CHARACTERIZING THE IMPACT OF STRESS EXPOSURE ON SURVIVAL OF FOODBORNE PATHOGENS

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Bacterial pathogens transmitted by the fecal-oral route endure several stresses during survival/growth in host and non-host environments. For foodborne pathogens, understanding the range of phenotypic responses to stressors and the environmental factors that impact survival can provide insights for the development of control measures. For example, the gastrointestinal system presents acidic, osmotic, and cell-envelope stresses and low oxygen levels, but *Listeria monocytogenes* can withstand these stresses, causing illnesses in humans. Survival/growth characteristics may differ among *L. monocytogenes* strains under these stressors due to their genetic diversities. Our knowledge of such phenotypic characteristics under bile and salt stresses are inadequate. In this dissertation, variation in growth characteristics was observed among *L. monocytogenes* strains under bile and osmotic stresses with no evidence of cross-protection, but rather an antagonistic effect was observed with the formation of filaments when pre-exposed to 1% bile and treated with 6% NaCl. This shows that variation in stress adaptability exists among *L. monocytogenes* strains with the ability to form filaments under these conditions. Similarly, *Salmonella* survival in soil is dependent on several factors, such as soil, amendment types, moisture, irrigation, and desiccation stress. In this study, the use of HTPP (heat-treated poultry pellets) was investigated as a soil amendment in the survival/growth of *Salmonella* in soil extracts mimicking runoff events, and in soil cultivated with spinach plants to assess its safety for use for an organic fertilizer. The presence of HTPP in soil increased *S. Newport* survival with a greater likelihood of its transfer to and survival on spinach plants. Increased microbial loads and *rpoS* mutant showed decreased growth/survival in soil extracts, however, *rpoS* was not important for survival in soil under the tested conditions showing possible lack of desiccation stress. These results show that HTPP provided nutrients to the *Salmonella* for increased growth

and survival in soil extracts and soil, respectively, which show that the use of treated BSAAO to soils may still require appropriate mitigation to minimize *Salmonella* Newport contamination of leafy greens in the pre-harvest environment. Overall, the results in this study increased our understanding of *L. monocytogenes* and *Salmonella* phenotypic adaptation to stressful environments.

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DEDICATION

I would like to dedicate this dissertation to my parents, late Lal Babu Sah and Urmila Devi Sah.

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Salmonella enterica and *Listeria monocytogenes* are two major foodborne pathogens accounting for the first and third most deaths, respectively, in the United Sates (1). Foods for human consumption can be contaminated at various stages during food production, such as from cultivation of crops on farms to processing and packaging, which upon consumption can make us sick potentially leading to hospitalizations or deaths. During cultivation, pathogens can be transmitted to crops from contaminated soil, water, or use of contaminated manure. Similarly, during food processing pathogens can be introduced from contaminated raw ingredients, via workers, insect vectors or improper manufacturing practices. Numerous animals, such as cattle, pigs, wild animals, birds, and poultry are known reservoirs of *Salmonella* (2). Similarly, *Listeria monocytogenes* are known saprophytes with soil, water and ruminants as their reservoirs (3-5). Contaminated soil, water or animal manure that harbor pathogens like *Salmonella enterica* and *L. monocytogenes* are known to be implicated in various foodborne disease outbreaks (6-9).

Fruits and vegetables, such as leafy greens are consumed raw, and if these foods get contaminated on farms, they may end up contaminated on our plates due to lack of an intermediate kill step. Use of contaminated water for irrigation purposes and the use of raw or untreated manure as soil amendment can act as source of produce contamination on the fields. While manure is a recognized source of contamination, it has also been shown to increase pathogen survival in manure amended soil possibly increasing the likelihood of pathogen transfer to crops. Similarly, manure runoff from cattle farms are a plausible explanation for contamination of water sources, including water in the irrigation canals or rivers. However, there are significant gaps in our knowledge on factors that impact survival of *Salmonella enterica* in runoff like matrices and soil that are amended with manure. Similarly, transfer and survival of

Salmonella from contaminated soil to leafy greens are unknown. In this dissertation, I used Heat-treated poultry pellets (HTPP) as our manure source. HTPP are heat-treated and pelletized poultry pellets used as a slow release fertilizer with a N-P-K of 3-2-3. The heat treatment process may eliminate any harmful pathogens from the manure fertilizer, however, subsequent contamination events on farms from the usage of contaminated irrigation water or from wildlife and birds may contaminate soil containing HTPP amendment. Also, if there are any pathogens surviving heat treatment, the nutrient rich condition in the soil by the addition of HTPP may help in increased pathogen survival. Role of HTPP as a soil amendment in the survival of *Salmonella* is also unknown. In this dissertation, I studied the impact of HTPP in the survival of *Salmonella* Newport wild type and *rpoS*-deficient strains in soil extracts (manure runoff like matrices) and in soil, leading to subsequent transfer and survival on spinach leaves while also accessing the impact of other factors that may be relevant during cultivation of leafy greens, such as irrigation events, soil nutrient characteristics, and formation of viable and non-viable cells.

Similarly, upon contamination of foods, *Listeria monocytogenes* are known to survive or even grow at conditions that present challenging stresses, such as at low temperatures and higher salt concentrations (10-12). Variation in survival and growth characteristics have also been observed among *L. monocytogenes* strains under various stresses (10). One thing to note is that the stress adaptive capability of *L. monocytogenes* to stresses encountered in foods may help it in increased survival through its passage through the gastrointestinal system. One of the major stresses encountered in the gastric system is exposure to bile salts. In this dissertation, I wanted to study the variation in growth characteristics among *L. monocytogenes* strains exposed to salt and bile stresses. There is evidence demonstrating stress response to one stressor can provide adaptation against another stressor (11, 13). Based on this premise, after short-adaptation to one

of these stresses (osmotic or bile stress), cross-protection mechanisms were also investigated for *L. monocytogenes* strains.

1.2. Literature Review

1.2.1. Epidemiology of Listeria monocytogenes

Listeria monocytogenes is a saprotroph but it can cause listeriosis in susceptible humans and animals through consumption of contaminated foods (14). Foods can be contaminated by the pathogen during cultivation and food processing by the use of contaminated irrigation water and manure, feces from animals, and contaminated equipment. These contaminated foods may be sold for human consumption, potentially causing foodborne disease outbreaks. Globally, *L. monocytogenes* causes 23,150 illnesses, 5,463 deaths, and a loss of 172,823 DALYs (Disability-adjusted life year) annually (15). In the United States, *L. monocytogenes* causes 1,591 illnesses, 1,455 hospitalizations, and 255 deaths annually (1). Outbreaks of *L. monocytogenes* have been attributed to various types of foods, such as dairy products, meat, ice-cream, fruits and vegetables (CDC, 2019). Some of these foods are preserved by storage at refrigeration temperatures without the need of additional cooking and the ability of *L. monocytogenes* to survive and grow at refrigeration temperature makes people sick when such foods are consumed. Recent foodborne disease outbreaks due to *Listeria monocytogenes* in pork products in 2018 caused four illnesses with all cases reported to be hospitalized. Similarly, another outbreak in deli ham in 2018 caused another four illnesses with all cases becoming hospitalized leading to one death. From the outbreak reports, it can be well understood that hospitalizations and death rates are high due to *Listeria monocytogenes* infections with reported mortality rate of as high as 30-40% (1). Infections due to *Listeria monocytogenes* are known to severely impact newborns,

pregnant women, elderly population aged 65 or older and people with weakened immune response.

1.2.2. Listeria monocytogenes pathogenesis

Listeria is a Gram-positive bacillus and a facultative anaerobe that does not produce endospores. Thus far, the genus *Listeria* is known to comprise 17 species with only *Listeria monocytogenes* associated with most listeriosis infections in humans. However, a rare case of infection due to *L. ivanovii* has been reported in a man associated with gastroenteritis and bacteremia (16). *L. monocytogenes* strains are grouped into 13 serotypes based on somatic (O) and flagellar (H) antigens, however, 90% of listeriosis in humans are due to serotypes 1/2a, 1/2b, and 4b (17). Strains from these serotypes are further classified into lineages based on phylogenetic analyses using microarray, MLST (Multi Locus Sequence Type) or cgMLST (core genome MLST). The serotypes (1/2a, 1/2b, and 4b) that cause most illnesses belong to lineage I (1/2b and 4b) and lineage II (1/2a) with the lineages III and IV consisting of isolates from ruminants, environment and sporadic cases of foodborne disease incidents (17, 18). Lineages are further classified into multiple clonal complexes in *L. monocytogenes* (19). Infections due to *L. monocytogenes* lead to clinical manifestations such as sepsis, meningitis, meningo-encephalitis, long-term bacteremia and perinatal infections leading to abortions in pregnant women.

The uptake of *Listeria monocytogenes* into human intestinal cells occurs via dissemination of epithelial cells or uptake by macrophages and spread via lymph and blood to other organs, such as the liver and spleen (20). Attachment and uptake of *L. monocytogenes* into the surface of the intestinal wall occur by internalin InlA that binds to E-cadherin in goblet cells, and by InlB that binds to hepatocyte growth factor (Met) on trophoblasts inducing its phosphorylation and ubiquitylation. *L. monocytogenes* enters these infected cells by receptor-

mediated endocytosis as observed by ruffling of the cell membrane (21). Upon entry into the cells, *L. monocytogenes* cells are enclosed in a vacuole where they are protected from vacuolar killing by MnSOD (cytoplasmic superoxide dismutase) (22) and from lysozyme by PgdA (23). In macrophages, *L. monocytogenes* can replicate in spacious *Listeria*-containing phagosomes (SLAPs). Exit from the vacuole is mediated by LLO (listeriolisin-O) (24), PlcA (phosphatidylinositol phospholipase C) and PlcB (phosphatidylcholine phospholipases C). Upon exit from the vacuole after 30 minutes, *L. monocytogenes* cells can replicate in the cytosol and they move about in the intracellular environment by actin based motility (ActA) (25). The same actin based polymerization leads to generation of membrane protrusions from the native cell to neighboring cells spreading the infection process. Many of these genes required for *Listeria* monocytogenes virulence, such as infection and spread are regulated by transcriptional activator PrfA (26). The infectious dose of *L. monocytogenes* is known to be as low as 7,400 cells and can vary by the type of food consumed and are dependent upon the well-being of patients with lower infectious doses observed in susceptible immunocompromised persons (27).

1.2.3. Adaptation of Listeria monocytogenes to stress

L. monocytogenes encounters various stresses, such as low pH, bile stress and low availability of oxygen during its passage through the gastrointestinal system. *L. monocytogenes* are able to withstand these stresses by regulation of genes, some of them mediated by Sigma B, which is also responsible for regulation of virulence genes required for infection (28). The first stress encountered by *L. monocytogenes* in the GI system is its exposure to low pH environments in stomach and small intestine, which are as low as 1.0-2.5 and 4.0-7.0, respectively. The next stress encountered by *L. monocytogenes* is its exposure to bile that is secreted by contraction of gall bladder stimulated by production of cholecystokinin upon consumption of food (29). As a

result, the pH in the duodenum is observed to rise as high as 7.0-8.5 due to the exposure to bile. Bile is composed of cholic acid and chenodeoxycholic acids that are synthesized from cholesterol in hepatocytes and conjugated with glycine or taurine before secretion from the liver. The pH of bile in gall bladder is 7.0 – 8.5, however, in the small intestine a lower pH of 4.0 – 7.0 is observed when it mixes with the food containing the gastric juice. Several studies have shown that bile can have antimicrobial properties at lower pH whereas growth of *L. monocytogenes* is observed to occur in bile at neutral pH, such as in the gall bladder (30, 31). In addition, anaerobic condition is also known to have an increased impact on the survival of *L. monocytogenes* during exposure to bile stresses (31-33).

Growth of *L. monocytogenes* has been observed at a low pH of 4.1 (34), at high salt concentrations >10% (12) and in gall bladder obtained from pigs (30). The robust stress adaptive mechanisms employed by *L. monocytogenes* are thought to contribute to resilience in non-host environments as well as survival during passage through the gastrointestinal tract. Several studies have been conducted to study *L. monocytogenes* survival under acidic stress, which shows the regulation of glutamate decarboxylase (*gad*) and arginine deiminase (*adi*) systems to withstand those stresses (35-37). Similarly, *L. monocytogenes* are known to cope with exposure to bile stresses via bile salt hydrolase coding gene-*bsh,* and *pva* and *btlB* (increased bile tolerance) eventually helping in survival through gastro intestinal tract before it leads to systemic infection (35-39). Several other genes are also known to be regulated by *L. monocytogenes* under other types of stresses, such as *csps*, *lisR*, *lhkA, yycJ* and *yycF* under cold stress (40-43) and *ppuC*, *bsh*, *DnaK, clpP,* and *gbuA* under osmotic stress (44, 45). Evidence of cross protection is known to exist when *L. monocytogenes* are subsequently exposed to multiple stresses and studies have shown that exposure to osmotic stress leads to cross-protection against other cell envelope

stresses, such as? (13, 46, 47). Exposure to osmotic stresses are known to regulate sodium antiporter and Sig B regulated genes, such as *betL*, *gbu*, and OpuC encoding for osmolyte transport leading to increased *L. monocytogenes* survival (48), which is also observed to be regulated under bile stresses. Also, short-term stress exposure to 6% NaCl included increased transcript levels of *sigB* and SigB-regulated genes, including transcription of a sodium/proton antiporter (*mrpABCDEFG*). The short term exposure (i.e. during the lag phase) to 6% NaCl also provided cross-protection against H_2O_2 exposure, which causes an oxidative stress to the cells (47). Similarly, pre-exposure of *L. monocytogenes* to a pH of 5.5 showed increased resistance to a lowered pH of 3.5. due to regulation of ATR (acid tolerance response) (49). These studies suggest that cross protection occurs among various stresses due to regulation of genes under one stress, helping under other stresses. However, cross-protection mechanisms across osmotic and cell envelope stressors are still unknown and need to be elucidated for *L. monocytogenes* survival.

