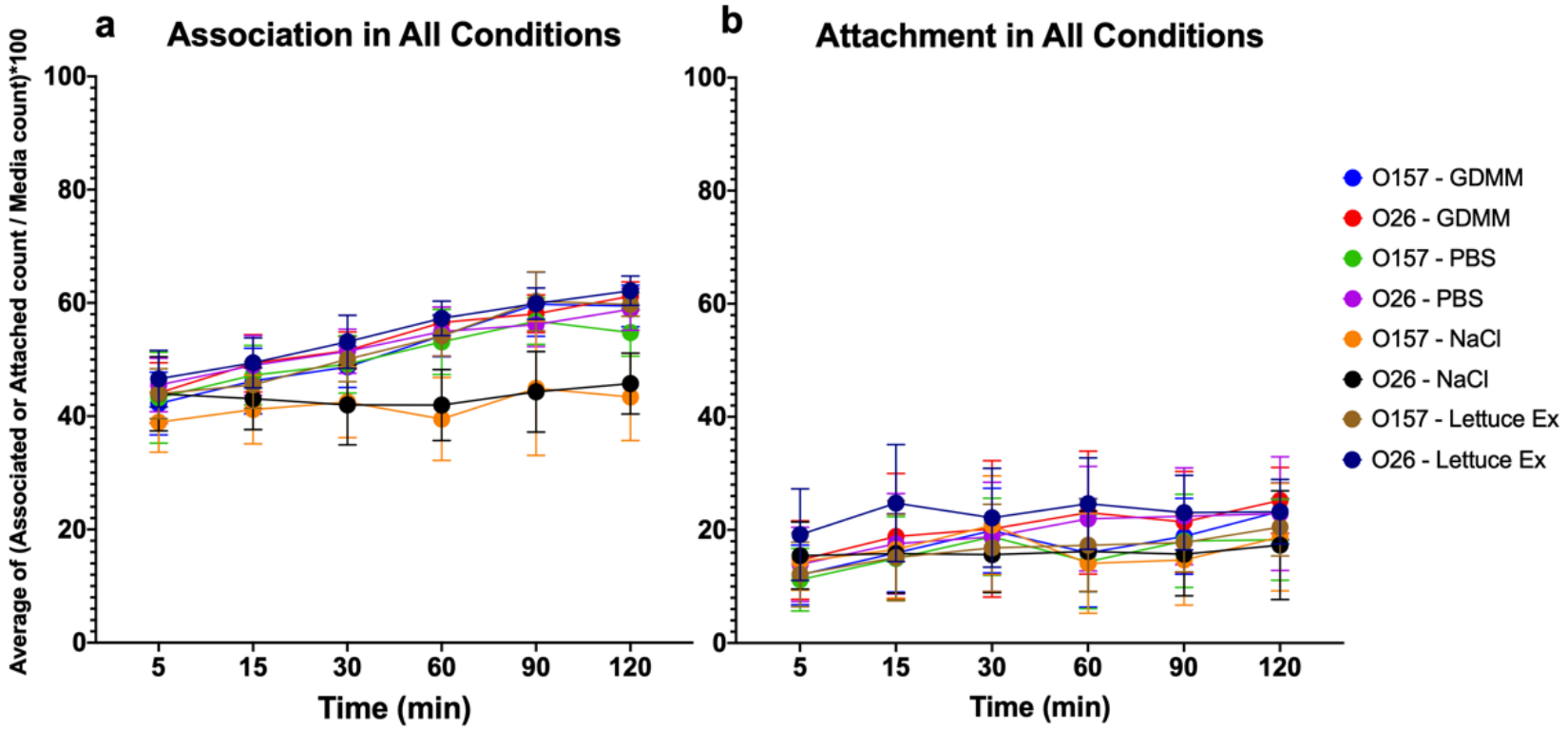

Figure 2. Proportion of (a) associated and (b) attached cell counts of EHEC O157 and O26 on stainless steel coupon in GDMM, PBS, 4.5% NaCl, and lettuce exudates over 120 min incubation. These points represent the averages of three biological replicates from eight strains (four belonging to each serogroup) with error bars signifying SD.

Attachment variation in lettuce exudates is driven by strain, not serogroup

Proportions of attached bacteria were determined in the same manner as the associated bacteria. Here, an initial two-way ANOVA highlighted significant differences between serogroups in lettuce exudates, GDMM, and PBS at several time points (Figure 3). However, further nested t-test analysis identified that this variation was due to significant differences among the strains within each serogroup rather than overall differences between the serogroups (Figure 4). At each time point, the nested t-test identified that no serogroups were significantly different. When strains were treated as a subgroup of serogroup, the pattern arose of some attached proportions being significantly different. For example, at the 120 min time point, the Jimmy John's strain attached proportion (17.1% ± 2.21) was significantly lower ($p<0.05$) than DA-10 (27.2% ± 1.60) and DA-22 (28.8% ± 1.35). The trends seen here, where variation is amongst strains and not serogroups, underlines the need to directly assess dynamics of attachment due to strains.

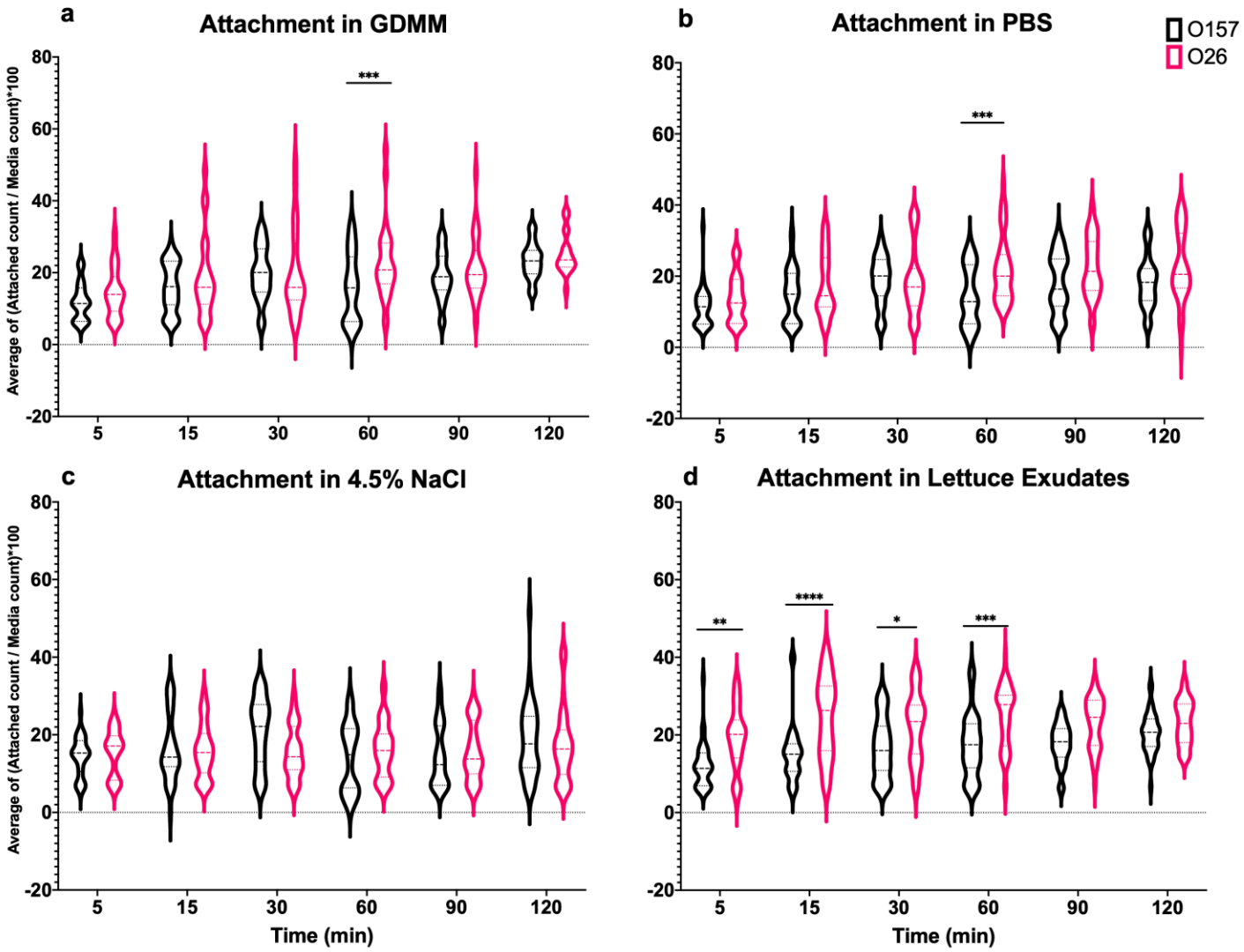

Figure 3. Comparison of EHEC serogroups attachment in (a) GDMM, (b) PBS, (c) 4.5% NaCl, and (d) lettuce exudate across time. Data is from eight EHEC strains (four belonging to each serogroup) and is an average of three biological replicates. ($p<0.01$ (*), $p<0.001$ (**), $p<0.0001$ (***), $p<0.00001$ (***)