Also, *L. monocytogenes* survival under various stressful conditions are known to differ among strains. For an example, Bergholz *et al*., showed that *L. monocytogenes* lineage I strains grew faster in BHI with 6% NaCl at 37°C than lineage II strains, however, the differences in growth among lineages were not found to paralleldifferences in transcripts levels for osmolyte transporter-encoding genes (*betL*, *gbuA*, and *opuCA*) (10). Variation in bile resistance has also been studied among *L. monocytogenes* strains and it was shown that the strain 2011L-2676 (1/2a) was more susceptible than the strains ScottA (4b) and F2365 (4b) after exposure to 1% bile for 3 h (31). The same study found decreased viability for all the strains at pH 5.5 than that at pH 7.5 (31). However, elaborate studies on variation in stress tolerance abilities under bile stresses among *L. monocytogenes* strains by lineages, serotypes and clonal complexes are

lacking. Other studies, such as by Hingston *et al*., found that significant differences in *L. monocytogenes* ability to withstand stresses (acid, cold and salt stresses) differed among clonal complex but not necessarily among lineages or serotypes (50). They also observed that strains possessing full length *inlA* or a plasmid showed enhanced stress tolerance abilities to cold and acidic stresses, respectively. Recent studies have discovered strain and clonal complex specific variations in genes, indels and Single Nucleotide Polymorphisms (SNPs) that may be associated to stress tolerant phenotypes in these strains. For example, environmental persistent strains (R479a-ST8, 4423 & 6179 from ST121) showed higher expression of stress resistance genes, such as Lmo2637 (membrane anchored lipoprotein), and proteins, such as NamA, Fhs and QoxA than the non-persistent strain (EGDe) (51). This is evidence of survival of some strains to abiotic niches, such as in presence of alkaline stresses or in presence of quaternary ammonium compounds in the food processing environments (51). Some other strains, such as *L. monocytogenes* Scott A was also shown to harbor *L. monocytogenes* genomic island-2 (LGI2) that is involved in arsenic and cadmium resistance (52). While these studies show variation in genes in specific strains, recent studies have shown differences in genetic variants among different clonal complexes. The distribution of stress survival Islet-1 (SSI-1), which is composed of five stress response genes (*lmo0444, lmo445, pva, gad1* and *gadT1*) is clonal (11). SSI-1 were present in CCs 3, 5, 7 and 9 whereas it was absent in CCs 2, 6, and 11. In this same study, deletion of SSI-1 from one of the clonal complexes, CC7 led to decreased survival in salt-induced nisin stress (11). Another study investigated 85 arsenic resistant *L. monocytogenes* strains and found that 70/71 of 4b strains belonged to three CCs 1, 2, and 4. Similar to previous findings, LGI2 was not found in many 1/2 serogroup (52). Moreover, lin0464 coding a putative transcriptional regulator and lin0465 encoding an intracellular PfpI protease are novel inserts

termed as Stress Survival Islet -2 (SSI-2) that were mainly found in food associated strains of sequence type ST12. These genes are known to be associated in alkaline and oxidative stress response (53).

In summary, *L. monocytogenes* are foodborne pathogens that are able to adapt well in the GI system causing the highest mortality rates among people with foodborne illnesses. The GI system presents several challenges to *L. monocytogenes* survival and exposure to one stress may lead to protection against other stresses, known as cross-protection. Also, variation in survival among strains are known to vary by lineages, serotypes, and clonal complexes, which may be due to differences in presence/absence of genes, single nucleotide polymorphisms, truncation in genes or differential regulation of genes.

1.2.4. Epidemiology of Salmonella

The Center for Disease Control (CDC) estimates that foodborne illnesses account for 48 million sick people annually with 128,000 hospitalizations, leading to 3,000 deaths (54). Foodborne disease outbreaks with known etiology from 1998-2008 were used to estimate the burden of illnesses by 30 major foodborne pathogens (1, 55). It was found that 1 million foodborne illnesses were caused by *Salmonella,* only second to Norovirus, resulting in most hospitalizations (19,336 cases, 35%), and deaths (378 cases, 28%) per year (1). Foodborne illnesses due to produce commodities were estimated to be 46% of total illnesses of which 22% were attributed to leafy vegetables. *Salmonella* accounted for 2.9% of total illnesses due to contaminated leafy greens (55). In recent years, several outbreaks have been observed due to *Salmonella* in many produce commodities. In 2018, two outbreaks were attributed to *Salmonella* Sandiego and *Salmonella* Adelaide in spring pasta salad and pre-cut melons, respectively, leading to a total of 171 illness and 61 hospitalizations (56). Outbreaks in cucumbers due to

Salmonella Newport, *Salmonella* Poona, and *Salmonella* Saintpaul have also been observed in multiple years 2015, 2014, and 2013, respectively. A retrospective investigation determined that the outbreak due to *Salmonella* Newport was found to originate from Maryland farms that used poultry litter as a soil amendment. The outbreaks in cucumbers led to a combined total of 1,266 illnesses, 269 hospitalizations, and 7 deaths (56).

Between 2016 and 2018, 116 food recalls have been made due to possible *Salmonella* contamination. In 2018, 40 food recalls were due to *Salmonella* with at least three recalls associated with produce commodities, such as roasted tomatoes, spinach and baked potato mixes (57). *Salmonella* infections are not only detrimental to the health and well-being of the affected individuals but also adversely affect the economy at a large scale. There are loss due to medical expenses, and lost productivity due to illnesses, hospitalizations, and deaths (58). Loss of QALY (Quality Adjusted Life in Years) also arise from the pain, sufferings and disability that may have occurred from Salmonellosis (59). Moreover, recalls of food products lead to economic loss to the manufacturers. Annually, foodborne illnesses due to non-typhoidal *Salmonella* is estimated to cause a loss of 11,391 million dollars making *Salmonella* the costliest foodborne pathogen in the USA (1, 58).

1.2.5. Salmonella pathogenesis

Salmonella is a rod-shaped, Gram-negative, facultative anaerobe from the family *Enterobacteriaceae* (60). When *Salmonella* was first isolated from pigs with viral swine fever by Salmon and Smith in 1886, it was considered to exist as a secondary pathogen (61). However, *Salmonella* is found to cause diseases in both humans and some animals, with many animals recognized to be asymptomatic carriers. Currently, the genus *Salmonella* is known to consist of two species: *Salmonella bongori* and *Salmonella enterica. Salmonella enterica* is further sub

divided into six subspecies: i) *S. enterica* subsp. *enterica*, ii) *S. enterica* subsp. *salamae*, iii) *S. enterica* subsp. *arizonae,* iv) *S. enterica* subsp. *diarizonae*, v) *S. enterica* subsp. *indica,* and vi) *S. enterica* subsp. *houtenae* (62). *Salmonella* are classified into serovars/serotypes based on their antigenic profile i.e. first by somatic O antigen and then by flagellar H antigens (63, 64). There are currently ~2600 serovars of *Salmonella enterica. Salmonella* causes two kinds of clinical outcomes one with fever and one without fever, which are classified as typhoidal and non-typhoidal *Salmonella,* respectively. Typhoidal *Salmonella* is caused by *Salmonella enterica* subsp. *enterica* serovar Typhi, which commonly causes illnesses in humans in the eastern part of the world. Non-typhoidal *Salmonella* such as, *Salmonella enterica* subsp. *enterica* serovar Enteritidis, and *Salmonella enterica* subsp. *enterica* serovar Newport are known to commonly cause gastroenteritis and are frequently associated with consumption of contaminated food.

When contaminated food or water is ingested, *Salmonella* cells infect the intestinal epithelium causing gastrointestinal disease. Upon invasion of the epithelial cells, infection is spread to subsequent cells and internalization within phagocytes occur. Pathogenesis in *Salmonella* is regulated by virulence factors that are encoded on multiple *Salmonella* Pathogenicity Islands (SPIs). Virulence genes located on *Salmonella* pathogenicity islands 1 to 5 are required for entry and survival inside the host cells (65, 66). Pathogenicity islands 1 and 2 encode for Type III secretion systems (TSS3) (67), which are molecular syringes designed to deliver effector proteins into hosts cells. When foods contaminated with *Salmonella* are ingested, they swim with the help of flagella and attaches to the M cells of the Peyer's patches in the intestinal epithelium by the means of adhesion encoded on SPI-3 and SPI-4 (68). Upon attachment, *Salmonella* injects its effector proteins SipA and SopE (encoded on SPI-1 and SPI-5) into M cells via a type III secretion system, which leads to perturbation of the host cells resulting

in the engulfment of *Salmonella.* Upon ingestion, *Salmonella* cells are protected inside a vacuole known as *Salmonella*-containing vacuole (SCV). Once inside the host cells, effector proteins such as SifA are ejected outside the vacuole to provide an extra layer of protective coating. This coating protects the enclosed *Salmonella* from host enzymes such as lysozyme. *Salmonella* cells start to replicate inside the *Salmonella* containing vacuole eventually leading to subsequent release from the vacuole and spread of *Salmonella* to other cells in the Peyer's patches. This leads to infection of numerous intestinal M-cells causing symptoms, such as intestinal inflammation and diarrhea (69). Also, dissemination of *Salmonella* cells into mesenteric lymph nodes occur via the intestinal lymph with spread mediated by dendritic cells. Dendritic cells are known to directly take up *Salmonella* cells from intestinal lumen, which are again phagocytosed and internalized within SCVs. *Salmonella* containing phagocytes mainly macrophages are disseminated systemically leading to spread of the pathogen to spleen and liver (70).

Gastroenteritis resulting from *Salmonella* is self-limiting alleviated by proper rehydration, however, it has been found that in about 5% of the population, *Salmonella* infections may lead to severe complications, such as bacteremia causing osteomyelitis, meningitis, and endovascular infections (71, 72). The infectious dose of *Salmonella* is estimated to be as low as 10-100 cells as observed in foodborne disease outbreaks (73-75). Infectious dose has been observed to vary by food types. Foods rich in fatty acids, such as chocolate, and peanut butter were found to contain low number of *Salmonella* cells in outbreak implicated food samples (73, 75). Also, risks of Salmonellosis are observed to be higher in immunocompromised patients, children and elderly population (76).

1.2.6. Salmonella contamination on plants

Consumption of fresh produce that may have been contaminated present a greater risk for foodborne illnesses because they are consumed raw without the existence of an intermediate kill step. Pathogens from contaminated soil can transfer to vegetable crops via several means, such as during rain or irrigation splash events (77, 78). Dust generated during application of manure in field can also be spread via wind to nearby farms. Spread of *E. coli* O157:H7 from animal feedlot surface manure to nearby lettuce farms was investigated. It was found that lettuce were positive for the same strains of *E. coli* up to a distance of 600 feet (79). Bird droppings and animal activities have also been found to be associated to spread of pathogens to produce commodities. Gardner *et al*., reported that peas were contaminated by feeding Sandhill cranes in farms leading to outbreaks in humans due to *Campylobacter jejuni* originating from feces of cranes (80). Similarly, strawberries were contaminated from feces of deer visiting the farms leading to an outbreak by the consumption of strawberries contaminated *E. coli* O157:H7 (81). Use of contaminated irrigation water is another possible route for transfer of pathogens to produce fields and sources of water used for irrigation are known to be contaminated with *Salmonella* and *E. coli* O157:H7 (82-84).

After transfer to leafy greens, such as spinach or lettuce, these pathogens can persist on the leaves for several days (77). Several factors are known to contribute to the survival of pathogens on spinach or lettuce leaves. One of the major factors is the exposure to UV light. Oni *et al.,* showed that the transfer of *Salmonella* by dried turkey manure dust on spinach leaves protected *Salmonella* cells (cocktails of three *S. enterica* strains: Typhimurium CVM-98 and LT-2 and Enteritidis 13076) from direct exposure to UV light leading to increased persistence (85). It is also possible that the *Salmonella* cells were pre-adapted to desiccation conditions in the

dried turkey manure, leading to enhanced stress survival on the leaves. In the same study, a significant reduction was observed on the adaxial surface of the leaves compared to the abaxial surfaces with a 5-log reduction obtained in 14 days. Increased relative humidity has also been shown to aid in the survival of *Salmonella* spp. on cantaloupes (86). Few other studies have investigated the survival of *E. coli* O157:H7 on spinach leaves and it was found that a 4 log reduction was observed within 24 h with no samples observed as positive for *E. coli* O157 by an enrichment method (87). Several other factors, such as increase in precipitation or transfer of nutrients from soil to leaves during a splash event has not been well studied. Mostly, transfer of pathogens from phosphate buffer or irrigation water to leafy greens have been studied, which may be significantly different from the survival of soil-adapted *Salmonella* on leaves. Pre-exposure to soil may lead to increased survival and hence transfer studies using soil-adapted pathogens are essential. Soil-adapted *Salmonella* spp. have been found to transfer to tuber crops, such as carrots and radishes during cultivation leading to numerous contaminated samples (88, 89). Persistence of *Salmonella* Montevideo on tomatoes and tomatillos were associated to formation of biofilm leading to increased attachment and it was found that relative humidity and temperature significantly affected the pathogen attachment (90, 91). Similar, studies are needed for pathogen survival on leafy greens.