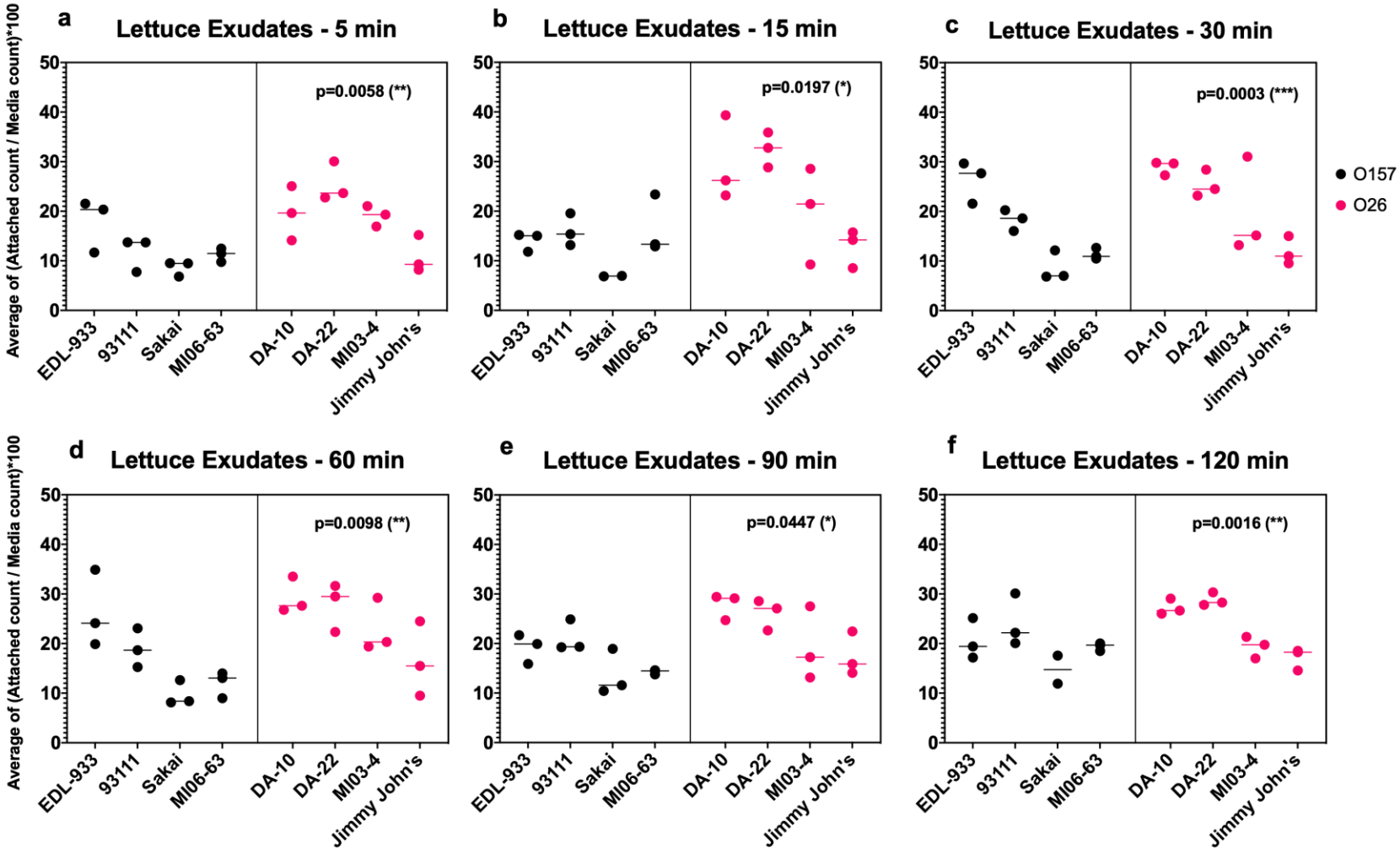

Figure 4. Nested analysis comparing individual strains within serogroups at (a) 5 min, (b) 15 min, (c) 30 min, (d) 60 min, (e) 90 min, and (f) 120 min post inoculation. Each point represents a biological replicate of the strain, and a nested t-test determined significant differences. ($p<0.1234$ (ns), $p<0.0332$ (*), $p<0.0021$ (**), $p<0.0002$ (***))

Correlations between association and attached proportions in GDMM, PBS, 4.5% NaCl, and lettuce exudates

Next, a simple linear regression was applied to the associated and attached proportions in order to identify if there was any correlation between these two factors. All strains were combined to make correlations solely between associated and attached proportions in each condition. Dots in figure 5 represent all strain attachment proportions across time and included is the line of best fit and r^2 values in each condition. Of all the conditions it appears that the lowest strength of correlation was observed in NaCl with an r^2 value of 0.2522. However, all conditions r^2 values are relatively close being greater than 0.25 and no higher than 0.3. While these regressions show that the variation in association proportions can explain for some of the variation seen in attached proportions, but the r^2 values indicate there are more explanatory variables.

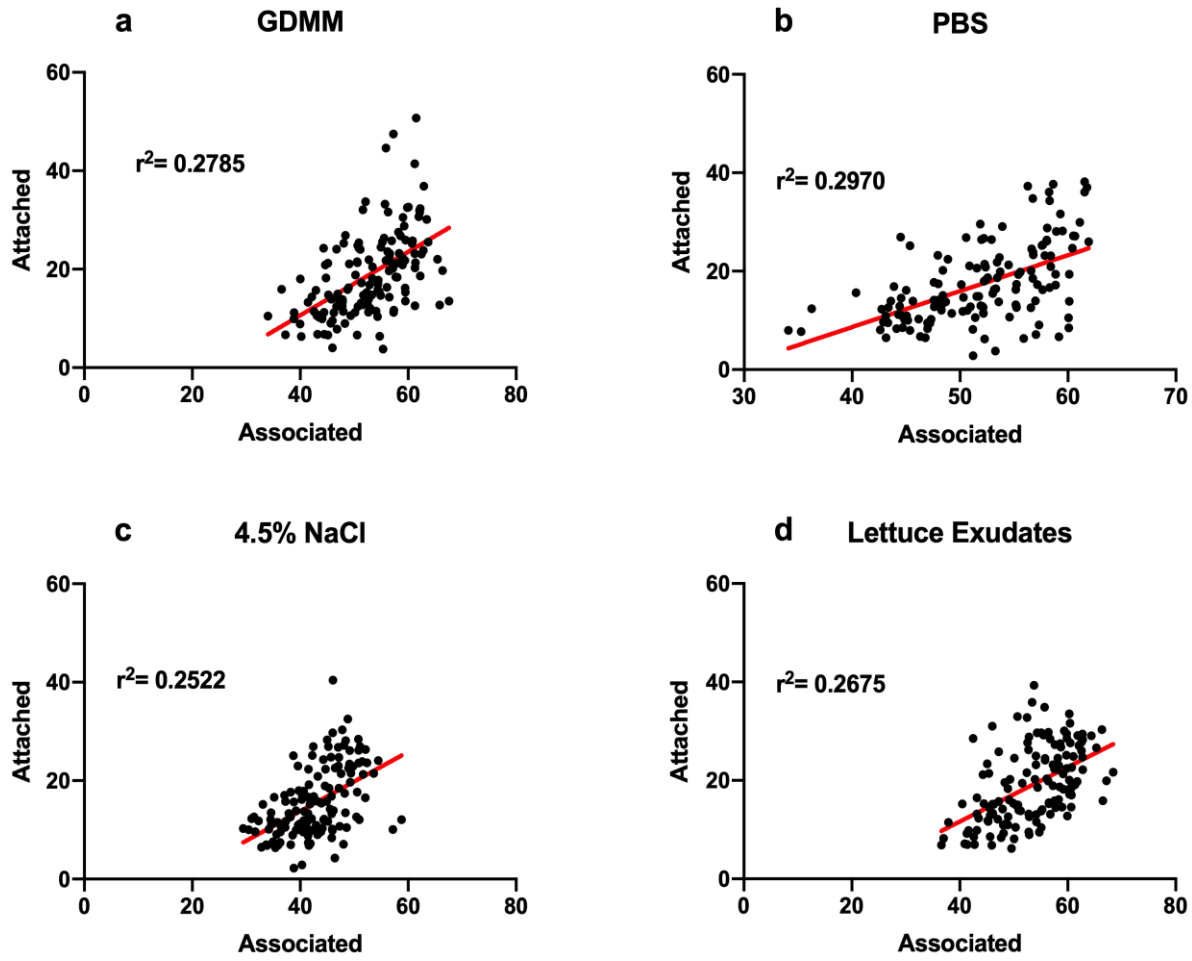

Figure 5. Simple linear regressions of attached and associated proportions in each condition: (a) GDMM, (b) PBS, (c) 4.5% NaCl, and (d) lettuce exudates. All three replicates for each of the eight strains across all six time points are included as individual dots in each graph.

Discussion

The effects of food processing-relevant environmental conditions on bacterial pathogen attachment to abiotic surfaces, like stainless steel, needs to be a better elucidated phenotype. This complex response of bacteria is influenced by environmental signals like temperature, osmotic pressure, pH, or even surface topography (Feng et al., 2014, Zulfakar et al., 2013). With that in mind, it is important to note that these are factors commonly present in the food processing environment. Here, if association to a food-contact surface

progresses to irreversible attachment, bacterial pathogens can remain on that surface and continue to be a food safety concern. This is why we wanted to uncover how specific environmental factors, which EHEC may encounter during food processing, impact attachment to a SS surface over time.

Initial association generally increases over time

With the attachment assay used in this study, we were first able to investigate initial association of EHEC to SS. Our analysis indicated that there was a steady increase in associated cells over time, but there were no significant differences amongst serogroups or between the GDMM, PBS, and lettuce exudate condition. This means the proportions of associated cells in these three conditions increased to similar levels at each sampling time. The same increase was not observed in the 4.5% NaCl condition. Overall, this phenomenon could be explained by the continuous settling of bacterial cells from suspension. Even with the gentle rotation of the 6-well plate containing the assay, cells will gradually drift down and settle on the SS coupon. This explanation is backed up by the lack of growth of the strains in the media (Table A1). Further experiments could expand on this by observing if cells retain their motility or if they have indeed become settled on the surface.

NaCl interrupts association of EHEC

As for association in the 4.5% NaCl, there is a deviation from this pattern. Overall there is no change in proportion of associated cells for both serogroups like was seen in the other conditions. But over time, the proportions of associated EHEC cells did not change in the NaCl like was seen in the other three conditions. With no significant decrease over time in the media, there must be some effect from the NaCl preventing association. Conversely, there was no paralleled decrease in attachment for EHEC in the 4.5% NaCl condition (Figure 2b). In fact, when comparing among all four conditions, there was no significant

differences in attachment observed. So, while association is lower in NaCl, this is not translating to a decrease in attachment. This may have to do with the hydrophobic properties and ionic charge of the surface and of the bacterial membrane. Previous work has identified that how the hydrophobic and electrokinetic nature of bacterial cell walls and abiotic surfaces can influence adhesion (van Loosdrecht et al., 1987a, van Loosdrecht et al., 1987b). In our assay, the NaCl may be interfering with the electrostatic properties of both the bacterial cell and the SS leading to the limited association. This does not explain why attachment is no different, so more work will need to be done to better understand this interaction.

Attachment not significantly impacted by any condition for all EHEC strains

While attachment did not vary amongst treatments, the same cannot be said about serogroups. With the 2-way ANOVA identifying significant differences in attachment between O26 and O157 strains, a deeper look was needed when the data were graphed (Figure 3). Initially, attachment of O26 appeared significantly higher in GDMM and PBS at 60 min and in lettuce exudates from 5 min to 60 min. After the nested t-test, it became clear that variation among strains was leading to the differences seen between serogroups. With that in mind, not enough is understood about how plant-derived materials, like the exudates here, affect EHEC attachment. Lettuce exudates provide EHEC with an alternative source of nutrients and plant-derived compounds have been observed to influence expression of genes related to carbohydrate metabolism, flagella, type 3 secretion system (T3SS), and chemotaxis (Bufe et al., 2019; Kyle et al. 2010). In these studies, researchers worked to understand changes in survival and gene expression in lettuce exudates, which was a simulated result of mechanical damage to plant tissue. Since these compounds would be easy to encounter in produce processing, the previous works in concert

with these data highlight an important gap. If lettuce exudates are leading to higher expression of genes known to be involved in attachment, it is likely leading to increased attachment when EHEC encounters damaged leafy green tissue. Another point of interest in this study was that the simple linear regression did not identify strong correlations between associated and attached proportions in the lettuce exudates. With these data from Bufe et al. and Kyle et al., the evidence points to increasing association and eventual attachment under these conditions due to increased expression of key structural genes. But this was not observed in this study. The increases seen in associated counts could only account for 26.8% of the variation in the attachment proportions in lettuce exudates (Figure 5). Other variables not accounted for here must be playing important roles in this phenotype. One particular piece that this study did not involve was the inclusion of native microbes that would be present under these same conditions. Since under natural conditions these pathogens would interact with native microbiome species, the fact that those populations are not present is likely impacting association and attachment. Further work could investigate expression of attachment-associated genes over time and under different exudate conditions simulating what happens during processing to better understand the responses seen in this work.

In conclusion, we assessed the impacts that GDMM, PBS, 4.5% NaCl, and lettuce exudates had on EHEC attachment to SS. Association varied only with the GDMM, PBS, and lettuce exudate proportions clustering higher than the NaCl proportions, with no differences observed between serogroups. Attachment variation required a nested analysis, which identified that variation from strains within serogroups caused significant differences at certain time points within GDMM, PBS, and lettuce exudates. In the future,

work exploring expression of attachment-related genes changing over time and in different lettuce exudates could shed more light on this important phenotype.

Funding acknowledgement

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4. SURVIVAL OF EHEC, *SALMONELLA*, AND *LISTERIA MONOCYTOGENES* IN HIGH- AND LOW-NUTRIENT SOIL EXTRACTS

Abstract

Survival of foodborne pathogens in agricultural water and soils is an important area of research due to the nature of these matrices as vehicles for contamination of the pre-harvest environment. While studies have revealed the impacts of biological soil amendments and specific soil chemistry components on pathogen survival, there is a need to further determine the impacts from chemical composition and native microbes on pathogen survival. Additionally, this should be assessed with multiple pathogens to see if impacts differ. We conducted two studies that looked into two chemically distinct extracts defined as low- and high-nutrient and what impact they had on the survival of enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella enterica*, and *Listeria monocytogenes*. The first phase established the pattern of survival was poorer in non-sterile low-nutrient extracts, while all pathogens experienced growth ranging from 1 to 2.4 Log CFU/ml over the two-week incubation. Chemistry analysis identified that total N, total P, and total organic carbon (TOC) were significantly higher ($p<0.01$). We expanded on this by assessing chemistry and survival together over time in order to make correlations. The trend of high-nutrient extracts having larger concentrations of chemical components continued for several tests, except for Ca (ppm) where low-nutrient had the larger concentration. A multi-linear regression identifying only Ca correlating with *Salmonella* survival in high-nutrient extract. With these data in mind, more work involving microbiome profiling and different chemistry analyses would expand on what specific factors impact pathogen survival in soil extracts.

Introduction

In recent years, an increased number of gastroenteritis outbreaks have been linked to fresh fruits and vegetables. Enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella,* and *Listeria monocytogenes* are ranked among the top 5 foodborne pathogens with the highest rates of illness and hospitalization (Scallan et al., 2011), and a number of outbreaks associated with these pathogens have been attributed to consumption of fresh produce (Self et al., 2019; Turner et al., 2019; Krishanasamy et al., 2019). The transmission of human pathogens to fresh produce is complex and can occur in the pre-harvest environment via several different routes. Use of contaminated irrigation water, application of raw manure, direct fecal deposition by wildlife, and improper worker hygiene are a few ways in which produce could become contaminated in the field (Brandl 2006). Manure runoff from animal farms to crop fields and eventually to water sources can lead to the spread of enteric pathogens on the farm (Liu et al., 2018). Flooding events can also impact the spread of pathogens in the agricultural environment (Bergholz et al., 2016).

Variables associated with manure application as well as irrigation water use and spread of foodborne pathogens have been examined. The presence of biological soil amendments (manure/poultry litter) in soils leads to greater survival of foodborne pathogens such as *Salmonella* (Shah et al., 2019a). Manure type can impact survival of enteric bacteria in soils, where soils amended with poultry manure supported longer survival of *E. coli* compared to soils amended with dairy manure (Neher et al., 2019) Under conditions designed to simulate runoff events or standing water after heavy rain or flooding, biological soil amendments along with soil in the aqueous environment have been investigated (Neher et al., 2019; Shah et al., 2019b). In Neher et al., survival of *E. coli* was greater when nutrients were plentiful, and the system was devoid of native microbes.

Specifically, in these systems, higher levels of organic carbon, phosphorous, and nitrogen extracted from amendments and soil correlated with growth of these organisms (Neher et al., 2019; Shah et al., 2019b). Alternatively, low pH and competition for nutrients in agricultural soils have shown to negatively impact the survival of EHEC (Xing et al., 2018). Many of these observations of pathogen growth are from soil/amendment extracts that have been sterilized, removing the native microbes originating from the soil and amendment. In cases where non-sterilized extracts are used, pathogen growth is reduced or eliminated (Shah et al., 2019b), highlighting the potential role native soil microbes play in controlling growth of pathogens.

Data are needed to make science-based recommendations for manure application and agricultural water quality standards to reduce the potential for spread of human foodborne pathogens in the agricultural environment. While soil and water chemistry data have been collected for soil extracts inoculated with foodborne pathogens, these features have rarely been assessed together over time. The microbiome in these soils and extracts is dynamic, and the available carbon, phosphorus, and nitrogen in a system is likely to change over time due to metabolic activity of that microbiome. The native soil/water microbes are direct competitors for nutrients in these environments and are likely to influence pathogen behavior and survival. Accounting for both variables will allow development of models for prediction of pathogen persistence and spread in agricultural systems.

For this study, we evaluated the survival of EHEC, *Salmonella*, and *L. monocytogenes* in distinctly different soil extracts that can be found in agricultural environments. There has been previous work showing that each of these pathogens can survive for prolonged periods of time in water (Budzinska et al., 2011; Shah et al., 2019a; Wang and Doyle, 1998). In preliminary work, we collected samples from different

agricultural soil sources and conducted a soil chemistry analysis. Two of those soil types, corn field and beef barn, showed the most distinct differences in concentration of nitrogen, phosphorous, and total organic carbon (Table 2). With this information, we chose extracts from these soils and inoculated them with the pathogen strains to evaluate survival over 14 days. This assessment of pathogens in differing aqueous environments would not only look at survival, but also would provide insight into changes in soil extract chemistry with testing at designated time points. To observe interactions between the microbiome and the extract chemistry, we also included a sterile and a non-sterile subset of the extracts for analysis to see if the native microbiome of the extracts caused any observable changes over time. With these approaches, we predicted that the differences and changes in soil extract chemistry would influence pathogen survival over a two-week period.

Materials and methods

Bacterial strains and preparation

For this study, we conducted an initial study using two strains each of EHEC, *Salmonella,* and *L. monocytogenes* (Table 5). For the next phase of the study, only one representative strain was chosen for each species. The *L. monocytogenes* strain, 10403S, belongs to the serogroup 1/2a and was resistant to 80 ug/ml streptomycin – a characteristic employed to selectively isolate the strain from the soil extracts. An O157:H7 EHEC strain, MI-0041B, was chosen and made resistant to 80 ug/ml rifampicin via random mutant selection. The *Salmonella* Newport strain, Mdd314, had been previously determined resistant to 80 ug/ml rifampicin (Shah et al., 2019b). To determine if these resistant mutants would suffer a disadvantage in the assay compared to their non-resistant mutants, the strains were compared and no difference in survival was observed. These mutants were stored at -80°C in Brain Heart Infusion broth (BHIB) with glycerol and freshly streaked for

isolation on growth media, with added antibiotics, as needed. Isolated colonies were picked and suspended in antibiotic-supplemented Luria Bertani broth (EHEC and *Salmonella,* 80ug/ml rifampicin) or BHIB (*L. monocytogenes*, 80ug/ml streptomycin) and incubated without shaking at 37°C for 16 h. The concentration after this incubation was confirmed via plate count to be ~10^9 CFU/ml for each strain. In preparation for the soil extract assays, the strains were diluted 1:10 five times in Butterfield's buffer with the final, working concentration of 10^4 CFU/ml in the soil extracts. Strains were prepared identically for both phases of the experiment.

Table 5. Strains used in this study

Strain	Species	Serogroup (serovar)	Source
10403S	*Listeria monocytogenes*	1/2 a	Skin Lesion
H7858	*Listeria monocytogenes*	4b	Hot Dog
MI-0041B	EHEC	O157	Human
DA-5	EHEC	O121	Human
FSL-S10-1646	*Salmonella enterica*	Enteriditis	Environmental, Produce
Mdd314	*Salmonella enterica*	Newport	Tomato

Soil extract preparation

Soils were collected in-bulk from a cornfield adjacent to and owned by NDSU and from the NDSU Beef Cattle Barn. The bulk soil samples were bagged and frozen at -80°C in ~6-7 kg portions. New batches of soil extracts were prepared from these frozen sample portions each time the assay was performed. The soil extracts fell into these categories for the assay: sterile low, non-sterile low, sterile high, and non-sterile high. These low (corn) and high (beef) designations were determined with preliminary chemistry work in the first phase of the study by quantifying total organic carbon (TOC), nitrogen, and phosphorous (Table 6). For the preliminary work, 25 g of soil was suspended in 50 ml of non-sterile Milli-

Q ultrapure water and the assays were conducted in 4.5 ml of extract with 0.5 ml of inoculum added. These suspensions were incubated at 4°C with gentile rotation for 24 h. Suspensions were centrifuged at 4°C and 5000 RPM with only the liquid fraction was taken for the extract assays. Half of these extracts were filter sterilized using a 22 μm filter to obtain the sterile samples.

Next in the large-scale soil extract preparation, 625 g of the two soil types were suspended in 1250 ml of non-sterile Milli-Q ultrapure water. This was done with enough soil+water to complete one biological replicate (~9.5 L/soil type). Liquid fraction was collected and sterilized as done previously. These extracts were aliquoted into 49.5 ml portions for the pathogen tests, while six 50 ml portions were not inoculated and set aside for soil chemistry testing.

Table 6. Preliminary soil chemistry results from three replicates.