1.2.7. Manure

Biological soil amendments of animal origin (BSAAO), such as manure, provide essential nutrients for plant growth (92-94) and also helps to maintain several soil properties, such as water retention, aeration, drainage, and structure (95). Such manure consists of feces or feces mixed with urine from cattle (beef, dairy), pigs, and horses that are collected in lagoons. Chicken litter are also used as manure and is usually a mixture of beddings (hay), left-over feed

and chicken feces. Cattle manure is also known to contain bedding materials, such as hay, straw or husks. However, BSAAO can be contaminated with human pathogens, such as *Salmonella*, enterohemorrhagic *E. coli*, and *Campylobacter* (96-98). These pathogens are normal inhabitants of animals and shedding via feces can lead to high pathogen levels in feces (97, 99, 100). Pathogenic bacteria at levels as high as 5 logs CFU/g can be found in manure with their number varying among manure types (96, 101-103). Use of untreated BSAAO (Biological Soil Amendment of Animal Origin) (i.e. manure that has not been composted or heat treated) can lead to contamination of soil with possible pathogen transfer from contaminated soil to fresh fruits and vegetables causing foodborne disease outbreaks in humans (98). Several other outbreaks due to *S.* Newport related to contaminated produce, such as cucumbers (2014) and tomatoes (2005) have been attributed to environmental reservoirs, potentially contaminated by animal manure (8, 9). Therefore, such untreated manure needs to be treated or composted properly to reduce the number of pathogens to an acceptable level. Besides reduction of pathogens, composting also helps in nutrient stabilization, reduces odor and potential attraction of vectors like insects to crop fields.

Proper composting of manure can be performed by following the guidelines and recommendations made by USDA National Organic Program (NOP) (104). The NOP requires the initial levels of carbon and nitrogen (C:N) in a ratio between 25:1 and 40:1 before composting process begins. The manure is then required to be maintained at a temperature of 55°C to 77°C for 3 days when using aerated static pile systems or a closed vessel. When using a windrow piling of raw manure, the temperature is to be maintained for 15 days with a minimum of five times of pile turning during the period. Such practice is known to reduce pathogens to low levels (105, 106), however, it is unclear if such strict practices are adhered to on all the farms.

When proper composting measures are followed compost samples have been found to be devoid of pathogens from composting operations (107). Farmers are still known to use untreated BSAAO (108), and use of untreated BSAAO are subjected to guidelines developed by the United States Department of Agriculture National Organic Program. According to the guidelines, farmers using untreated BSAAO can harvest crops only 120 days after the application of manure if the edible portion of the plants are in contact with the soil. If the edible portions of plants are not in direct contact with the soil, the crop can be harvested after 90 days of application of manure. However, 58% of farmers producing organic crops who participated in a recent nationwide survey was found to be using raw manures and 10% of these farmers were not found to comply with the NOP guidelines (109). While the Food and Drug Administration (FDA) has no objection to the NOP rule, the FDA has yet to provide their guidelines regarding the use of raw manure. FDA is waiting for the collection of more scientific evidence and data to support the release of a more robust guidelines (110).

The quantity of manure generation is on the rise due to the increasing number of cattle and chicken farms. The huge quantity of manure generation is being recognized as an emerging environmental issue leading to pollution of land and water sources. Therefore, alternative methods are being investigated to properly utilize manure. Besides composting, manures, such as poultry litter can be heat-treated to reduce the number of pathogens. Poultry litter is generated in large quantities in the USA estimated to be ~550 million tons annually (111). If utilized correctly, these litter could be used as BSAAO to fertilize farmlands.

Poultry litter can be heat-treated and pelletized to kill pathogens and stabilize nutrients, such as nitrogen. These HTPP (Heat-treated poultry pellets) are known to contain nitrogen, phosphorous and potassium (N, P, K) in 3:2:3 ratio that can be essential for nutrient supply to

plants. HTPP is also a slow release chemical fertilizer that can provide nutrients to soil over a long period of time. However, heat treatment used to produce HTPP may lead to reduction of native microbial population in HTPP. Lower prevalence of native microbiota in HTPP may be unable to compete against downstream *Salmonella* contamination problems when used in soil as a BSAAO. Also, slow release of chemical nutrients may provide nutrient supply for pathogen survival over an increased period. Previously, it has been observed that sterile amendments (biosolids) lead to increased *Salmonella* growth as compared to non-sterile biosolids (112). Holly et al, showed that soil containing heat-treated poultry compost had increased death rate of *Salmonella*, which may have been due to higher concentrations of heavy metals as reported but not necessarily the use of heat-treated compost (113). Assessment of HTPP as a soil amendment is yet to be investigated. It is important to understand the impact of HTPP in the survival of *Salmonella* in contaminated soil with its potential transfer and survival on leafy greens.

1.2.8. Manure runoff

Manure from animal farms or cattle feed lot operations can runoff during a rain or storm event to water sources or crop fields. Increased concentration of coliform have been observed previously after such events in water bodies (114). Leaching of phosphorous and nitrogen from farms containing poultry litter amendments to water streams have been investigated after rain events leading to eutrophication (115). Use of water from these sources for irrigation purposes can lead to contamination of produce (81, 116, 117). For an example, irrigation water canals used for cultivation was found to contain the same strain of *Escherichia coli* O157:H7 associated in romaine lettuce outbreak in 2018 that caused 96 hospitalizations and 5 deaths. In this outbreak, nearby upstream cattle feedlot operations were hypothesized to be the likely source of contamination for the irrigation canals. In 2006, a similar *E. coli* O157:H7 outbreak in lettuce

was attributed to use of irrigation water possibly contaminated from cattle manure (116). Other several outbreaks have been attributed to possible manure runoff. A Shiga toxin-producing *E. coli* outbreak in lettuce in 2005 in Sweden caused 135 illnesses and 11 cases of hemolytic uremic syndrome, which was also associated to a contamination arising from an upstream cattle farm (117). Similarly, public drinking water wells in Walkerton, Canada were possibly contaminated with cattle manure runoff containing *E. coli* O157:H7 from nearby farms following rainfall leading to hundreds of illnesses and multiple deaths (118). Survival and growth of pathogens in manure runoff is inadequately studied. It is likely that pathogens can persist or grow in such matrices over a long period of time. However, their survival and growth characteristics in such matrices are completely unknown. Manure runoff can serve as a potential reservoir for pathogen survival or growth leading to contamination of crops during splash or irrigation events.

1.2.9. Salmonella survival in soil

Salmonella survival in soil is dependent on several abiotic and biotic factors, such as soil and amendment types, irrigation, humidity, temperature, seasons, and geographic locations (119-121). Several studies have shown that *Salmonella* can survive for several months in manure-amended soils (119, 122, 123). Islam et al, showed *Salmonella* Typhimurium survival for 231 days in soil containing several types of manure composts when inoculated with contaminated irrigated water (124). Similarly, dairy manure amended soil stored at 25°C with high moisture content of ~80 % showed *Salmonella* Newport survival for 107 days (123). A three strain mixture of *Salmonella enterica* strains (Enteritidis, Heidelberg, and Typhimurium) survived for 77 and 14 days, respectively, in poultry compost and poultry compost that were heat-treated when stored at 22°C (125). Usually, higher moisture content and addition of manure has been shown to significantly increase *Salmonella* survival in clay soil (113). Some studies have shown

increase in *Salmonella* population in soil containing various types of amendments. In almond orchard soil containing almond hull extracts, *Salmonella* Enteritidis PT30 populations was shown to increase by ~3.0 log CFU (126). Similarly, Reynnels et al, showed increase in *Salmonella* spp. populations by 1.5 log CFU during storage at 25°C over 3 days period in various composts types (biosolids, manure and yard waste) (127). Growth of *Salmonella* can also occur during storage of manure compost and mainly when the compost is sterile and subsequently contaminated with *Salmonella*. Kim et al showed that storage of autoclaved composts with high moisture content (40 and 50%) led to increase in *Salmonella* spp. counts by 4 log CFU over 3 days period (128). This indicates that improper storage of manure that may be exposed to *Salmonella* contamination lead to increase in their numbers due to reduced competition from native microbiota but increased availability of nutrients.

1.2.10. Role of rpoS in Salmonella survival in soil

Bacteria survival under stressful conditions require regulation of stress response genes that may help cope with the encountered stressors in their environments. Transcription of genes are needed for bacterial growth under normal or stressful conditions. RNA polymerase binds to the promoter of genes that needs to be transcribed. Various sigma factors (σ) interacts with RNA polymerase to form a holoenzyme to initiate transcription (129). *Salmonella* has six known sigma factors with σ^D (σ^{70}) responsible for transcription of housekeeping genes during normal growth and other sigma factors, such as σ^E (σ^{24}), σF (σ^{28}), σH (σ^{32}), σS (σ^{38}), and σ^N (σ^{54}) are known to regulate transcription of genes associated with extracytoplasmic extreme heat stress, the flagellar expression, heat shock, starvation or stationary phase, and nitrogen limitation, respectively (130). Sigma factor σS (σ^{38}), which encodes the gene *rpoS* is also known to regulate

as many as 500 genes that are recognized as part of the general stress response (131). *Salmonella* utilizes *rpoS* gene for increased survival under several environmental conditions.

Survival of pathogens in the soil can be challenging for several reasons, such as low availability of nutrients, competition from native microbes and predation from protozoa, exposure to desiccation, low pH, and the presence of heavy metals. Stress response genes may play an important role for pathogen survival during these stressful conditions as evidenced by role of *rpoS* under osmotic stress and low temperature (132). Previously, presence of *rpoS* gene have been attributed to prolonged *Salmonella* survival in soil amended with aged broiler litter (133, 134). Similar role of *rpoS* has also been observed for *E. coli* O157:H7 survival in manure-amended soil where a mutation in *rpoS* was observed with a decreased survival (<155 days) as compared to populations without mutations (>200 days) (133, 134). On the other hand, mutation in *rpoS* in *Salmonella* Dublin, Oranienburg, and Typhimurium LT2 strains were attributed to rapid induction of viable but non culturable state in 7% NaCl (135). However, the role of *rpoS* in the survival of *Salmonella* Newport in soil amended with HTPP mimicking farm-like conditions is unknown. Cells when under physiological stress are known to enter viable but non-culturable (VBNC) state and role of *rpoS* in the formation of VBNC state under a growth chamber condition in soil or soil amended with HTPP is unknown.

1.2.11. Viable but non-culturable Salmonella

Bacteria are present in a myriad of environments and only recently many bacteria have been known to exist in environments that were not considered to harbor any microorganisms. Advancements in molecular methods have been able to classify some of these organisms into specific genus or species. However, only a handful of them have been successfully grown in the laboratories on a media culture. This explains that cells are viable in the environment but are

unable to grow in a media culture. This may be due to lack of nutrients or required metabolic components in the media cultures that are not able to support the growth of these microorganisms. However, some bacteria, such as, *Vibrio cholera*, *Salmonella* and *E. coli* have been well studied and they are easily grown on media cultures. But, these bacteria upon exposure to certain stressful conditions or nutrient limitations are unable to grow on media cultures even when they are shown to be viable under microscopic observations, by polymerase chain reactions (PCR), or other physiological tests (136). This state of bacterial cells is known as viable but non-culturable (VBNC). The existence of VBNC cells were debated for a long period of time. However, currently more than 60 microbial species have been studied to enter into such as state (137). The reason for induction of VBNC is shown to differ among bacteria. Xu et al., showed that *Escherichia coli* and *Vibrio cholera* were found to be in VBNC state in estuarine and marine environment (138). CJ et al., showed that the *Vibro parahaemolyticus* cells entered VBNC state in six weeks when exposed to Morita mineral salt-0.5% NaCl medium and incubated at 4°C (139). Besides, irregular temperatures and osmotic stresses, several other stressors, such as acid stress, oxygen concentrations, food preservatives, and heavy metals have been shown to cause VBNC state in microorganisms (140, 141). It is unknown as to how these microbes enter such a state of being. A very few studies have considered studying and understanding the molecular mechanisms of such phenomenon. Lai CJ et al., (2009) showed increased expression of protein profiles, such as alpha transcription (subunit of RNA polymerase), translation (ribosomal protein S1, elongation factor TU and EF-G), gluconeogenesis-related metabolism, ATP synthase, and antioxidants during VBNC state for *Vibrio parahaemolyticus* (139).

Survival of *Salmonella* in soil can be stressful leading to difficulty in recovering of physiologically stressed cells on traditional agar plates. Also, lack of *rpoS* may result in the

induction of VBNC as observed previously under osmotic condition (135). Recovery of cell that are non-viable on agar plates may be quantified using quantitative polymerase chain reaction (qPCR) or using live/dead staining kit. Studies have recovered higher number of cells compared to traditional methods using qPCR (120, 142). In addition, Propidium-monoazide dye is used with qPCR (PMA-qPCR) to quantify live cells but exclude dead cells (143-145). However, use of PMA dye can be erroneous due to various other chemical present in the soil. So, many studies have quantified *Salmonella* in soil using qPCR but without the addition of PMA dye. Garcia et. al., showed significant *Salmonella* recovery using qPCR as compared to plate count methods from both soil amended with dairy cow manure or unamended soil stored at temperatures of 5, 15 and 25°C (120). However, in their study, quantification dead cells could have likely contributed to greater differences between their two methods of *Salmonella* quantification. Recovery of viable but non-culturable cells have been quantified for other pathogens, such as *E. coli* O157:H7 and *Listeria monocytogenes* (144, 145). These studies showed greater recovery of *L. monocytogenes* using PMA-qPCR from contaminated pig manure stored at 8 and 20°C as compared to the plate count method (144). Similarly, survival of *E. coli* O157:H7 on spinach leaves were observed to be higher by as much as 4 log CFU when quantified by PMA-qPCR as compared to plate count methods (143).

1.3. In Summary

We know that foodborne pathogens encounter several stresses through its passage to the gastrointestinal (GI) system, however, they are able to survive these challenges and cause foodborne infections. *Listeria monocytogenes* is one of these foodborne pathogens. Various strains of *L. monocytogenes* may show differences in survival or growth in the GI system due to their genetic diversities. Knowledge of such differences in phenotypic characteristics under bile

stresses are lacking for *L. monocytogenes* strains. My experiments will quantify survival/growth characteristics of six *L. monocytogenes* strains in bile, one of the stresses encountered in the GI system, to understand variability among *L. monocytogenes* strains to survive/grow in bile. In addition, *L. monocytogenes* may have been pre-exposed to salt stress in foods, and upon passage through the GI system they encounter bile stress. We wanted to understand if cells of *L. monocytogenes* pre-exposed to salt show increased growth in bile stress. This would provide increased knowledge on variation in survival/growth among *L. monocytogenes* strains. Moreover, it is possible that *L. monocytogenes* encounter both salt and bile stresses in the GI system, bile from the gall bladder and salt stress from the consumed food. We wanted to understand cross-protection phenotypes from pre-exposure to bile and treatment with salt stress while exploring changes in cell morphology to understand *L. monocytogenes* physiological mechanism to stress adaptation.

Similarly, *Salmonella enterica* is another foodborne pathogen that is able to survive in various stressful environments leading to contamination of food during cultivation. Consumption of such contaminated foods, mainly leafy greens which may be consumed raw, is known to cause illnesses in humans. However, there are adequate gaps in our knowledge about *Salmonella* survival in soil leading to transfer and survival on plants. In this study, I wanted to determine the number of days *S.* Newport could survive in soil while investigating the role of *rpoS* gene, irrigation events, formation of viable and non-culturable cells, and HTPP- a as biological soil amendment. This would improve our understanding on implementation of proper measures for control of *Salmonella* at pre-harvest conditions on the farms. Prolonged survival of *Salmonella* in soil may lead to increased possibility of *Salmonella* transfer to plants during cultivation. I also investigated the transfer of *Salmonella* from inoculated soil to spinach plants grown in the same

soil. In addition, I studied the survival of *Salmonella* on spinach leaves while investigating roles of *rpoS* genes, the presence of HTPP on the leaves, and increased moisture on leaves-mimicking rain events. The results from this study shall enhance our knowledge on understanding factors that affect survival and transfer of *Salmonella* to leafy greens. Insights from these studies shall provide a strong foundation for further research and present a strong case for the implementation of new guidelines that may be beneficial for safe production of leafy green vegetables, such as spinach and lettuce.

1.4. Specific Aims and Organization of the Dissertation

There were three major specific aims explored in this dissertation project that are presented in chapters II, III and IV with Chapter I consisting of general introduction, summary of dissertation structure, and literature review. The first aim of this dissertation was to understand survival and cross-protection phenotypes under salt and bile stresses for multiple strains of *Listeria monocytogenes,* which are discussed in chapter II. The second aim of this dissertation was to quantify the survival and growth of *Salmonella* Newport in soil extracts prepared using Heat-treated poultry pellets that are discussed in Chapter III. The third and final aims of this dissertation were to understand the impact of abiotic and biotic factors in the survival of *S.* Newport in HTPP amended soil and subsequent transfer to spinach plants that are discussed in Chapter IV.

1.5. References

1. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis 17:7-15.
2. RA G. Medical Microbiology. Chapter: 21, *Salmonella*, 4th Edition. ed.

3. Linke K, Rückerl I, Brugger K, Karpiskova R, Walland J, Muri-Klinger S, Tichy A, Wagner M, Stessl B. 2014. Reservoirs of *Listeria* species in three environmental ecosystems. Appl Environ Microbiol 80:5583-92.
4. Ferreira V, Wiedmann M, Teixeira P, Stasiewicz MJ. 2014. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J Food Prot 77:150-70.
5. Chlebicz A, Śliżewska K. 2018. Campylobacteriosis, Salmonellosis, Yersiniosis, and Listeriosis as Zoonotic Foodborne Diseases: A Review. Int J Environ Res Public Health 15.
6. Auld H, MacIver D, Klaassen J. 2004. Heavy rainfall and waterborne disease outbreaks: the Walkerton example. J Toxicol Environ Health A 67:1879-87.
7. Klontz KC, Klontz JC, Mody RK, Hoekstra RM. 2010. Analysis of tomato and jalapeño and Serrano pepper imports into the United States from Mexico before and during a National Outbreak of *Salmonella* serotype Saintpaul infections in 2008. J Food Prot 73:1967-74.
8. Greene SK, Daly ER, Talbot EA, Demma LJ, Holzbauer S, Patel NJ, Hill TA, Walderhaug MO, Hoekstra RM, Lynch MF, Painter JA. 2008. Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005. Epidemiol Infect 136:157-65.
9. Angelo KM, Chu A, Anand M, Nguyen TA, Bottichio L, Wise M, Williams I, Seelman S, Bell R, Fatica M, Lance S, Baldwin D, Shannon K, Lee H, Trees E, Strain E, Gieraltowski L, (CDC) CfDCaP. 2015. Outbreak of *Salmonella* Newport infections linked to cucumbers--United States, 2014. MMWR Morb Mortal Wkly Rep 64:144-7.

10. Bergholz TM, den Bakker HC, Fortes ED, Boor KJ, Wiedmann M. 2010. Salt stress phenotypes in *Listeria monocytogenes* vary by genetic lineage and temperature. Foodborne Pathog Dis 7:1537-49.

11. Malekmohammadi S, Kodjovi KK, Sherwood J, Bergholz TM. 2017. Genetic and environmental factors influence *Listeria monocytogenes* nisin resistance. J Appl Microbiol.

12. Shabala L, Lee SH, Cannesson P, Ross T. 2008. Acid and NaCl limits to growth of *Listeria monocytogenes* and influence of sequence of inimical acid and NaCl levels on inactivation kinetics. Journal of Food Protection 71:1169-1177.

13. Begley M, Gahan CG, Hill C. 2002. Bile stress response in *Listeria monocytogenes* LO28: adaptation, cross-protection, and identification of genetic loci involved in bile resistance. Applied and environmental microbiology 68:6005-6012.

14. Freitag NE, Port GC, Miner MD. 2009. *Listeria monocytogenes* - from saprophyte to intracellular pathogen. Nat Rev Microbiol 7:623-8.

15. de Noordhout CM, Devleesschauwer B, Angulo FJ, Verbeke G, Haagsma J, Kirk M, Havelaar A, Speybroeck N. 2014. The global burden of listeriosis: a systematic review and meta-analysis. Lancet Infect Dis 14:1073-1082.

16. Guillet C, Join-Lambert O, Le Monnier A, Leclercq A, Mechaï F, Mamzer-Bruneel M-F, Bielecka MK, Scortti M, Disson O, Berche P. 2010. Human listeriosis caused by *Listeria ivanovii.* Emerging infectious diseases 16:136.

17. Zhang C, Zhang M, Ju J, Nietfeldt J, Wise J, Terry PM, Olson M, Kachman SD, Wiedmann M, Samadpour M, Benson AK. 2003. Genome diversification in phylogenetic

lineages I and II of *Listeria monocytogenes*: identification of segments unique to lineage II populations. J Bacteriol 185:5573-84.

18. Orsi RH, den Bakker HC, Wiedmann M. 2011. *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. Int J Med Microbiol 301:79-96.

19. Chen Y, Gonzalez-Escalona N, Hammack TS, Allard MW, Strain EA, Brown EW. 2016. Core Genome Multilocus Sequence Typing for Identification of Globally Distributed Clonal Groups and Differentiation of Outbreak Strains of *Listeria monocytogenes*. Appl Environ Microbiol 82:6258-6272.

20. Cossart P. 2011. Illuminating the landscape of host-pathogen interactions with the bacterium *Listeria monocytogenes*. Proc Natl Acad Sci U S A 108:19484-91.

21. Radoshevich L, Cossart P. 2018. *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. Nat Rev Microbiol 16:32-46.

22. Archambaud C, Nahori M-A, Pizarro-Cerda J, Cossart P, Dussurget O. 2006. Control of Listeria superoxide dismutase by phosphorylation. Journal of Biological Chemistry.

23. Boneca IG, Dussurget O, Cabanes D, Nahori M-A, Sousa S, Lecuit M, Psylinakis E, Bouriotis V, Hugot J-P, Giovannini M. 2007. A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system. Proceedings of the National Academy of Sciences 104:997-1002.

24. Dramsi S, Cossart P. 2002. Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite. The Journal of cell biology 156:943-946.

25. Tilney LG, Portnoy DA. 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. The Journal of cell biology 109:1597-1608.

26. Sheehan B, Klarsfeld A, Msadek T, Cossart P. 1995. Differential activation of virulence gene expression by *PrfA*, the *Listeria monocytogenes* virulence regulator. Journal of bacteriology 177:6469-6476.

27. Pouillot R, Klontz KC, Chen Y, Burall LS, Macarisin D, Doyle M, Bally KM, Strain E, Datta AR, Hammack TS, Van Doren JM. 2016. Infectious Dose of *Listeria monocytogenes* in Outbreak Linked to Ice Cream, United States, 2015. Emerg Infect Dis 22:2113-2119.

28. Toledo-Arana A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, Balestrino D, Loh E, Gripenland J, Tiensuu T, Vaitkevicius K, Barthelemy M, Vergassola M, Nahori MA, Soubigou G, Régnault B, Coppée JY, Lecuit M, Johansson J, Cossart P. 2009. The *Listeria* transcriptional landscape from saprophytism to virulence. Nature 459:950-6.

29. Ridlon JM, Kang DJ, Hylemon PB. 2006. Bile salt biotransformations by human intestinal bacteria. J Lipid Res 47:241-59.

30. Dowd GC, Joyce SA, Hill C, Gahan CG. 2011. Investigation of the mechanisms by which *Listeria monocytogenes* grows in porcine gallbladder bile. Infect Immun 79:369-79.

31. White SJ, McClung DM, Wilson JG, Roberts BN, Donaldson JR. 2015. Influence of pH on bile sensitivity amongst various strains of *Listeria monocytogenes* under aerobic and anaerobic conditions. J Med Microbiol 64:1287-96.

32. Sewell D, Allen SCh, Phillips CA. 2015. Oxygen limitation induces acid tolerance and impacts simulated gastro-intestinal transit in *Listeria monocytogenes* J0161. Gut Pathog 7:11.

33. Wright ML, Pendarvis K, Nanduri B, Edelmann MJ, Jenkins HN, Reddy JS, Wilson JG, Ding X, Broadway PR, Ammari MG, Paul O, Roberts B, Donaldson JR. 2016. The Effect of Oxygen on Bile Resistance in *Listeria monocytogenes*. J Proteomics Bioinform 9:107-119.

34. Cornu M, Beaufort A, Rudelle S, Laloux L, Bergis H, Miconnet N, Serot T, Delignette-Muller M. 2006. Effect of temperature, water-phase salt and phenolic contents on *Listeria monocytogenes* growth rates on cold-smoked salmon and evaluation of secondary models. International Journal of Food Microbiology 106:159-168.

35. Cotter PD, Gahan CG, Hill C. 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Molecular microbiology 40:465-475.

36. Cotter PD, Hill C. 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiology and molecular biology reviews 67:429-453.

37. Ryan S, Begley M, Gahan CG, Hill C. 2009. Molecular characterization of the arginine deiminase system in *Listeria monocytogenes*: regulation and role in acid tolerance. Environmental microbiology 11:432-445.

38. Begley M, Sleator RD, Gahan CG, Hill C. 2005. Contribution of three bile-associated loci, b*sh*, *pva,* and *btlB*, to gastrointestinal persistence and bile tolerance of Listeria monocytogenes. Infection and Immunity 73:894-904.

39. Sleator RD, Watson D, Hill C, Gahan CG. 2009. The interaction between *Listeria monocytogenes* and the host gastrointestinal tract. Microbiology 155:2463-2475.

40. Chan YC, Raengpradub S, Boor KJ, Wiedmann M. 2007. Microarray-based characterization of the *Listeria monocytogenes* cold regulon in log- and stationary-phase cells. Appl Environ Microbiol 73:6484-98.