Soil extract type	Total N (ppm)	Total P (ppm)	Total Organic C (ppm)
High nutrient level	134.5 ± 25.6	22.4 ± 4.4	248.7 ± 58.8
Low nutrient level	30.1 ± 8.5	2.8 ± 0.1	27.5 ± 2.7

Soil extract assay

Each aliquot of extract (high or low) was inoculated with each pathogen to achieve a concentration of 10^3 CFU/ml and placed in a 15°C incubator without shaking for the duration of the assay. Initially, the assay was run with sterile and non-sterile extracts from either high or low and was sampled at 0, 24, 48, 72, 96, 144, 196, 240, 288, and 336 hours after inoculation. In the second phase, samples were taken at fewer times at 0, 24, 96, 144, 192, 240, and 336 hours post inoculation. These sets of samples for each time point included extracts for the chemistry analysis (extracts not inoculated with pathogens) and plating for

enumeration of pathogens and total microbes present. To isolate pathogens from the extracts, we used selective media for each species supplemented with antibiotics. This included MacConkey's agar (BD Diagnostics, Berkshire, UK) with rifampicin for EHEC, xylose lysine deoxycholate agar (XLD, BD Diagnostics) with rifampicin for *Salmonella*, and Rapid LM (Bio-Rad Laboratories, Inc., Hercules, CA) media with streptomycin for *L. monocytogenes.* To determine total microbes present, samples were diluted and plated on LB. All plates were incubated at 30°C for 24 h and enumerated using the Q-count (Color QCount, Spiral Biotech Inc., Norwood, MA).

Soil chemistry analysis

To evaluate the change in soil chemistry over time for each of the extract types, samples were submitted to the NDSU Soil Testing Lab for each time point. The preliminary work focused only on nitrogen, phosphorous, and TOC, but there was a need to see how other soil chemistry characteristics in these extracts differed and changed over the 14-day assay. In the second round, the previous three tests were performed again along with pH, alkalinity, and the ppm of Cl-, Mg, K, Na, Ca, Cu, Fe, Mn, and Zn. Due to the number of tests, water samples were frozen at -20°C until testing could be conducted by the Soil Testing Lab.

Data analysis

For both phases of this study, strains were prepared 4 individual times (biological replicates). This resulted in each extract type having one pathogen for four different replicates with two technical replicates collected for plating. The first phase involved inoculating high non-sterile, high sterile, low non-sterile, and low sterile extracts while in the second phase only the non-sterile extracts were inoculated. Total microbes were enumerated only in phase two. Counts of pathogens and total microbes present were log-

transformed and averaged for each strain, extract type, and time point and graphed. The average log CFU/ml from 0 to 336 h were calculated, graphed, and the differences between the beginning point and end point were calculated and we used a one-way analysis of variance (ANOVA) in Prism 8 (GraphPad, San Diego, CA), with $p<0.05$ to determine significant changes. Chemistry was collected only at the beginning of phase one, but during phase two samples were taken at each time point to correlate with pathogen counts. Chemistry results were used in multi-variable regression analyses to determine correlations between pathogen or total microbe changes and the changes in chemistry (Prism 8).

Results

Soil chemistry and native microbiome influence pathogen survival

The data presented in figure 6 represent the average log CFU/ml of each pathogen collected from each of the four soil extract treatments in the first phase of this study. Initial inoculum levels in all extracts ranged from 3.39 to 3.78 log CFU/ml depending on pathogen (Figure 6). In the low-nutrient extracts, there was no change in the concentration *L. monocytogenes* strains (10403S and H7858) in the sterile condition (Fig 6a). However, in the non-sterile condition a decrease was seen until 288 h post-inoculation (Fig 6b). At 336 h, a sharp increase to 3.98 ± 0.23 log CFU/ml for 10403S and 3.16 ± 0.39 log CFU/ml for H7858 showed a deviation from the trend. A similar pattern was not seen for either EHEC or *Salmonella* strains, as they all plateaued at 144 h post inoculation just below 2 log CFU/ml (Fig 6b). Additionally, in the low sterile extract, the EHEC and *Salmonella* strains behaved differently than *L. monocytogenes* by continuing to decrease over time (Fig 6a). Overall, it was evident in these low-nutrient extracts that the inclusion of native microbes impacted the pathogens' survival. This trend only strengthened when looking at the high-

nutrient extracts. When placed in the high-nutrient extract, all pathogens grew when competition was absent (Fig 6c). This aligns with previous studies showing the presence of nitrogen, phosphorous, and TOC can lead to pathogen growth while under these conditions (Neher et al., 2019; Shah et al., 2019). While overall there was a decrease in the high-nutrient non-sterile extract, a slight phase of growth was observed for the first 72 h (Fig 6d). Beyond that time point, there was a continued decrease until the end of the assay. These data point to some impact on survival from the available nutrients and interactions with the native soil extract microbiome.

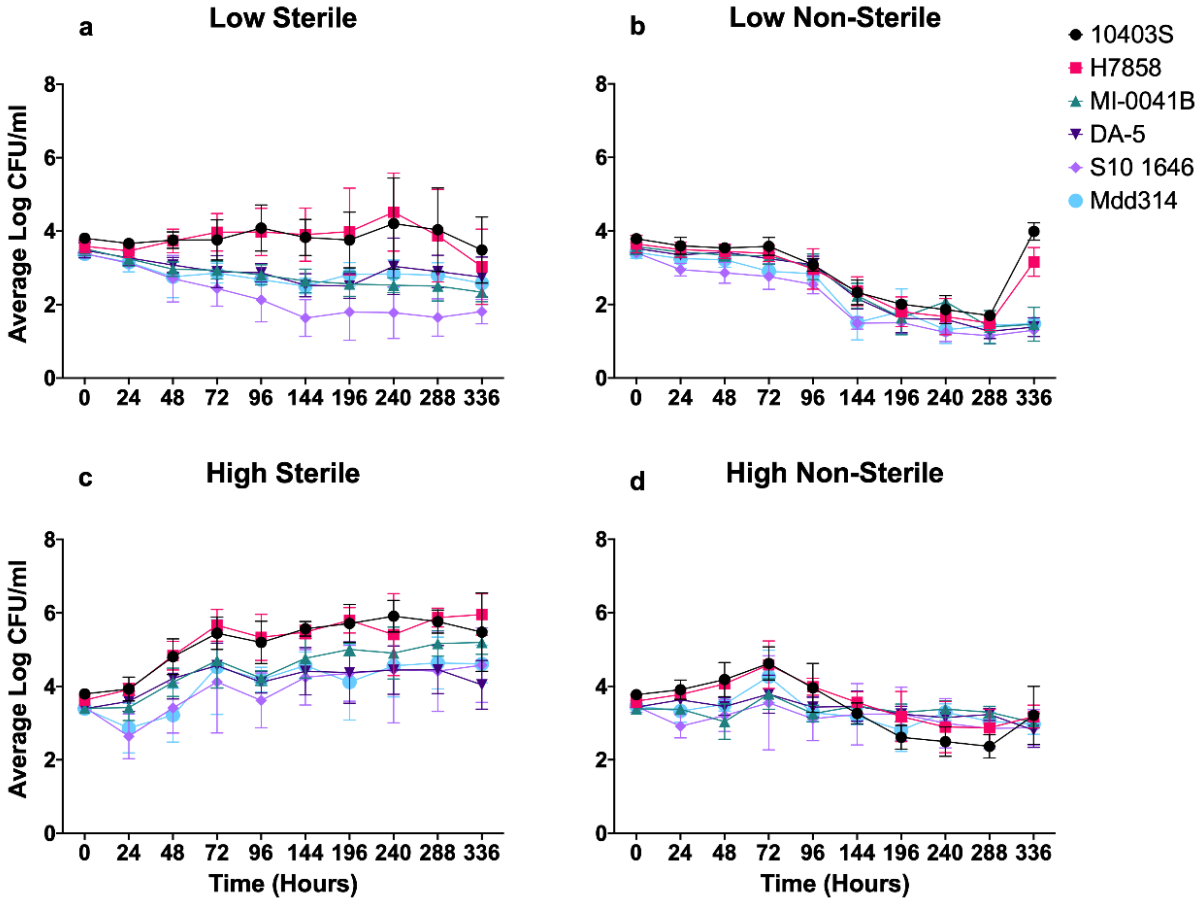

Figure 6. Survival of *L. monocytogenes*, EHEC, and *Salmonella* strains in (a) low-nutrient sterile, (b) low-nutrient non-sterile, (c) high-nutrient sterile, and (d) high-nutrient non-sterile soil extracts over a two-week period. Each point represents four replicates of each strain and error bars denote SD.

A comparison of changes from 0 to 336 h was made within each treatment to see if there were significant differences between strains (Figure 7). Here, the previously discussed trends are summarized by showing either growth (Fig 7a) or decline (Fig 7b, c, d) of each strain. The *L. monocytogenes* strains grew 1.7 to 2.4 log CFU/ml higher over time – values that were significantly higher than the two EHEC strains ($p<0.0002$, Fig 7a). The largest increase was observed for the *L. monocytogenes* strain H7858, while the *Salmonella* strain Mdd314 saw the smallest at 0.65 ± 0.18 log CFU/ml after 336 h. The changes in pathogen populations in high-nutrient non-sterile extracts affirm that, while that small increase was observed early on (Fig 6d), there was an overall decrease for all strains between 0.37 and 0.62 log CFU/ml (Fig 7b). Next, the low-nutrient sterile followed a similar trend with minor decreases for almost all strains, besides MI-0041B experiencing a nearly 2 log reduction (Fig 7c). In the final panel, the trend from figure 7a is inverted with *L. monocytogenes* seeing the lowest reduction (1-1.2 log), EHEC dropping nearly 3 logs, and *Salmonella* falling in between with a 2.3 log decrease (Fig 7d). It is evident from these data that the presence of native microbes and limited nutrients in soil extracts leads to a decreased survival, while the lack of competition and increased availability of N, P, and TOC can allow for growth.