41. Chan YC, Hu Y, Chaturongakul S, Files KD, Bowen BM, Boor KJ, Wiedmann M. 2008. Contributions of two-component regulatory systems, alternative sigma factors, and negative regulators to *Listeria monocytogenes* cold adaptation and cold growth. J Food Prot 71:420-5.

42. Liu X, Basu U, Miller P, McMullen LM. 2014. Stress response and adaptation of *Listeria monocytogenes* 08-5923 exposed to a sublethal dose of carnocyclin A. Appl Environ Microbiol 80:3835-41.

43. Chassaing D, Auvray F. 2007. The *lmo1078* gene encoding a putative UDP-glucose pyrophosphorylase is involved in growth of *Listeria monocytogenes* at low temperature. FEMS Microbiol Lett 275:31-7.

44. Abram F, Starr E, Karatzas KA, Matlawska-Wasowska K, Boyd A, Wiedmann M, Boor KJ, Connally D, O'Byrne CP. 2008. Identification of components of the sigma B regulon in *Listeria monocytogenes* that contribute to acid and salt tolerance. Appl Environ Microbiol 74:6848-58.

45. Fraser KR, Sue D, Wiedmann M, Boor K, O'Byrne CP. 2003. Role of sigmaB in regulating the compatible solute uptake systems of *Listeria monocytogenes*: osmotic induction of opuC is sigmaB dependent. Appl Environ Microbiol 69:2015-22.

46. Bergholz TM, Tang S, Wiedmann M, Boor KJ. 2013. Nisin resistance of *Listeria monocytogenes* is increased by exposure to salt stress and is mediated via LiaR. Applied and environmental microbiology 79:5682-5688.

47. Bergholz TM, Bowen B, Wiedmann M, Boor KJ. 2012. *Listeria monocytogenes* shows temperature-dependent and -independent responses to salt stress, including responses that induce cross-protection against other stresses. Appl Environ Microbiol 78:2602-12.

48. Sleator RD, Gahan CG, Hill C. 2003. A postgenomic appraisal of osmotolerance in *Listeria monocytogenes*. Applied and Environmental Microbiology 69:1-9.

49. O'Driscoll B, Gahan CG, Hill C. 1996. Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl Environ Microbiol 62:1693-8.

50. Hingston P, Chen J, Dhillon BK, Laing C, Bertelli C, Gannon V, Tasara T, Allen K, Brinkman FS, Truelstrup Hansen L, Wang S. 2017. Genotypes Associated with *Listeria monocytogenes* Isolates Displaying Impaired or Enhanced Tolerances to Cold, Salt, Acid, or Desiccation Stress. Front Microbiol 8:369.

51. Rychli K, Grunert T, Ciolacu L, Zaiser A, Razzazi-Fazeli E, Schmitz-Esser S, Ehling-Schulz M, Wagner M. 2016. Exoproteome analysis reveals higher abundance of proteins linked to alkaline stress in persistent *Listeria monocytogenes* strains. Int J Food Microbiol 218:17-26.

52. Lee S, Ward TJ, Jima DD, Parsons C, Kathariou S. 2017. The Arsenic Resistance-Associated *Listeria* Genomic Island LGI2 Exhibits Sequence and Integration Site Diversity and a Propensity for Three *Listeria monocytogenes* Clones with Enhanced Virulence. Appl Environ Microbiol 83.

53. Harter E, Wagner EM, Zaiser A, Halecker S, Wagner M, Rychli K. 2017. Stress Survival Islet 2, Predominantly Present in *Listeria monocytogenes* Strains of Sequence Type 121, Is Involved in the Alkaline and Oxidative Stress Responses. Appl Environ Microbiol 83.

54. CDC. 2018. Foodborne Illnesses and Germs. https://www.cdc.gov/foodsafety/foodborne-germs.html. Acessed 2019.

55. Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM. 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. Emerg Infect Dis 19:407-15.

56. *CDC. Updated 2018. Reports of Selected Salmonella Outbreak Investigations.* https://www.cdc.gov/salmonella/outbreaks.html. *Accessed*

57. FDA. 2018. Recalls, Market Withdrawals, & Safety Alerts.

58. Scharff RL. 2012. Economic burden from health losses due to foodborne illness in the United States. J Food Prot 75:123-31.

59. Goepfert JM, Iskander IK, Amundson CH. 1970. Relation of the heat resistance of *Salmonellae* to the water activity of the environment. Appl Microbiol 19:429-33.

60. Farmer JJI. 1995. *Enterobacteriaceae*: Introduction and identification. Manual of clinical microbiology, Sixth edition:438-449.

61. Dolman CE. 1969. Theobald Smith, 1859-1934. Life and work. N Y State J Med 69:2801-16.

62. Popoff MY, and L. Le Minor. 1997. Antigenic formulas of the *Salmonella* serovars, 7th revision. W.H.O. Collaborating Centre for Reference and Research on *Salmonella*.

63. Baron EJ. 1996. Classification. In S. Baron et al., eds. Baron's Medical Microbiology, 4th edition. University of Texas Medical Branch. ISBN 0963117211.

64. Judicial Commission of the International Committee on Systematics of Prokaryotes. 2005. The type species of the genus *Salmonella* Lignieres 1900 is *Salmonella enterica* (ex Kauffmann and Edwards 1952) Le Minor and Popoff 1987, with the type strain

LT2T, and conservation of the epithet enterica in *Salmonella enterica* over all earlier epithets that may be applied to this species. Opinion 80. Int J Syst Evol Microbiol 55:519-20.

65. Galán JE. 1996. Molecular and cellular bases of *Salmonella* entry into host cells. Curr Top Microbiol Immunol 209:43-60.

66. Shea JE, Hensel M, Gleeson C, Holden DW. 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. Proc Natl Acad Sci U S A 93:2593-7.

67. Galán JE, Wolf-Watz H. 2006. Protein delivery into eukaryotic cells by type III secretion machines. Nature 444:567-73.

68. Fàbrega A, Vila J. 2013. *Salmonella enterica* serovar Typhimurium skills to succeed in the host: virulence and regulation. Clin Microbiol Rev 26:308-41.

69. Zhang, S., Kingsley, R. A., Santos, R. L., Andrews-Polymenis, H., Raffatellu, M., Figueiredo, J., ... & Bäumler, A. J. (2003). Molecular pathogenesis of *Salmonella enterica* serotype Typhimurium-induced diarrhea. Infection and immunity 71:1-12.

70. Ohl ME, Miller SI. 2001. *Salmonella*: a model for bacterial pathogenesis. Annu Rev Med 52:259-74.

71. Chiu CH, Su LH, Chu C. 2004. *Salmonella enterica* serotype Choleraesuis: epidemiology, pathogenesis, clinical disease, and treatment. Clin Microbiol Rev 17:311-22.

72. Acheson D, Hohmann EL. 2001. Nontyphoidal salmonellosis. Clinical Infectious Diseases 32:263-269.

73. Kasuga F, Hirota M, Wada M, Yunokawa T, Toyofuku H, Shibatsuji M, Michino H, Kuwasaki T, Yamamoto S, Kumagai S. 2004. Archiving of food samples from restaurants and caterers--quantitative profiling of outbreaks of foodborne salmonellosis in Japan. J Food Prot 67:2024-32.

74. Teunis PF, Kasuga F, Fazil A, Ogden ID, Rotariu O, Strachan NJ. 2010. Dose-response modeling of *Salmonella* using outbreak data. Int J Food Microbiol 144:243-9.

75. Hockin JC, Daoust JY, Bowering D, Jessop JH, Khanna B, Lior H, Milling ME. 1989. An International outbreak of *Salmonella* Nima from imported chocolate. Journal of Food Protection 52:51-54.

76. Braden C. 2012. Suspecting Foodborne illnesses in special populations: Quick Facts for providers. CDC expert commentary. Atlanta, Georgia: USA.

77. Waitt JA, Kuhn DD, Welbaum GE, Ponder MA. 2014. Postharvest transfer and survival of *Salmonella enterica* serotype enteritidis on living lettuce. Lett Appl Microbiol 58:95-101.

78. Olaimat AN, Holley RA. 2012. Factors influencing the microbial safety of fresh produce: a review. Food microbiology 32:1-19.

79. Berry ED, Wells JE, Bono JL, Woodbury BL, Kalchayanand N, Norman KN, Suslow TV, López-Velasco G, Millner PD. 2014. Effect of proximity to a cattle feedlot on *Escherichia coli* O157: H7 contamination of leafy greens and evaluation of the potential for airborne transmission. Applied and environmental microbiology:AEM. 02998-14.

80. Gardner TJ, Fitzgerald C, Xavier C, Klein R, Pruckler J, Stroika S, McLaughlin JB. 2011. Outbreak of campylobacteriosis associated with consumption of raw peas. Clinical Infectious Diseases 53:26-32.

81. Laidler MR, Tourdjman M, Buser GL, Hostetler T, Repp KK, Leman R, Samadpour M, Keene WE. 2013. *Escherichia coli* O157:H7 infections associated with consumption of locally grown strawberries contaminated by deer. Clin Infect Dis 57:1129-34.

82. Duffy E, Lucia L, Kells J, Castillo A, Pillai S, Acuff G. 2005. Concentrations of *Escherichia coli* and genetic diversity and antibiotic resistance profiling of *Salmonella* isolated from irrigation water, packing shed equipment, and fresh produce in Texas. Journal of food protection 68:70-79.

83. Gannon VP, Graham T, Read S, Ziebell K, Muckle A, Mori J, Thomas J, Selinger B, Townshend I, Byrne aJ. 2004. Bacterial pathogens in rural water supplies in southern Alberta, Canada. Journal of Toxicology and Environmental Health, Part A 67:1643-1653.

84. Johnson JY, Thomas JE, Graham TA, Townshend I, Byrne J, Selinger LB, Gannon VP. 2003. Prevalence of *Escherichia coli* O157:H7 and *Salmonella spp.* in surface waters of southern Alberta and its relation to manure sources. Can J Microbiol 49:326-35.

85. Oni RA, Sharma M, Buchanan RL. 2015. Survival of *Salmonella enterica* in Dried Turkey Manure and Persistence on Spinach Leaves. J Food Prot 78:1791-9.

86. Stine SW, Song I, Choi CY, Gerba CP. 2005. Effect of relative humidity on preharvest survival of bacterial and viral pathogens on the surface of cantaloupe, lettuce, and bell peppers. J Food Prot 68:1352-8.

87. Markland SM, Shortlidge KL, Hoover DG, Yaron S, Patel J, Singh A, Sharma M, Kniel KE. 2013. Survival of pathogenic *Escherichia coli* on basil, lettuce, and spinach. Zoonoses Public Health 60:563-71.

88. Islam M, Doyle MP, Phatak SC, Millner P, Jiang X. 2004. Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley

grown in fields treated with contaminated manure composts or irrigation water. J Food Prot 67:1365-70.

89. Natvig EE, Ingham SC, Ingham BH, Cooperband LR, Roper TR. 2002. *Salmonella enterica* serovar Typhimurium and Escherichia coli contamination of root and leaf vegetables grown in soils with incorporated bovine manure. Appl Environ Microbiol 68:2737-44.

90. Iturriaga MH, Escartin EF, Beuchat LR, Martinez-Peniche R. 2003. Effect of inoculum size, relative humidity, storage temperature, and ripening stage on the attachment of *Salmonella* Montevideo to tomatoes and tomatillos. Journal of food protection 66:1756-1761.

91. Iturriaga MH, Tamplin ML, Escartín EF. 2007. Colonization of tomatoes by *Salmonella* Montevideo is affected by relative humidity and storage temperature. J Food Prot 70:30-4.

92. Tewolde H, McLaughlin MR, Way TR, Jenkins JN. 2016. Optimum Poultry Litter Rates for Maximum Profit versus Yield in Cotton Production. Crop Science 56:3307-3317.

93. Tewolde H, Sistani KR, McLaughlin MR. 2016. Residual Effect of Poultry Litter Applications on No-Till Cotton Lint Yield. Agronomy Journal 108:1405-1414.

94. Flavel TC, Murphy DV. 2006. Carbon and nitrogen mineralization rates after application of organic amendments to soil. J Environ Qual 35:183-93.

95. Rosen CJ, Allan DL. 2007. Exploring the benefits of organic nutrient sources for crop production and soil quality. HortTechnology 17:422-430.

96. Chinivasagam HN, Redding M, Runge G, Blackall PJ. 2010. Presence and incidence of food-borne pathogens in Australian chicken litter. Br Poult Sci 51:311-8.

97. Fukushima H, Seki R. 2004. High numbers of Shiga toxin-producing *Escherichia coli* found in bovine faeces collected at slaughter in Japan. FEMS Microbiol Lett 238:189-97.

98. Mukherjee A, Speh D, Dyck E, Diez-Gonzalez F. 2004. Preharvest evaluation of coliforms, *Escherichia coli, Salmonella*, and *Escherichia coli* O157:H7 in organic and conventional produce grown by Minnesota farmers. J Food Prot 67:894-900.

99. Mughini-Gras L, Enserink R, Friesema I, Heck M, van Duynhoven Y, van Pelt W. 2014. Risk factors for human salmonellosis originating from pigs, cattle, broiler chickens and egg laying hens: a combined case-control and source attribution analysis. PloS one 9:e87933.

100. Wells J, Shipman L, Greene K, Sowers E, Green J, Cameron D, Downes F, Martin M, Griffin P, Ostroff S. 1991. Isolation of *Escherichia coli* serotype O157: H7 and other Shiga-like-toxin-producing *E. coli* from dairy cattle. Journal of Clinical Microbiology 29:985-989.

101. Létourneau V, Duchaine C, Côté C, Letellier A, Topp E, Massé D. 2010. Presence of zoonotic pathogens in physico-chemically characterized manures from hog finishing houses using different production systems. Bioresour Technol 101:4048-55.

102. Volkova VV, Bailey RH, Wills RW. 2009. *Salmonella* in broiler litter and properties of soil at farm location. PLoS One 4:e6403.

103. Shepherd MW, Liang P, Jiang X, Doyle MP, Erickson MC. 2010. Microbiological analysis of composts produced on South Carolina poultry farms. J Appl Microbiol 108:2067-76.

104. Anonymous. 2011. National Organic Program.

105. Lung A, Lin C-M, Kim J, Marshall M, Nordstedt R, Thompson N, Wei C. 2001. Destruction of *Escherichia coli* O157: H7 and *Salmonella* Enteritidis in cow manure composting. Journal of food protection 64:1309-1314.

106. Wichuk KM, McCartney D. 2007. A review of the effectiveness of current time–temperature regulations on pathogen inactivation during composting. Journal of Environmental Engineering and Science 6:573-586.

107. Edrington TS, Fox WE, Callaway TR, Anderson RC, Hoffman DW, Nisbet DJ. 2009. Pathogen prevalence and influence of composted dairy manure application on antimicrobial resistance profiles of commensal soil bacteria. Foodborne pathogens and disease 6:217-224.

108. Harrison JA, Gaskin JW, Harrison MA, Cannon JL, Boyer RR, Zehnder GW. 2013. Survey of food safety practices on small to medium-sized farms and in farmers markets. J Food Prot 76:1989-93.

109. Pires AF, Millner PD, Baron J, Jay-Russell MT. 2018. Assessment of Current Practices of Organic Farmers Regarding Biological Soil Amendments of Animal Origin in a Multi-regional US Study. Food Protection Trends 38:347-362.

110. FDA. 2016. United States Food and Drug Administration. 2016. Risk Assessment of foodborne illness associated with pathogens from produce grown in fields amended with untreated biological soil amendments of animal origin; request for scientific data, information, and comments. Fed. Reg. Vol. 81, No. 43/Friday, March 4, 2016, 11572–11574.

111. Coker C. 2017. Poultry Litter Digestion. https://www.biocycle.net/2017/09/07/poultry-litter-digestion/. Accessed 2019

112. Sidhu J, Gibbs RA, Ho GE, Unkovich I. 2001. The role of indigenous microorganisms in suppression of *Salmonella* regrowth in composted biosolids. Water Res 35:913-20.

113. Holley RA, Arrus KM, Ominski KH, Tenuta M, Blank G. 2006. *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure. J Environ Qual 35:1170-80.

114. Kunkle SH. Concentrations and cycles of bacterial indicators in farm surface runoff, p. *In* (ed),

115. Schroeder P, Radcliffe D, Cabrera M. 2004. Rainfall timing and poultry litter application rate effects on phosphorus loss in surface runoff. Journal of Environmental Quality 33:2201-2209.

116. Sodha SV, Lynch M, Wannemuehler K, Leeper M, Malavet M, Schaffzin J, Chen T, Langer A, Glenshaw M, Hoefer D, Dumas N, Lind L, Iwamoto M, Ayers T, Nguyen T, Biggerstaff M, Olson C, Sheth A, Braden C. 2011. Multistate outbreak of *Escherichia coli* O157:H7 infections associated with a national fast-food chain, 2006: a study incorporating epidemiological and food source traceback results. Epidemiol Infect 139:309-16.

117. Söderström A, Osterberg P, Lindqvist A, Jönsson B, Lindberg A, Blide Ulander S, Welinder-Olsson C, Löfdahl S, Kaijser B, De Jong B, Kühlmann-Berenzon S, Boqvist S, Eriksson E, Szanto E, Andersson S, Allestam G, Hedenström I, Ledet Muller L, Andersson Y. 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. Foodborne Pathog Dis 5:339-49.

118. Kondro W. 2000. *E. coli* outbreak deaths spark judicial inquiry in Canada. Lancet 355:2058.

119. Sharma M, Millner PD, Hashem F, Camp M, Whyte C, Graham L, Cotton CP. 2016. Survival and Persistence of Nonpathogenic *Escherichia* coli and Attenuated *Escherichia coli* O157:H7 in Soils Amended with Animal Manure in a Greenhouse Environment. J Food Prot 79:913-21.

120. García R, Baelum J, Fredslund L, Santorum P, Jacobsen CS. 2010. Influence of temperature and predation on survival of *Salmonella enterica* serovar Typhimurium and expression of invA in soil and manure-amended soil. Appl Environ Microbiol 76:5025-31.

121. Palacios MP, Lupiola P, Tejedor MT, Del-Nero E, Pardo A, Pita L. 2001. Climatic effects on *Salmonella* survival in plant and soil irrigated with artificially inoculated wastewater: preliminary results. Water Sci Technol 43:103-8.

122. Semenov AV, van Overbeek L, Termorshuizen AJ, van Bruggen AH. 2011. Influence of aerobic and anaerobic conditions on survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in Luria-Bertani broth, farm-yard manure and slurry. J Environ Manage 92:780-7.

123. You Y, Rankin SC, Aceto HW, Benson CE, Toth JD, Dou Z. 2006. Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. Appl Environ Microbiol 72:5777-83.

124. Islam M, Morgan J, Doyle MP, Phatak SC, Millner P, Jiang X. 2004. Persistence of *Salmonella enterica* serovar typhimurium on lettuce and parsley and in soils on which

they were grown in fields treated with contaminated manure composts or irrigation water. Foodborne Pathog Dis 1:27-35.

125. Chen Z, Kim J, Jiang X. 2018. Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* in animal waste-based composts as influenced by compost type, storage condition and inoculum level. J Appl Microbiol 124:1311-1323.

126. Danyluk MD, Nozawa-Inoue M, Hristova KR, Scow KM, Lampinen B, Harris LJ. 2008. Survival and growth of *Salmonella* Enteritidis PT 30 in almond orchard soils. J Appl Microbiol 104:1391-9.

127. Reynnells R, Ingram DT, Roberts C, Stonebraker R, Handy ET, Felton G, Vinyard BT, Millner PD, Sharma M. 2014. Comparison of U.S. Environmental Protection Agency and U.S. Composting Council microbial detection methods in finished compost and regrowth potential of Salmonella spp. and *Escherichia coli* O157:H7 in finished compost. Foodborne Pathog Dis 11:555-67.

128. Kim J, Jiang X. 2010. The growth potential of *Escherichia coli* O157:H7, *Salmonella spp.* and *Listeria monocytogenes* in dairy manure-based compost in a greenhouse setting under different seasons. Journal of Applied Microbiology 109:2095-2104.

129. Borukhov S, Nudler E. 2003. RNA polymerase holoenzyme: structure, function and biological implications. Curr Opin Microbiol 6:93-100.

130. Gruber TM, Gross CA. 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. Annu Rev Microbiol 57:441-66.

131. Hengge R. 2009. Proteolysis of sigmaS (*RpoS*) and the general stress response in Escherichia coli. Res Microbiol 160:667-76.

132. McMeechan A, Roberts M, Cogan TA, Jørgensen F, Stevenson A, Lewis C, Rowley G, Humphrey TJ. 2007. Role of the alternative sigma factors sigmaE and sigmaS in survival of *Salmonella enterica* serovar Typhimurium during starvation, refrigeration and osmotic shock. Microbiology 153:263-9.

133. Chen Z, Jiang X. 2017. Thermal Resistance and Gene Expression of both Desiccation-Adapted and Rehydrated *Salmonella enterica* Serovar Typhimurium Cells in Aged Broiler Litter. Appl Environ Microbiol 83.

134. van Hoek AH, Aarts HJ, Bouw E, van Overbeek WM, Franz E. 2013. The role of *rpoS* in *Escherichia coli* O157 manure-amended soil survival and distribution of allelic variations among bovine, food and clinical isolates. FEMS Microbiol Lett 338:18-23.

135. Kusumoto A, Asakura H, Kawamoto K. 2012. General stress sigma factor RpoS influences time required to enter the viable but non-culturable state in *Salmonella enterica*. Microbiol Immunol 56:228-37.

136. Lleò MM, Pierobon S, Tafi MC, Signoretto C, Canepari P. 2000. mRNA detection by reverse transcription-PCR for monitoring viability over time in an *Enterococcus faecalis* viable but nonculturable population maintained in a laboratory microcosm. Appl Environ Microbiol 66:4564-7.

137. Oliver JD, Bockian R. 1995. In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Appl Environ Microbiol 61:2620-3.

138. Xu HS, Roberts N, Singleton FL, Attwell RW, Grimes DJ, Colwell RR. 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb Ecol 8:313-23.

139. Lai CJ, Chen SY, Lin IH, Chang CH, Wong HC. 2009. Change of protein profiles in the induction of the viable but nonculturable state of *Vibrio parahaemolyticus*. Int J Food Microbiol 135:118-24.

140. Kana BD, Gordhan BG, Downing KJ, Sung N, Vostroktunova G, Machowski EE, Tsenova L, Young M, Kaprelyants A, Kaplan G, Mizrahi V. 2008. The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth in vitro. Mol Microbiol 67:672-84.

141. Ghezzi JI, Steck TR. 1999. Induction of the viable but non-culturable condition in *Xanthomonas campestris* pv. campestris in liquid microcosms and sterile soil. FEMS Microbiol Ecol 30:203-208.

142. Bech TB, Johnsen K, Dalsgaard A, Laegdsmand M, Jacobsen OH, Jacobsen CS. 2010. Transport and distribution of *Salmonella enterica* serovar Typhimurium in loamy and sandy soil monoliths with applied liquid manure. Appl Environ Microbiol 76:710-4.

143. Moyne AL, Harris LJ, Marco ML. 2013. Assessments of total and viable *Escherichia coli* O157:H7 on field and laboratory grown lettuce. PLoS One 8:e70643.

144. Desneux J, Biscuit A, Picard S, Pourcher AM. 2016. Fate of Viable but Non-culturable *Listeria monocytogenes* in Pig Manure Microcosms. Front Microbiol 7:245.

145. Zhao X, Zhong J, Wei C, Lin CW, Ding T. 2017. Current Perspectives on Viable but Non-culturable State in Foodborne Pathogens. Front Microbiol 8:580.

2. VARIATION IN GROWTH AND EVALUATION OF CROSS-PROTECTION IN *LISTERIA MONOCYTOGENES* IN SALT AND BILE STRESS

2.1. Abstract

Exposure of *Listeria monocytogenes* to osmotic stress can induce increased resistance to subsequent lethal exposure to cell envelope stressors, such as nisin and bile salts. We wanted to determine if similar cross-protection phenotypes could occur when *L. monocytogenes* strains were treated with osmotic stress and exposed to sub-lethal levels of the cell envelope stressor, bile. Growth phenotypes were measured for six *L. monocytogenes* strains exposed to 6% NaCl, 0.3 and 1% bile in BHI. To evaluate cross protection, cells were pre-exposed to 6% NaCl, followed by exposure to BHI + 1% bile for 26 h and vice-versa. Significant increases in λ (lag phase) and doubling time were observed under salt and bile stresses compared to BHI alone. Average λ and N_{max} (maximum cell density) in 0.3 and 1% bile for all strains were significantly lower than that in 6% NaCl. Pre-exposure to 6% NaCl followed by exposure to 1% bile significantly increased λ ($p<0.05$) whereas pre-exposure to 1% bile followed by exposure to 6% NaCl led to formation of filamentous cells, with no changes in cell density over 26 h. Variation in growth characteristics were observed among strains exposed to bile. Exposure to osmotic stress did not lead to increased resistance to bile. Exposure to bile significantly impacted the ability of *L. monocytogenes* to adapt to grow under osmotic stress, where cells did not multiply but formed filamentous cells. Pre-exposure to a cell envelope stress and subsequent exposure to an osmotic stress may have the potential to control *L. monocytogenes* growth.

2.2. Keywords

Listeria monocytogenes, Variation in growth phenotype, salt and bile stresses, Cross-protection, filament formation

2.3. Introduction

Listeria monocytogenes is a saprotroph that can cause listeriosis in susceptible humans and animals through consumption of contaminated foods (1). Globally, *L. monocytogenes* is estimated to cause 23,150 illnesses, 5,463 deaths, and a loss of 172,823 Disability-adjusted life year (DALYs) annually (2). In the United States, *L. monocytogenes* is estimated to cause 1,591 illnesses, 1,455 hospitalizations, and 255 deaths annually (3). Infections in humans are predominantly acquired via oral exposure, and *L. monocytogenes* must be able to survive transit through the gastrointestinal tract to reach the initial site of infection in the intestinal epithelium (4).