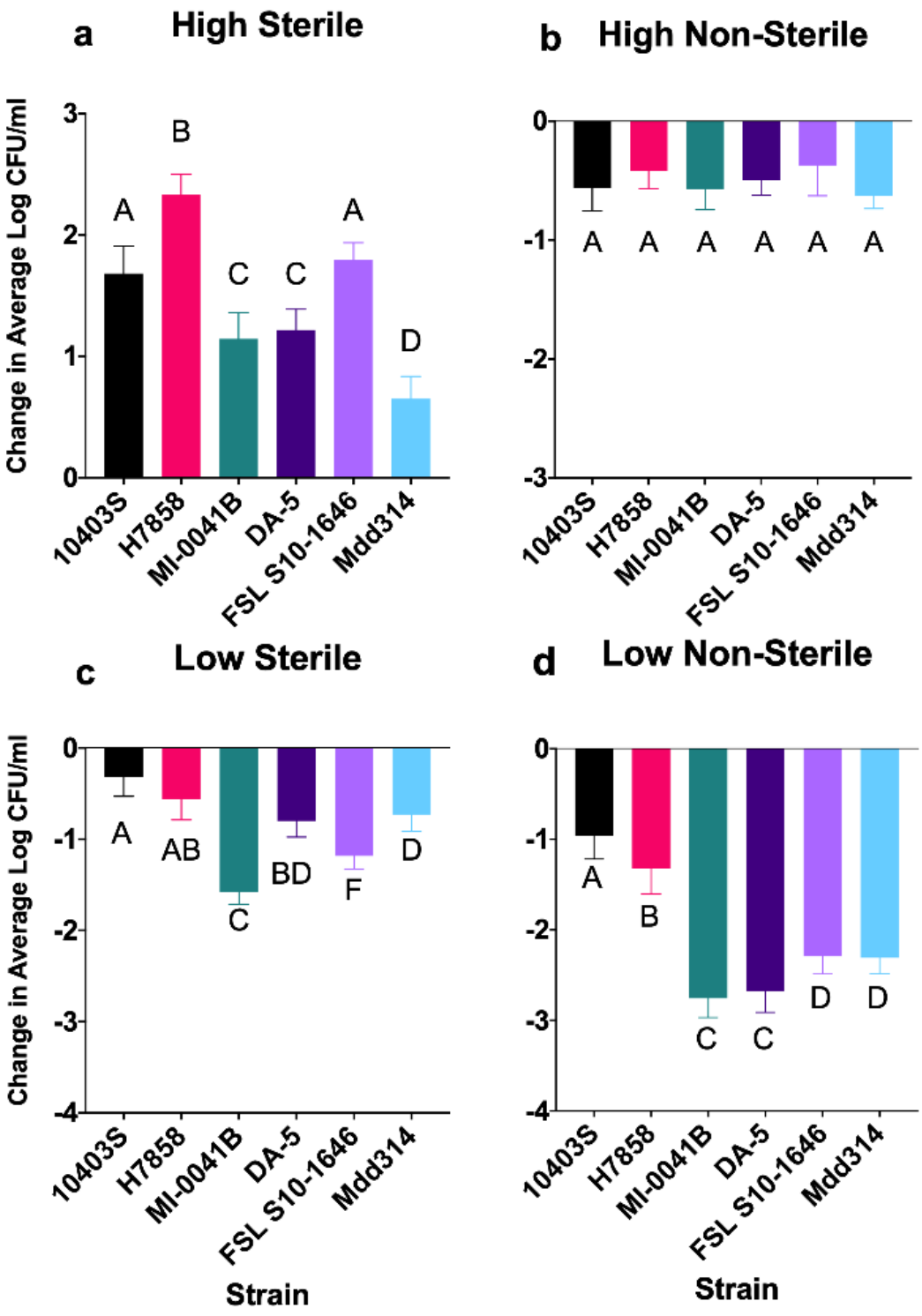

Figure 7. Changes over time in each condition for all six strains in (a) high-nutrient sterile, (b) high-nutrient non-sterile, (c) low-nutrient sterile, and (d) low-nutrient non-sterile extracts. Bars represent the difference between average CFU/ml at 0 to 336 h. Capital letters denote significant differences amongst strains based on Tukey's multiple comparison test ($p<0.05$).

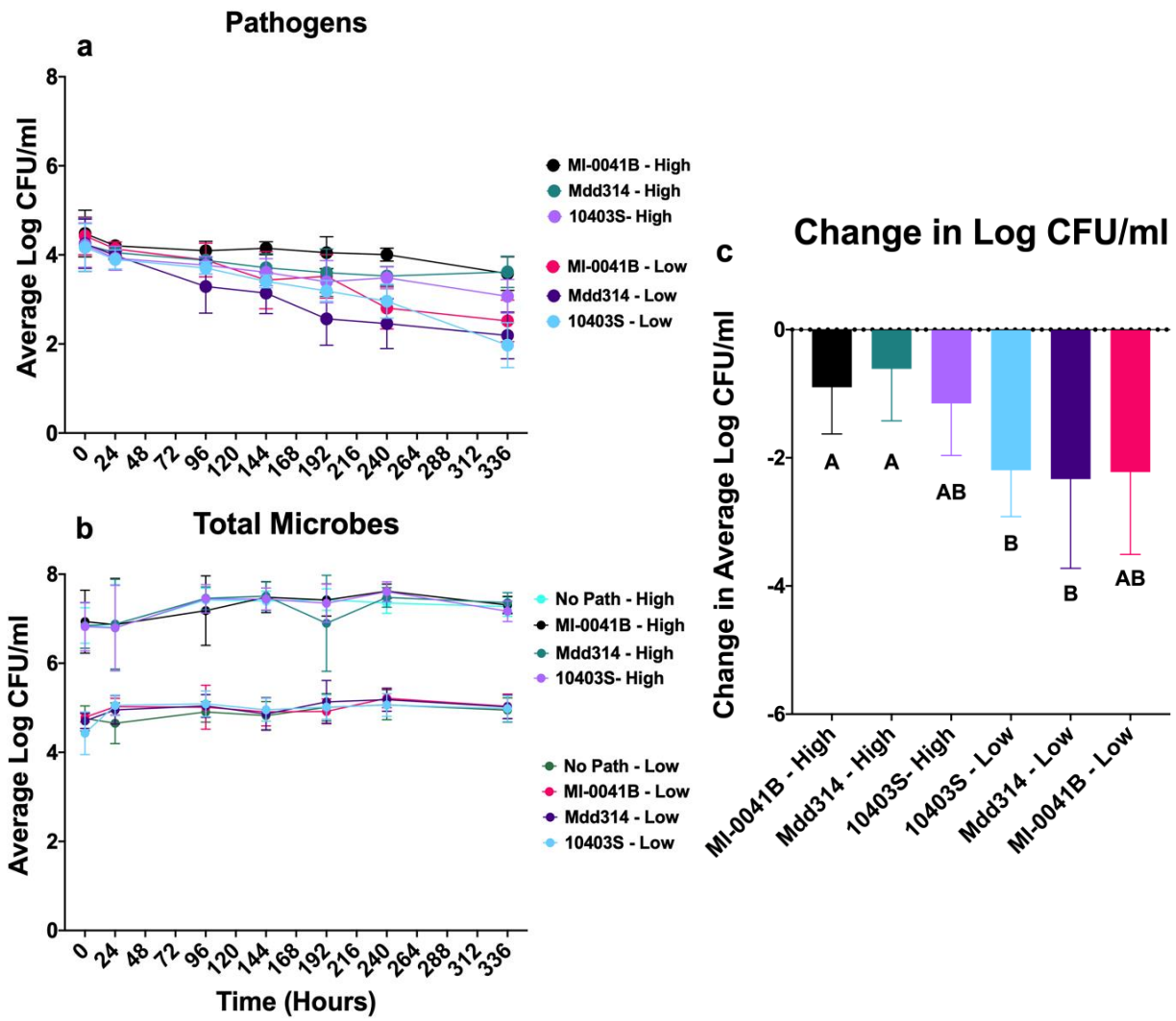

Figure 8. Average Log CFU/ml of (a) MI-0041B, Mdd314, and 10403S and (b) total microbes present in non-sterile high- and low-nutrient extracts over a two-week period. Each point represents average of four replicates with error bars denoting SD. (c) Changes in average Log CFU/ml of each strain in high and low nutrient extracts. Capital letters denote significant differences based on Tukey's multiple comparisons test ($p<0.05$).

Pathogens continue to decrease in presence of native extract microbes

Since survival was similar between strains in non-sterile extracts in the first phase, only one of each pathogen was chosen for the second phase. Other than the reduction in strains, handling of extract samples was similar to the first phase but strains were suspended only in non-sterile extracts and in larger volumes (4.5 to 45.9 ml) than the first phase so enough extract was available for downstream analysis. Pathogen counts over time are displayed in their log-transformed form over the two-week incubation period (Fig 8a). In addition to pathogen enumeration, the total aerobic plate count provided a partial look at the native microbiome in the low and high extracts over time (Fig 8b). This method does not allow for enumeration of all microorganisms present, but still provided data on the culturable, aerobic members of the microbiome. Not only was total aerobic microbes present measured in each pathogen-inoculated extract, but also in an un-inoculated control set of extracts to see if the presence of the pathogens interacted with the native microbes. Also, change in Log CFU/ml over time was calculated and the decreases were compared between high and low extracts and amongst strains (Fig 8c).

As seen in the first phase, pathogens' initial concentration began around ~4 Log CFU/ml in both high and low and gradually decreased in the low-nutrient extract for all strains over time. For the first 24 h, all strains remained at approximately the same concentration but deviation amongst strains began after 96 h. Mdd314 in the low-nutrient extract dropped lower than the other strains to ~3.5 Log CFU/ml while in the high-nutrient extract it held near 4 Log CFU/ml. The MI-0041B started seeing stronger decreases at 240 h post inoculation, with the final concentration near 2 Log CFU/ml reached to match the other two pathogens. The *L. monocytogenes* strain, 10403S, saw a more gradual decline in the low-nutrient extract than either Mdd314 or MI-0041B. All strains had marginal

decreases in the high-nutrient extract, and this is made evident in the difference calculations made in fig 8c.

As for the total microbes present, there were only clear distinction in population size when comparing high and low extracts (Fig 8b). In high extract, the population density was around 7 Log CFU/ml while in the low extract the population stayed near 5 Log CFU/ml. There were no differences detected amongst strain treatments, so it appeared from this perspective that the population size did not change over time regardless of what pathogen was present. Additionally, there was no significant increase or decrease in population size over time, even with pathogen numbers declining in the low-nutrient extract. Overall, this evaluation of population dynamics amongst these pathogens and the native microbes only serves as a partial look at what is occurring in these extracts, and a more in-depth look is necessary.

Extract chemistry stays constant over time, but is distinctly different between high and low

Due to the differences in N, P, and TOC concentrations seen in the first phase, we wanted to expand this soil extract chemistry analysis to see if a chemistry profile correlated to pathogen survival could be identified. The NDSU soil testing lab provided an array of tests that allowed for comparisons between sterile and non-sterile sets within the high and low extracts. No significant changes over the incubation time was observed in any chemistry test result (Table A2). Moreover, no differences were seen between sterile and non-sterile treatments of either high or low extracts.