The robust stress adaptive mechanisms employed by *L. monocytogenes* contribute to its ability not only to survive but grow under a variety of suboptimal conditions (5-8). These conditions include those that *L. monocytogenes* may encounter in the extra-host as well as in the host environment. For example, growth of *L. monocytogenes* has been observed at pH of 4.1 (9), at salt concentrations >10% (10), and in bile extracted from porcine gall bladders (11). During its passage through the gastrointestinal system, *L. monocytogenes* encounters acidic stress, osmotic stress, bile stress, and low availability of oxygen. As *L. monocytogenes* may experience these stresses sequentially during GI transit, exposure to one of these stresses has the potential to increase resistance to a subsequent stressor.

Phenotypes evident of cross protection have been observed for *L. monocytogenes* exposed to a range of stressors. For example, exposure to low pH and organic acids can increase resistance to compounds that target the cell envelope, such as lauric arginate, poly-lysine, and nisin (12-14). Prior exposure to low pH, however, did not lead to cross-protection against bile, which can also be considered a cell envelope stressor (15). Osmotic stress has the potential to

provide increased resistance to subsequent lethal levels of bile (16) possibly because adaptation to both stresses are known to involve *sigB* and SigB regulated genes (7, 17, 18).

Bile is composed of cholic acid and chenodeoxycholic acids that are synthesized from cholesterol in hepatocytes and conjugated with glycine or taurine before secretion from the liver. The pH of bile in gall bladder is 7.0 – 8.5, however, in the small intestine a lower pH of 4.0 – 7.0 is observed when it mixes with the food containing the gastric juice. Several studies have shown that bile can have antimicrobial properties at lower pH whereas growth of *L. monocytogenes* is observed to occur in bile at neutral pH, such as in the gall bladder (11, 15). In addition, anaerobic conditions can increase survival of *L. monocytogenes* during exposure to bile stresses (15, 19, 20).

L. monocytogenes strains are grouped into 13 serotypes, 4 lineages, and many clonal complexes. However, 90% of listeriosis in humans are caused by serotypes 1/2b and 4b (lineage I) and 1/2a (lineage II) (21) with lineages III and IV consisting of isolates from ruminants, environment and sporadic cases of foodborne illnesses (21-23). Several studies have investigated differences in stress tolerances among these groups (22, 24). For example, it was previously observed that lineage I strains were able to grow significantly faster than lineage II strains under salt stress (25), and that *L. monocytogenes* serotype 4b strains were more salt tolerant than serotypes 1/2a and 1/2b (26). In another study, tolerances to cold (4°C), salt (6%) and pH (5.0) was investigated for a total of 166 isolates to examine associations between stress tolerances, serotypes, and clonal complexes (8). On average, serotypes 1/2a and 1/2b were found to be more resistant to cold (4°C) than serotypes 4b and 1/2c but no significant differences were observed between different serotypes under salt stress 6%. In the same study, however, there were multiple clonal complexes among which significant differences in stress tolerances could be

observed for all stresses (8). These studies show that stress tolerances may vary among different strains belonging to serotypes, lineages and clonal complexes.

It has been observed that pre-exposure to salt provides resistance against other stresses. For example, exposure to salt stress lead to cross protection against low temperature (27) and bile salts (16). Pre-exposure to NaCl has also shown increased resistance against nisin, another cell-envelope stressor (28). However, the effect of salt and bile stresses on growth kinetics of *L. monocytogenes* strains has not been characterized. Variation in phenotypic characteristics among *L. monocytogenes* have been studied under salt stress and nisin stress (7, 25, 29) whereas it is lacking under various concentrations of bile. The objectives of this study were to i) characterize the growth parameters of multiple *L. monocytogenes* strains exposed to salt and bile stress, and ii) examine if cross-protection occurs upon pre-exposure to salt and treatment with bile and vice-versa.

2.4. Materials and Methods

2.4.1 Bacterial strains

Six bacterial strains, three each from lineage I (G6054, FSL J1-0194, and H7858) and lineage II (FSL R2-0559, J0161, and FSL F2-0515) were obtained from the Food Safety Lab at Cornell University (Table 1). The bacterial strains were stored at -80ºC in brain heart infusion broth (BHI) with 15% glycerol.

2.4.2. Growth and survival assays

The six strains were obtained from -80ºC freezer stocks and were streaked for isolation onto Brain Heart Infusion agar (BHIA) plates and incubated at 37ºC overnight. For each strain, an isolated colony was transferred to 5 ml BHI broth and incubated at 37ºC without shaking for 16 h.

Table 1. *L. monocytogenes* strains used in this study

strains	serotype	lineage	clonal complex
G6054	1/2b	I	CC3
FSL J1-0194	1/2b	I	CC88
H7858	4b	I	CC6
FSL R2-0559	1/2a	II	CC11
J0161	1/2a	II	CC11
FSL F2-0515	1/2a	II	NA

-NA' indicates not available

This culture was inoculated into flasks containing 75 ml of i) BHI, ii) BHI with 6% sodium chloride (NaCl) (BD Difco, Franklin Lakes, NJ), iii) BHI with 0.3% bile, and iv) BHI with 1% bile (Porcine bile extract, Sigma-Aldrich, St. Louis, MO) at pH 7.0 to obtain a final *L. monocytogenes* concentration of ~10^4 log CFU/ml. The flasks were incubated non-shaking at 37ºC for 26 h. For enumeration of bacterial density, flasks were sampled periodically at regular intervals through 26 h incubation. 100 µl samples were diluted in Butterfield's dilution buffer and plated in duplicate on BHIA plates which were incubated at 37ºC for 24-48 h. The colonies were counted using Q-count model 350 (Spiral Biotech). For cross-protection studies, strains were i) first exposed to 6% NaCl in BHI for 30 min at 37ºC then transferred to 0.3 or 1 % bile in BHI and ii) first exposed to BHI + 1% bile for 30 min at 37ºC then transferred to BHI + 6% NaCl. Briefly, overnight culture was inoculated into flasks containing 75 ml of BHI + 6% NaCl at 1/100,000 dilution and incubated at 37ºC for 30 min. After 30 min, 7.5 ml of cultures from BHI with 6% NaCl was transferred into flasks containing 67.5 ml of BHI with 0.3 and 1% bile. Similarly, all the strains were exposed to BHI + 1% bile for 30 min and then transferred to BHI with 6% NaCl. The flasks were retrieved at regular intervals and 100 µl samples were diluted

appropriately in Butterfield's dilution buffer. The samples were plated on BHIA plates and were incubated at 37ºC for 24-48 h. Exposure to BHI + 6% NaCl after pre-exposure to BHI + 1% bile did not lead to a change in the count of *L. monocytogenes* and probable injury to these cells were investigated by enumeration of *L. monocytogenes* cells by plating on Modified Oxford *Listeria* agar (MOX) plates (Difco). The MOX plates were incubated at 30ºC for 48 h and the colonies were counted manually.

2.4.3. Scanning electron microscopy

Cell morphology were investigated by taking images of *L. monocytogenes* H7858 cells using scanning electron microscope (SEM) at three conditions i) 6% NaCl with BHI, b) 1% bile with BHI, and iii) pre-exposure to 1% bile for 30 min and treatment at 6% NaCl in BHI. To obtain higher concentration of cells for SEM imagining at an hour post-treatment, *L. monocytogenes* H7858 was inoculated at ca. 10^7 in 6% NaCl in BHI and 1% bile in BHI. For the pre-exposure study, *L. monocytogenes* cells were pre-exposed to 1% bile in BHI for 30 min and treated at 6% NaCl in BHI in duplicates as previously described but inoculated to obtain a final count of ca. 10^6 CFU/ml in 6% NaCl. Samples in the volumes of 75 ml were obtained at 1 h post-treatment for all three conditions. For SEM imaging at 22 h post-treatment, 6% NaCl in BHI and 1% bile in BHI were inoculated at ca. 10^4 CFU/ml resulting in counts of ca. 10^8 CFU/ml at 22h. For the pre-exposure study, duplicate samples prepared as described as above were incubated at 37ºC for 22 h. The samples were centrifuged (Allegra X 30-R Centrifuge) at 5,796g for 10 min. The resulting pellets were washed by suspension in 30 ml PBS and centrifugation for another 10 min. The obtained pellets were suspended in 1 ml PBS and transferred to a 2-ml micro centrifuge tube, which was then centrifuged at 16,000g for one min. The supernatant was discarded and the pellet was topped with 2.5% glutaraldehyde phosphate

buffer as a fixative (Tousimis, Rockville, MD, USA). The supernatant was removed from the fixed pelleted bacteria and they were re-suspended in deionized water and centrifuged at 10,000 rpm for five min. The wash step was performed twice after which 2% osmium tetroxide (OsO_4) was added and samples were refrigerated for two h. The OsO_4 was removed and the bacteria was washed twice with water as described above. After the final wash, most of the supernatant was removed and the bacteria was suspended in ~0.5 ml water. A few drops of each bacterial suspension was pipetted on to a 10-mm round glass coverslip and allowed to air dry at room temperature.

The glass coverslips were attached to cylindrical aluminum mounts using silver paint (Structure Probe Inc., West Chester, PA, USA) and then coated with a conductive layer of carbon in a high-vacuum evaporative coater (Cressington 208c, Ted Pella Inc., Redding, CA, USA). Images and measurements were obtained with a JEOL JSM-7600F SEM (JEOL USA, Inc., Peabody, MA, USA) operating at 2 kV. Each sample was imaged at 5 locations and a minimum of 10 cells in each image were measured for their lengths and widths. The experiment was repeated twice for each of the three conditions described above.

2.4.4. Statistical analysis

The experiment at each growth condition was performed in three replicates for each strain. The plate counts obtained in CFU/ml were transformed into log CFU/ml counts, which were used as input for the Baranyi model in R version 3.4.2 using the 'nlsmicrobio' package to obtain growth parameters: growth rate (μmax), average lag phase (λ), doubling time (DT) and N_{max} (final density) (30) using the equation [$(y_{(t)} = \ln X_{(t)}$] where $x_{(t)}$ is the cell concentration (CFU/ml) at time (t) , and $y_{(t)}$ is the growth rate. The growth parameters over 26 h for all strains were categorized by lineages providing nine data points for each lineage at each condition. Each

growth condition and lineage types I and II were used as the independent variable whereas the growth parameters were used as the dependent variables. Analysis of Variance (ANOVA) was conducted using Proc GLM in SAS V.9.4 (SAS Institute, Cary, NC) and based on adjusted LS means, a p value of <0.05 was used for significant statistical differences. Similarly, ANOVA was conducted for measurements of lengths and width (dependent variables) on two replicates of data imaged at 5 locations at each three conditions: in 6% NaCl, 1% bile, and pre-exposure to 1% bile and treatment with 6% NaCl at two time points (1 and 22 h post treatment) as independent variables in SAS. Similarly, for the analysis of cell measurements, cell lengths and widths were considered as dependent variables whereas the three treatment conditions were treated as independent variables. Based on adjusted LS means, a p value of <0.05 was used for significant statistical differences.

2.5. Results

2.5.1. Increased lag phase and decreased cell densities were observed under salt and bile stresses

Growth of all six *L. monocytogenes* strains in BHI broth alone was used as a control for comparison of growth parameters (Fig. 1A). The average doubling time in BHI broth for lineage I and II strains was 0.65±0.02 and 0.66±0.01 h, respectively, and were not significantly different ($p>0.05$). Similarly, no significant differences were observed in average maximum cell densities for lineage I (9.3 ± 0.1 $\log_{10}$ CFU/ml) and II (9.2 ± 0.1 $\log_{10}$ CFU/ml) strains in BHI broth (Table 2). However, in BHI + 6% NaCl, there was a lag phase of 6.25 ± 2.49 h for lineage I strains and 5.32 ± 0.54 h for lineage II strains ($p>0.05$) with a doubling time of 1.16 ± 0.11 h for lineage I strains and 1.23 ± 0.02 h for lineage II strains (Fig. 1B, Table 2). The maximum cell densities for both lineages in BHI + 6% NaCl were similar to that in BHI ($p>0.05$) (Table 2). Compared to

growth in BHI, all strain in BHI + 6% NaCl had a significantly greater doubling time ($p<0.05$) but similar maximum cell densities ($p>0.05$) (Table 2).