The next point of comparison was seeing if differences were maintained between high and low extracts — a trend identified in the first phase of the study. Significant differences were seen with higher levels in high extracts for the following chemical

analyses: alkalinity (mg/kg $CaCO_3$), Cl^- (ppm), K (ppm), Na (ppm), Fe (ppm), P (ppm), N (ppm), and TOC (ppm, Table 7). Interestingly, Ca (ppm) was significantly higher in the low-nutrient extracts, diverting from the previously highlighted trend (Table 7).

Table 7. Average of significantly different chemistry results at 336 h time point with comparisons made between high and low-nutrient extracts. Values consist of the average and SD of both sterile and non-sterile conditions, which represent eight replicates (four from each condition).

Chemistry Test	High	Low	p-value
Alkalinity mg/kg $CaCO_3$	435 ± 79.5	55.8 ± 42.7	<0.0001
Cl^- (ppm)	183 ± 55.4	3.47 ± 1.04	0.0003
K (ppm)	616 ± 197	18.1 ± 5.28	0.0005
Na (ppm)	53.4 ± 15.9	9.48 ± 3.45	0.0007
Ca (ppm)*	4.69 ± 0.59	37.4 ± 19.7	0.0199
Fe (ppm)	4.59 ± 2.71	0.248 ± 0.34	0.0232
Total P (ppm)	20.1 ± 2.43	1.91 ± 0.77	<0.0001
Total N (ppm)	53.3 ± 11.2	11.7 ± 5.12	<0.0001
TOC (ppm)	158 ± 40.7	8.54 ± 3.44	0.0001

*Significantly higher in low-nutrient extract.

Correlations between survival and chemistry minimal

We used multi-linear regression to identify significant correlations between pathogen survival and chemical composition. After separating the analyses based on strain, only one significant correlation was made using this regression method. In the high-nutrient extract, Mdd314 survival was significantly correlated with the Ca concentration in the extract (Fig 9a). Contrast this with the linear regression in the low-nutrient extract where no same correlation was made (Fig 9b). So, here we can assume that the variation

was due in-part to the concentration of Ca in the high-nutrient extract. However, the lack of correlations between the other chemistry tests and pathogen survival leads to more questions about what other characteristics not accounted for here that could be influencing the differences we observed in both phases of this study.

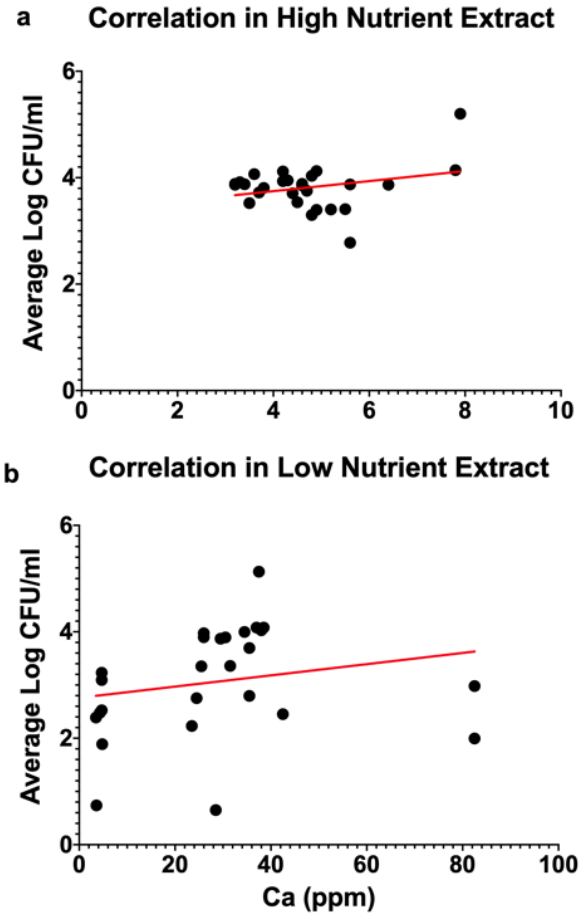

Figure 9. Linear regression showing the correlations between average Log CFU/ml for Mdd314 and Ca (ppm) in non-sterile (a) high and (b) low nutrient extracts. Line of best fit calculated based on four biological replicates of Mdd314 and Ca with p=0.0358 and an r^2 value of 0.907 in high and p=0.839 and an r^2 value of 0.499.

Discussion

Source, microbiome of soil extract impacts pathogen survival

A clear pattern emerged in the first phase of this study – pathogens will survive in these extracts to varying degrees. When placed in an extract with low available TOC, N,

and P the survival of EHEC, *Salmonella*, and *L. monocytogenes* decreased significantly highlighting the likelihood that these chemical components are important for these pathogens (Table 6, Figure 6, 7). And in the same study, the alternative was true as well with higher levels of these elements supporting pathogens for the two-week incubation period (Table 6, Figure 6,7). This outcome is in-line with previous studies looking at influence of amended soils on pathogen survival (Shah et al., 2019a; You et al., 2006). Here, *Salmonella* survival was measured in soil that had been amended with an animal-derived fertilizer. There was significant correlation between the higher N and TOC available when the soil was amended versus when it lacked amendments, with different *Salmonella* serovars surviving 91 (Shah, et al. 2019a), 107, and 231 days (You et al., 2006). Even more support for these phenomena comes from a study conducted in soil extracts that either contained or lacked amendments (Shah et al., 2019b). Again, the presence of higher levels of TOC, N, and P from the amended soil extracts lead to greater survival of *Salmonella*. With this, it seems that the source of these nutrients plays an important role. Something that was not done here that may be important to investigate is identification of the available sources of carbon in these extracts. In manure amended soils, the carbon source has been highly correlated with survival of EHEC (Franz et al., 2011). Our study only tested total available organic carbon en masse, and no profiling of carbon sources in the extracts was done. In alignment with this idea, it has been shown that survival differs when pathogens are in soils containing dairy manure or poultry litter (Neher et al., 2019). It's been proposed that chemical properties besides N, P, and TOC, like metals and carbon sources that are present in amendments, may be what is causing these differences.

In addition to soil extract chemistry, a different factor of these soils and extracts that appears to be playing a significant role is the presence of native microbes. The previous

studies have made note of this, with *Salmonella* survival decreasing when facing competition and limited nutrients in soil extracts (Shah et al., 2019a). Even in amended soil extracts, growth still occurred when native microbes were present, but that growth was significantly lower than in sterile conditions. So even with the abundance of key chemical components, the microbiome was playing a prominent role in limiting pathogen growth. In our initial study, a similar result was observed when microbes were present or absent (Figure 6, 7). We observed growth of ~1-2 Log CFU/ml when the pathogens were put in sterile high-nutrient extracts, which was noticeably different than when microbes were present and there was no overall change (Fig 7b). Under non-sterile low-nutrient conditions we saw the largest decreases of ~1.5 Log CFU/ml in the *Listeria* strains, 2.5 Log CFU/ml in the *Salmonella*, and nearly 3 Log CFU/ml for the two EHEC strains. This again signifies the impact native microbes have on pathogen survival. The impacts from soil microbiomes could explain the differences seen in amendments from different animal-derived fertilizers (Neher et al., 2019). Since this study compared waste products from different animals, they determined that chemical components and distinct microbial communities were driving the differences in survival. This could explain what is occurring in the extracts we studied here, as previous work has backed up this reduced survival when pathogens interact with native microbes (Sidhu et al., 2001). Profiling of the microbial communities in the low- and high-nutrient extracts could provide a more complete picture of what is impacting pathogen survival.

Pathogen survival not strongly correlated with specific chemical components of soil extracts

With the evidence we gathered in the first phase, we proceeded into the second phase of the study with the intent to find connections, if any, between specific chemical

components and pathogen survival. The original phase found the difference between the high- and low-nutrient extracts based on their concentration of P, N, and TOC (Table 6). These data were collected only at the beginning of the two-week incubation, so we could not correlate pathogen survival to any of these components over time. Additionally, we wondered if different soil chemistry analyses could reveal other important components impacting survival. Characteristics like pH of soil and aqueous environments have been investigated (Liang et al., 2019; Xing et al., 2019), but research has been limited to associate specific chemicals or concentrations with survival of EHEC, *Salmonella,* or *L. monocytogenes*.

With survival in non-sterile high- and low-nutrient extracts in the second phase behaving similar to the first, there is further support for the conclusions made previously (Figure 8). So next, we looked to the chemistry data collected parallel to bacterial counts to see if any associations existed. With no significant changes in chemistry over the entire time of the assay (Table A2), it was clear that the pathogens' presence did not cause observable shifts in chemistry over time. The only obvious trend was the higher concentrations of select components in the high-nutrient extract, except for the low-nutrient extract containing more Ca (Table 7). Ca is an important element in crop production and is in relatively high concentrations in agricultural soils (Norton 2013). The concentration of Ca in soils is impacted most notably by pH, but the pH did not significantly differ between high- and low-nutrient extracts. Interestingly, of all the soil chemistry tests the only result that correlated with *Salmonella* survival was Ca in the high-nutrient extract (Fig 9a). This could ultimately be due to the low levels of Ca available in the high-nutrient extract, as the same correlation was not made between *Salmonella* and low-nutrient extract (Fig 9b). When looking to previous studies, not much has been done concerning Ca concentrations in

soils or soil extracts and the effects on survival of pathogens that end up in those environments. This element has been shown to function in ion channels and metabolic pathways possessed by *Salmonella* (Brass 1986). If Ca is important in these metabolic systems, its absence could be detrimental to *Salmonella* survival.

Soil extracts and the concerns with VBNC

These studies of pathogens in soil extracts raise more questions about what specific mechanisms facilitate survival. An important mechanism to investigate would be the induction into a viable-but-not-culturable (VBNC) state. VBNC is described as a state of metabolic functioning where cells continue to survive dormant but are not recoverable by traditional laboratory culture methods (Smith et al., 1994). All three of the pathogens in this study have been shown to enter VBNC (Cunningham et al., 2009; Han et al., 2020; Roszak et al., 1984; Rowan 2004). Outside of the inability to culture these cells, one of the most concerning aspects is the potential infectivity of VBNC pathogens (Cappelier et al., 2007; Highmore et al., 2018). These studies indicated that virulence can be maintained by *L. monocytogenes* and *Salmonella* after entering VBNC, with Highmore et al. finding that this was observed after chlorine treatment. With chlorine commonly used as a sanitizer in food processing, this raises concerns of other triggers from the environment leading to VBNC in foodborne pathogens.

In soil extracts, VBNC could be induced in pathogens that have experienced distinct stress conditions. The most basic obstacle to overcome is limited nutrients. If necessary nutritional elements are not available, the pathogens will quickly die off. Evidence has shown that EHEC encounters starvation conditions in the phyllosphere of produce and in aqueous environments, which triggers entry into the VBNC state (Aurass et al., 2011; Cook and Bolster, 2007; Dinu and Bach, 2013). Similar to EHEC, *Salmonella* and *L.*

monocytogenes have also been shown to respond to starvation by entering VBNC (Gião and Keevil 2014; Gupte et al., 2003; Habimana et al., 2014; Rowan 2004). When living in a host, nutrients are much more accessible and after leaving, pathogens must adapt to survive. Induction into VBNC is not limited to starvation conditions, as the transition has been noted under food processing-relevant conditions. For instance, *L. monocytogenes* has shown to enter VBNC when encountering food preservatives (Rowan 2004), osmotic pressure from NaCl (Besnard et al., 2002), low pH (Cunningham et al., 2009), and chlorine sanitizers (Highmore et al., 2018). *Salmonella* also enters VBNC when encountering similar environmental conditions, which underlines the concerning trend with these pathogens (Han et al., 2020; Highmore et al., 2018; Suehr et al., 2020). If the conditions present in soil extracts lead to a VBNC state, this can make detecting these pathogens by common culture-based methods more challenging. This state could even perpetuate from pre-harvest into post-harvest environments. The combination of VBNC and maintenance of virulence signifies the main concern: these pathogens can avoid detection methods and still cause disease. Work should be done to expand on the VBNC state and identify properties of soil extracts that could be inducing this state and contributing to pathogen survival.

In conclusion, the results in both phases of this study indicated that survival of EHEC, *Salmonella,* and *L. monocytogenes* is influenced by the chemical composition of soil extracts. However, the exact components of the soil extracts that leads to this difference in survival is not clear. Native soil microbes also impacted pathogen survival, and more work could define the population composition of these microbiomes. The mechanisms contributing to pathogen survival were not studied here, and work should be done to identify if VBNC is an important component. With more work, characteristic profiles of

these soil extracts, like specific microbiome or chemical compositions, could inform pathogen control methods for standing water and add to surveillance methods.

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5. OVERALL CONCLUSIONS

While the knowledge around foodborne bacterial pathogens is improving, there continue to be numerous, wide-spread outbreaks associated with foods like fresh produce (Painter et al., 2012; Scallan et al., 2011). As consumption of these foods increases in the US, the concerns with contamination from pathogens like EHEC, *Salmonella*, and *L. monocytogenes* persist.

In order to reduce infections from foodborne pathogens like STEC, isolation and detection methods must be effective in multiple, varying sample matrices. Some methods have been consistent in identifying some serotypes of STEC, but prominent methods do not work the same for all serotypes. Isolation of non-O157 STEC via IMS was influenced strongly by multiple factors. When using the IMS beads in distinctly different sample matrices showed that accurate isolation decreased the more complex the matrix became. This likely was due to chemical properties, physical structures, or native microbes in the samples interfering with the binding of IMS beads to the appropriate STEC antigen. Binding affinity between the antibodies and the antigens of O111 and O145 STEC was also lower, as percent correct identification was consistently lower for those serogroups. These results indicate that improvements should be made with the IMS beads and isolation methods used in this study. With a more sensitive and accurate method, IMS can be more reliable for isolating STEC.

Influences on bacterial attachment are important to investigate since this phenotype is a response to conditions encountered in the post-harvest processing of leafy greens. Stainless steel (SS) is a common food-contact surface, and bacterial attachment to these surfaces can lead to biofilm formation. This protects pathogens and causes them to be difficult to remove, leading to prolonged adherence in unwanted areas. Our work involved

immersing strains of EHEC in processing-relevant conditions and observe any impacts on initial association and attachment to SS. Results indicated that initial association of EHEC was most affected by 4.5% NaCl, but this did not translate to reduced attachment. Attachment varied most prominently in lettuce lysate, and differences were observed amongst strains. With these results, we can infer that it is possible to interrupt initial association of EHEC to SS, but strain specific mechanisms need to be assessed in more detail. If other conditions show signs of influencing EHEC association or even attachment, this could aid in improving methods of reducing bacterial association/attachment in food processing environments.

Survival is one of the most important phenotypes to understand since it allows foodborne pathogens to travel from farm-to-fork. Monitoring agricultural water can prevent this common vehicle for transmission from continuing to cause contamination events like use as irrigation water. Other bodies of standing water, like ditches, small ponds, or flooding events, also pose a risk as vehicles but modeling pathogen survival in these systems would require additional studies. Survival of EHEC, *Salmonella*, and *L. monocytogenes* in these aqueous environments was influenced by the chemical compositions and presence of native microbes. Growth in soil extracts was observed when nutrients were in abundance and competition was absent. Alternatively, when nutrients were limited and pathogens had to compete with native microbes, the survival decreased more severely. In the future, it will be necessary to profile the microbiomes of these soil extracts and eventually work to develop water monitoring applications.

An important trend to take from these studies is that environmental influences on key bacterial phenotypes are not easily parsed, and changes in survival, attachment, or isolation are due to a combination of factors. With such dynamic systems, it is necessary to

use rigorous approaches to study these phenotypes and make well-informed modifications to pathogen control methods.

6. FUTURE STUDIES

While we identified these trends in our studies, there were still questions surrounding the impacts of environmental factors on these phenotypes. Isolation of STEC for the purpose of identification can be the last line of defense in preventing foodborne outbreaks. The IMS study conducted here could be expanded to test in different foodstuffs to make more comparisons and identify any consistent problem areas. Improving the efficacy of isolation methods will require this more intensive study, with the potential for testing the effects of outer membrane disruption on the accuracy of IMS. When pathogens encounter the right set of environmental signals, they progress through the process of attachment. From the work we conducted here, a deeper look should be taken into influences from NaCl concentrations or from other salts along with plant-derived exudates. The use of transcriptome studies could highlight what genes are important in these processes and help understand ways to reduce bacterial attachment to SS. In the case of survival, influence of specific chemical components, like carbon sources and Ca, and soil extract microbiomes will need to be investigated more thoroughly. With the potential for these pathogens to enter a VBNC state within soil extracts, triggers of this complex response should be studied under these conditions. Ultimately, there is a potential with conducting studies like these to identify trends in microbiome composition that could help in controlling pathogens in agricultural water.

APPENDIX

Table A1. Average Log CFU/ml, standard deviation, and 95% confidence interval of media counts for all eight EHEC strains in all conditions at each time point. Result of three biological replicates.

Strain	Condition	Time	Average CFU/ml	Standard Deviation	95% Confidence Interval
EDL-933	GDMM	5	6.582	0.4532	± 0.0882
		15	6.353	0.1565	± 0.0304
		30	6.413	0.17105	± 0.0333
		60	6.458	0.1598	± 0.0311
		90	6.544	0.1564	± 0.0304
		120	6.614	0.1722	± 0.0335
	PBS	5	6.131	0.2283	± 0.0444
		15	6.238	0.4159	± 0.0809
		30	6.216	0.2362	± 0.0459
		60	6.157	0.2377	± 0.0462
		90	6.291	0.3266	± 0.0635
		120	6.163	0.2355	± 0.0458
	4.5% NaCl	5	5.614	0.6327	± 0.1232
		15	5.759	0.6389	± 0.1243
		30	6.115	0.2932	± 0.05709
		60	5.957	0.3089	± 0.0601
		90	6.006	0.3978	± 0.0774
		120	6.286	0.4116	± 0.0801
	Lettuce Exudates	5	6.558	0.1218	± 0.0237
		15	6.653	0.1275	± 0.0248
		30	6.663	0.1978	± 0.0385
		60	6.735	0.08701	± 0.0169
		90	6.789	0.0934	± 0.0181
		120	6.818	0.0944	± 0.0183

Table A1. Average Log CFU/ml, standard deviation, and 95% confidence interval of media counts for all eight EHEC strains in all conditions at each time point (continued). Result of three biological replicates.

Strain	Condition	Time	Average CFU/ml	Standard Deviation	95% Confidence Interval
93111	GDMM	5	6.201	0.2137	± 0.0416
		15	6.208	0.1297	± 0.0252
		30	6.226	0.1677	± 0.0326
		60	6.326	0.1616	± 0.0314
		90	6.486	0.1823	± 0.0354
		120	6.504	0.1461	± 0.0284
	PBS	5	6.039	0.14804	± 0.0288
		15	6.079	0.09904	± 0.0192
		30	6.082	0.0824	± 0.01605
		60	6.088	0.09306	± 0.0181
		90	6.077	0.1167	± 0.0227
		120	6.075	0.1253	± 0.0244
	4.5% NaCl	5	5.373	0.6422	± 0.12504
		15	5.677	0.4913	± 0.0956
		30	5.719	0.2649	± 0.0515
		60	5.299	0.44408	± 0.0864
		90	5.424	0.1859	± 0.0362
		120	5.773	0.2543	± 0.0495
	Lettuce Exudates	5	6.508	0.1417	± 0.0275
		15	6.495	0.1487	± 0.0289
		30	6.509	0.2067	± 0.0402
		60	6.586	0.1118	± 0.0217
		90	6.643	0.0814	± 0.0158
		120	6.738	0.0744	± 0.0144

Table A1. Average Log CFU/ml, standard deviation, and 95% confidence interval of media counts for all eight EHEC strains in all conditions at each time point (continued). Result of three biological replicates.

Strain	Condition	Time	Average CFU/ml	Standard Deviation	95% Confidence Interval
DA-10	GDMM	5	6.582	0.4532	± 0.0882
		15	6.353	0.1565	± 0.0304
		30	6.413	0.17105	± 0.0333
		60	6.458	0.1598	± 0.0311
		90	6.544	0.1564	± 0.0304
		120	6.614	0.1722	± 0.0335
	PBS	5	6.131	0.2283	± 0.0444
		15	6.238	0.4159	± 0.0809
		30	6.216	0.2362	± 0.0459
		60	6.157	0.2377	± 0.0462
		90	6.291	0.3266	± 0.0635
		120	6.163	0.2355	± 0.0458
	4.5% NaCl	5	5.614	0.6327	± 0.1232
		15	5.759	0.6389	± 0.1243
		30	6.115	0.2932	± 0.05709
		60	5.957	0.3089	± 0.0601
		90	6.006	0.3978	± 0.0774
		120	6.286	0.4116	± 0.0801
	Lettuce Exudates	5	6.558	0.1218	± 0.0237
		15	6.653	0.1275	± 0.0248
		30	6.663	0.1978	± 0.0385
		60	6.735	0.08701	± 0.0169
		90	6.789	0.09346	± 0.0181
		120	6.818	0.09447	± 0.0183

Table A1. Average Log CFU/ml, standard deviation, and 95% confidence interval of media counts for all eight EHEC strains in all conditions at each time point (continued). Result of three biological replicates.

Strain	Condition	Time	Average CFU/ml	Standard Deviation	95% Confidence Interval
DA-22	GDMM	5	6.201	0.2137	± 0.0416
		15	6.208	0.1298	± 0.0252
		30	6.226	0.1677	± 0.0326
		60	6.326	0.1616	± 0.0314
		90	6.486	0.1823	± 0.0354
		120	6.504	0.1461	± 0.0284
	PBS	5	6.039	0.14804	± 0.0288
		15	6.079	0.09904	± 0.0192
		30	6.082	0.08243	± 0.0161
		60	6.088	0.09306	± 0.0181
		90	6.077	0.11678	± 0.0227
		120	6.075	0.12538	± 0.0244
	4.5% NaCl	5	5.373	0.64221	± 0.12504
		15	5.677	0.49131	± 0.0956
		30	5.719	0.26492	± 0.0515
		60	5.299	0.44408	± 0.0864
		90	5.424	0.18595	± 0.0362
		120	5.773	0.25439	± 0.0495
	Lettuce Exudates	5	6.508	0.14172	± 0.0275
		15	6.495	0.14878	± 0.0289
		30	6.509	0.20675	± 0.0402
		60	6.586	0.11183	± 0.0217
		90	6.643	0.081401	± 0.0158
		120	6.738	0.07442	± 0.0144

Table A1. Average Log CFU/ml, standard deviation, and 95% confidence interval of media counts for all eight EHEC strains in all conditions at each time point (continued). Result of three biological replicates.

Strain	Condition	Time	Average CFU/ml	Standard Deviation	95% Confidence Interval
Sakai	GDMM	5	6.568	0.4769	± 0.03005
		15	6.261	0.2277	± 0.0284
		30	6.286	0.2038	± 0.0293
		60	6.353	0.2198	± 0.03105
		90	6.405	0.2109	± 0.0499
		120	5.363	2.5129	± 0.3649
	PBS	5	6.3607	0.6037	± 0.1477
		15	6.268	0.1129	± 0.0411
		30	6.255	0.1693	± 0.0272
		60	6.209	0.2123	± 0.0403
		90	6.203	0.2012	± 0.0533
		120	6.269	0.3345	± 0.3258
	4.5% NaCl	5	6.5402	0.3624	± 0.0431
		15	6.013	0.2969	± 0.3343
		30	5.982	0.6703	± 0.3134
		60	5.952	0.4407	± 0.0472
		90	6.124	0.2808	± 0.3148
		120	6.1304	0.2902	± 0.0631
	Lettuce Exudates	5	5.803	0.0938	± 0.0163
		15	5.845	0.1386	± 0.0196
		30	5.765	0.1277	± 0.0153
		60	5.876	0.0896	± 0.0192
		90	6.072	0.2063	± 0.0223
		120	6.11003	0.23502	± 0.0227

Table A1. Average Log CFU/ml, standard deviation, and 95% confidence interval of media counts for all eight EHEC strains in all conditions at each time point (continued). Result of three biological replicates.

Strain	Condition	Time	Average CFU/ml	Standard Deviation	95% Confidence Interval
MI03-4	GDMM	5	6.568	0.4769	± 0.03005
		15	6.261	0.2277	± 0.0284
		30	6.286	0.2038	± 0.0293
		60	6.353	0.2198	± 0.0311
		90	6.405	0.2109	± 0.0499
		120	5.363	2.512	± 0.3649
	PBS	5	6.361	0.6037	± 0.1477
		15	6.268	0.1129	± 0.0411
		30	6.255	0.1693	± 0.0272
		60	6.209	0.2123	± 0.0403
		90	6.203	0.2012	± 0.0533
		120	6.269	0.3345	± 0.3258
	4.5% NaCl	5	6.5402	0.3624	± 0.0431
		15	6.013	0.2969	± 0.3343
		30	5.982	0.6703	± 0.3134
		60	5.952	0.4407	± 0.0472
		90	6.124	0.2808	± 0.3148
		120	6.1304	0.2902	± 0.0631
	Lettuce Exudates	5	5.803	0.0938	± 0.0163
		15	5.845	0.1386	± 0.0196
		30	5.765	0.1277	± 0.0153
		60	5.876	0.0896	± 0.0192
		90	6.072	0.2063	± 0.0223
		120	6.11003	0.23502	± 0.0227

Table A1. Average Log CFU/ml, standard deviation, and 95% confidence interval of media counts for all eight EHEC strains in all conditions at each time point (continued). Result of three biological replicates.

Strain	Condition	Time	Average CFU/ml	Standard Deviation	95% Confidence Interval
MI06-63	GDMM	5	6.421	0.3958	± 0.0301
		15	6.239	0.1993	± 0.0284
		30	6.602	0.4798	± 0.0293
		60	6.574	0.3592	± 0.0311
		90	6.492	0.3159	± 0.0499
		120	6.494	0.35704	± 0.3649
	PBS	5	6.231	0.4512	± 0.1477
		15	6.388	0.4243	± 0.0411
		30	6.2404	0.5459	± 0.0272
		60	6.253	0.2464	± 0.0403
		90	6.401	0.3998	± 0.0533
		120	6.321	0.1789	± 0.3258
	4.5% NaCl	5	6.717	0.5332	± 0.0431
		15	6.486	0.6107	± 0.3343
		30	6.225	0.5805	± 0.3134
		60	6.417	0.5507	± 0.0472
		90	6.174	0.46102	± 0.3148
		120	6.397	0.3714	± 0.0631
	Lettuce Exudates	5	6.176	0.1047	± 0.0163
		15	6.183	0.0846	± 0.0196
		30	6.227	0.1172	± 0.0153
		60	6.194	0.1229	± 0.0192
		90	6.385	0.1231	± 0.0223
		120	6.385	0.1252	± 0.0227

Table A1. Average Log CFU/ml, standard deviation, and 95% confidence interval of media counts for all eight EHEC strains in all conditions at each time point (continued). Result of three biological replicates.

Strain	Condition	Time	Average CFU/ml	Standard Deviation	95% Confidence Interval
Jimmy John's	GDMM	5	6.834	0.5624	± 0.0301
		15	6.593	0.3883	± 0.0284
		30	6.851	0.4623	± 0.0293
		60	6.781	0.3826	± 0.0311
		90	7.393	0.2669	± 0.0499
		120	6.966	0.37403	± 0.3649
	PBS	5	6.778	0.8554	± 0.1477
		15	6.589	0.6111	± 0.0411
		30	6.777	0.7525	± 0.0272
		60	6.831	0.6746	± 0.0403
		90	6.846	0.7432	± 0.0533
		120	6.691	0.3358	± 0.3258
	4.5% NaCl	5	6.619	0.7886	± 0.0431
		15	6.678	0.7364	± 0.3343
		30	6.631	0.6725	± 0.3134
		60	6.826	0.6738	± 0.0472
		90	7.205	0.4284	± 0.3148
		120	6.429	0.1991	± 0.0631
	Lettuce Exudates	5	5.966	0.1701	± 0.0163
		15	6.015	0.1585	± 0.0196
		30	5.999	0.1374	± 0.0153
		60	6.061	0.1301	± 0.0192
		90	6.206	0.1073	± 0.0223
		120	6.259	0.1121	± 0.0227

Table A2. Averages of the results of each soil chemistry test performed at the beginning and end of the soil extract assay. Each is the average of four replicates with the SD. Comparisons were made by two-way ANOVA using Sidak's multiple comparison test and $p<0.05$ determined significance.

	High Non-Sterile		High Sterile		Low Non-Sterile		Low Sterile	
Chemistry Test	**0**	**336**	**0**	**336**	**0**	**336**	**0**	**336**
pH	8.14 ± 0.193	8.17 ± 0.227	8.05 ± 0.34	8.128 ± 0.232	7.66 ± 0.471	7.44 ± 0.071	7.73 ± 0.67	7.74 ± 0.31
Alkalinity*	450 ± 112	445 ± 99.2	414 ± 101	423 ± 67.8	41.0 ± 14.1	40.5 ± 8.89	47.3 ± 20.2	71.0 ± 59.6
Cl- **	183 ± 39.3	187 ± 55.4	195 ± 60.0	179 ± 63.7	4.37 ± 1.67	3.19 ± 0.675	3.67 ± 1.23	3.76 ± 1.36
K	543 ± 167	709 ± 213	503 ± 132	521 ± 148	16.4 ± 3.54	18.4 ± 7.49	24.6 ± 9.46	17.9 ± 2.96
Na	46.3 ± 19.3	59.9 ± 21.6	45.9 ± 6.96	46.9 ± 3.54	7.56 ± 1.42	9.74 ± 4.35	8.56 ± 3.03	9.23 ± 2.494
Ca	4.65 ± 2.21	5.03 ± 0.427	4.20 ± 0.432	4.35 ± 0.592	32.8 ± 5.52	42.5 ± 27.1	30.6 ± 8.78	32.3 ± 10.1
Mg	23.2 ± 10.9	30.6 ± 10.7	20.2 ± 1.14	21.1 ± 2.69	15.7 ± 2.30	16.8 ± 6.31	14.6 ± 3.62	14.8 ± 2.01
Fe	6.67 ± 4.19	5.40 ± 2.72	4.52 ± 2.62	3.78 ± 2.83	0.39 ± 0.49	0.35 ± 0.48	0.133 ± 0.107	0.148 ± 0.096
Total P	20.7 ± 3.78	20.4 ± 1.64	20.4 ± 3.66	19.9 ± 3.48	2.18 ± 0.48	1.69 ± 0.41	2.29 ± 0.497	2.13 ± 1.04
Total N	56.0 ± 17.6	55.1 ± 13.1	53.5 ± 17.6	51.6 ± 10.6	11.2 ± 2.5	13.4 ± 5.2	10.7 ± 2.75	9.99 ± 5.21
TOC	174 ± 68.1	141 ± 37.8	180 ± 58.3	175 ± 40.8	9.04 ± 2.23	7.70 ± 2.75	15.1 ± 12.1	9.38 ± 4.27
Cu	0.08 ± 0.018	0.073 ± 0.037	0.09 ± 0.031	0.084 ± 0.023	0.032 ± 0.032	0.03 ± 0.021	0.038 ± 0.038	0.029 ± 0.015

*mg/kg $CaCO_3$

**concentrations in ppm – all following chemical tests