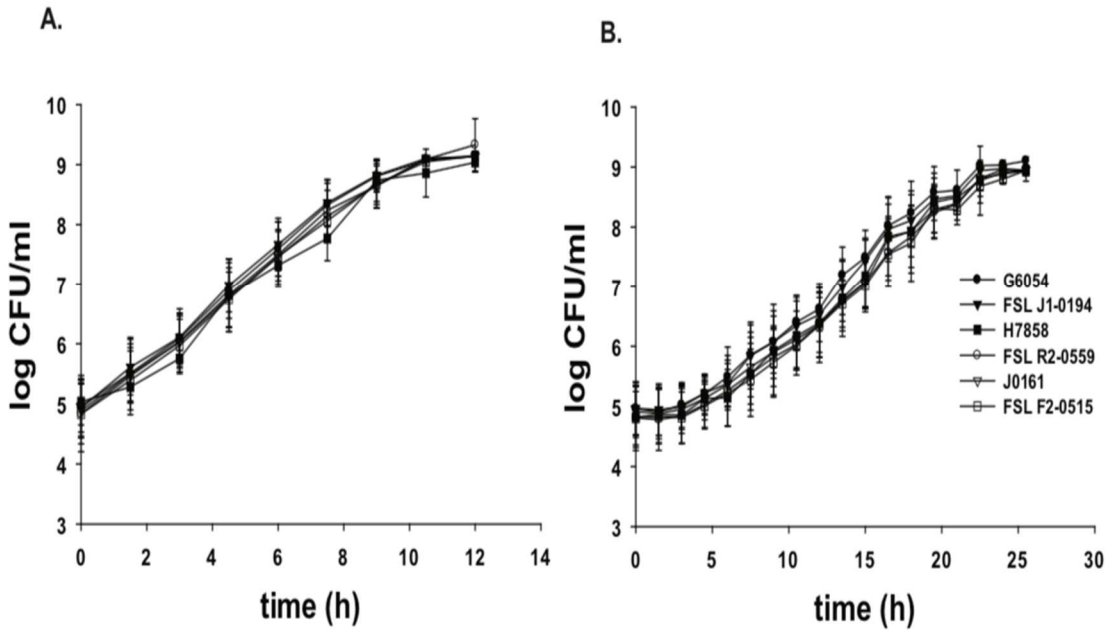

Figure 1. Growth curves of *Listeria monocytogenes* strains lineage I (closed symbols) and lineage II (open symbols) A. in Brain Heart Infusion Broth and B. in BHI with 6% NaCl. The error bars indicate standard deviation from three experimental replicates for each strain.

Exposure to 0.3% and 1% bile in BHI resulted in significantly shorter lag phases than those observed in BHI + 6% NaCl for all strains (Fig. 2A and B, Table 2). During exposure to BHI + 0.3% bile, similar lag phases of 1.24 ± 0.43 h (lineage I) and 1.17 ± 0.49 h (lineage II) were observed (Fig. 2A) ($p>0.05$) whereas significantly longer lag phases were observed for lineage II (3.71±0.76 h) compared to lineage I (1.89 ± 0.64 h) strains in BHI + 1% bile (Fig. 2B) ($p<0.05$). However, exposure to bile led to significantly lower maximum cell densities by c.a. 1-2 log CFU/ml compared to those observed in BHI and BHI + 6% NaCl (Table 2) ($p<0.05$). The observed maximum cell densities in BHI + 0.3% bile were similar for lineage I (8.4 ± 0.1 $\log_{10}$ CFU/ml) and lineage II (8.1 ± 0.2 $\log_{10}$ CFU/ml) strains (Fig. 2A, Table 2) ($p>0.05$). However,

the maximum cell densities in BHI + 1% bile were significantly higher for Lineage I (7.8 ± 0.6 $\log_{10}$ CFU/ml) than lineage II (6.5 ± 0.6 $\log_{10}$ CFU/ml) strains (Fig. 2A, Table 2) ($p<0.05$). In contrast to the difference in maximum cell densities, doubling times were similar for both lineages at both concentrations of bile (Table 2, $p>0.05$). However, the doubling times for both lineages in BHI + 1% bile and only for lineage II in BHI + 0.3% bile were significantly greater than their respective doubling times in BHI + 6% NaCl (Table 2) ($p<0.05$).

Table 2. Growth parameters estimated using the Baranyi model on three replicates of *Listeria monocytogenes* strains under various conditions

	lineage	DT	LOG_{10}NMAX	lag	RSS
BHI	I	0.65 ± 0.02^{A}	9.26 ± 0.09^{A}	NA	0.07±0.08
	II	0.66 ± 001^{A}	9.23 ± 0.08^{A}	NA	0.05±0.02
BHI + 6% NaCl	I	1.16 ± 0.11^{B}	9.12 ± 0.24^{A}	6.25 ± 2.49^{A}	0.23±0.11
	II	$1.23\pm0.02C^{B}$	9.17 ± 0.10^{A}	5.32 ± 0.54^{AB}	0.20±0.14
BHI + 0.3% bile	I	1.38 ± 0.02^{BCD}	8.41 ± 0.09^{BC}	1.24 ± 0.43^{FG}	0.06±0.02
	II	1.53 ± 0.09^{DE}	8.09 ± 0.17^{BC}	1.17 ± 0.49^{G}	0.09±0.05
BHI + 1% bile	I	1.63 ± 0.14^{EC}	7.84 ± 0.55^{D}	1.89 ± 0.64^{EFG}	0.12±0.05
	II	1.32 ± 0.33^{BCD}	6.50 ± 0.61^{F}	3.71 ± 0.76^{CD}	0.35±0.25
BHI + 6% NaCl to BHI + 0.3% bile	I	1.43 ± 0.02^{CDE}	8.5 ± 0.08^{BCD}	1.69 ± 0.52^{EFG}	0.07±0.02
	II	1.48 ± 0.05^{DE}	8.00 ± 0.33^{CD}	2.70 ± 0.61^{DEF}	0.09±0.02
BHI + 6% NaCl to BHI + 1% bile	I	1.61 ± 0.09^{E}	7.38 ± 0.19^{DE}	2.91 ± 0.55^{DE}	0.17±0.09
	II	$1.36\pm0.28B^{CD}$	6.26 ± 0.35^{F}	4.76 ± 0.95^{BC}	0.54±0.37

'NA', indicates Not applicable. Within each column, different superscript letters indicate significantly different values at p=0.05.

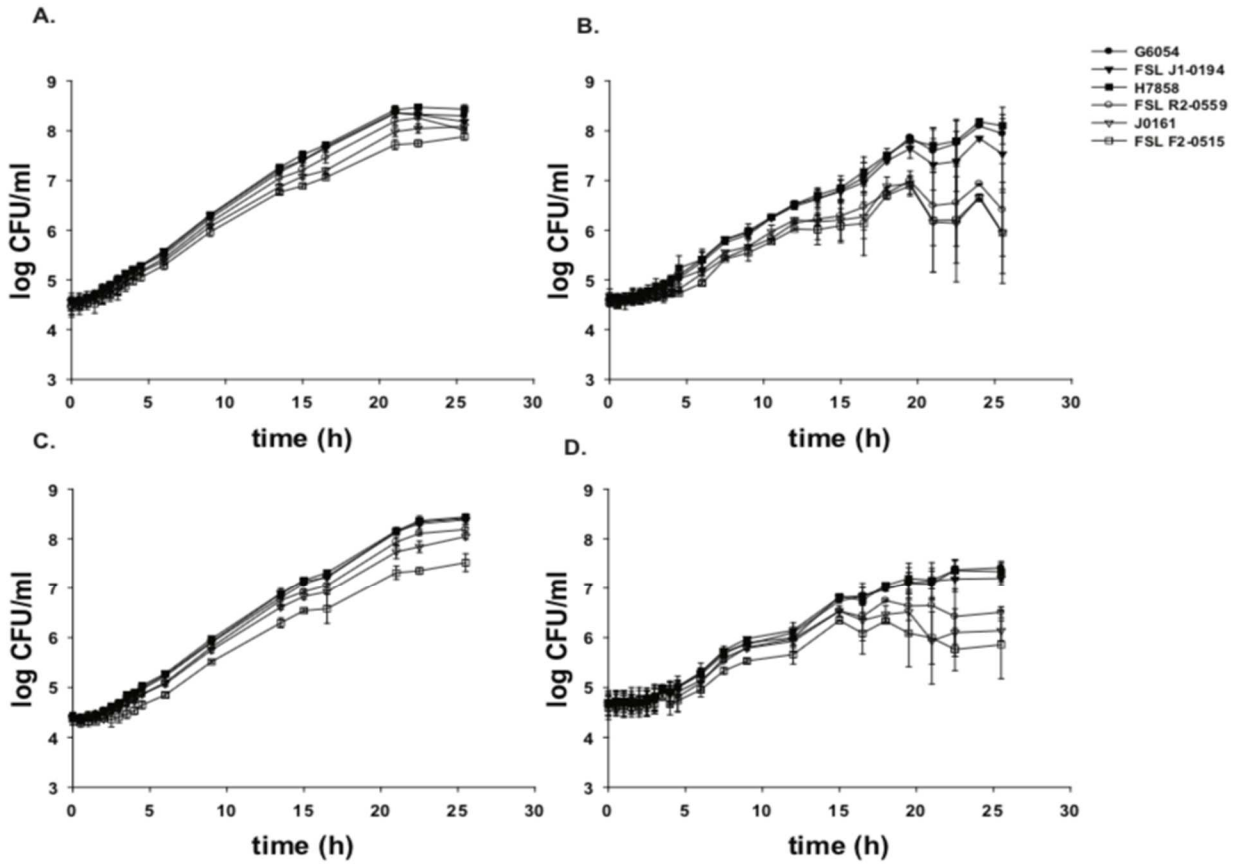

Figure 2. Growth curves of *Listeria monocytogenes* strains lineage I (closed symbols) and lineage II (open symbols) A. in BHI with 0.3% bile, B. in BHI with 1% bile, C. in 0.3% bile in BHI after pre-exposure to 6% NaCl in BHI, and D. in 1% bile in BHI after pre-exposure to 6% NaCl in BHI. The error bars indicate standard deviation from three experimental replicates for each strain.

2.5.2. Pre-exposure to osmotic stress and treatment with bile stresses led to increased lag phase but similar cell densities

To determine if pre-exposure to salt stress influenced bile resistance, *L. monocytogenes* strains were exposed to BHI + 6% NaCl for 30 min and then transferred to 0.3 or 1% bile in BHI at 37°C and cell densities were monitored over time (Fig. 2C and D). Pre-exposure to 6% NaCl followed by exposure to 0.3% bile significantly increased lag phase for lineage II strains (2.70 ± 0.61 h; $p<0.05$) compared to the length of lag phase in BHI + 0.3% bile without pre-exposure to BHI + 6% NaCl. Average lag phase (1.69 ± 0.52 h) for lineage I strains was similar to those

observed in BHI + 0.3% bile without pre-exposure to BHI + 6% NaCl ($p>0.05$). Pre-exposure to 6% NaCl then treatment with 0.3% bile resulted in doubling times of 1.43 ± 0.02 and 1.48 ± 0.05 h for lineage I and II strains, respectively, ($p>0.05$) which were not significantly different from those in BHI + 0.3% bile ($p>0.05$) (Table 2). Similarly, maximum cell densities of 8.5 ± 0.1 (lineage I) and 8.0 ± 0.3 log CFU/ml (lineage II) measured after pre-exposure to 6% NaCl and treatment with 0.3 % bile were not significantly different than those in BHI + 0.3% bile ($p>0.05$) (Fig. 2C).

Pre-exposure to 6% NaCl followed by exposure to 1% bile did not significantly increase the length of lag phase compared to those observed in BHI + 1% bile (Fig. 2D, Table 2) ($p>0.05$). Significant differences in lag phases between lineages I and II were observed in pre-exposure to salt and treatment with 1% bile ($p<0.05$) similar to that in 1% bile (Fig. 2D, Table 2). Pre-exposure to 6% NaCl followed by exposure to BHI + 1% bile did not significantly affect doubling times or maximum cell densities, which were similar to those observed in BHI + 1% bile ($p>0.05$) (Fig. 2D). Significantly longer lag phase (4.76 ± 0.95 h) and lower maximum cell densities (6.3 ± 0.4 log10 CFU/ml) were observed for lineage II strains pre-exposed to BHI + 6% NaCl followed by 1% bile compared to those that were transferred to 0.3 % bile after pre-exposure ($p<0.05$) whereas the values for lineage I strains were similar ($p>0.05$) (Table 2).

2.5.3. Pre-exposure to bile and treatment with NaCl exhibited a bacteriostatic effect on L. monocytogenes

To determine if pre-exposure to bile led to increased resistance to salt stress, *L. monocytogenes* strains were exposed to BHI + 1% bile for 30 min at 37°C followed by transfer to BHI + 6% NaCl at 37 °C and cell densities were monitored over time (Fig. 3). Surprisingly, no changes in cell densities were observed for all strains over 26 h with similar counts of c.a 3-4.0

log CFU/ml observed at 26 h (Fig. 3). To see if the cells were injured under this condition differences in *L. monocytogenes* counts were determined between MOX and BHI agar plates. No differences in counts between MOX and BHIA plates were observed for all six strains pre-exposed to 1% bile and treated with 6% NaCl, indicating that the cells were not injured (Supplementary Table 1). Scanning Electron microscopy was conducted to examine changes in the morphology of *L. monocytogenes* cells (Fig. 4 and 5).

The average lengths of *L. monocytogenes* cells were measured to be 1.80 ± 0.13, 1.65 ± 0.23, and 1.4 ± 0.14 μm in BHI + 6% NaCl, BHI + 1% bile, and pre-exposure to 1% bile for 30 min and treatment with 6% NaCl, respectively at 1 h post treatment (Fig. 4 and 5). At 22 h post treatment, similar average cell lengths of 2.01 ± 0.32 and 2.09 ± 0.15 μm were measured in BHI + 6% NaCl and BHI + 1% bile, respectively ($p>0.05$). However, average cell lengths of 2.87 ± 0.29 μm were observed after pre-exposure to 1% bile for 30 min and treatment with 6% NaCl, which was significantly longer than those in BHI + 6% NaCl or BHI + 1% bile (Fig. 4) ($p<0.05$). Cell sizes as long as 5.12 μm was observed after pre-exposure to 1% bile for 30 min and treatment with 6% NaCl at 22 h post-treatment. All measurements for width remained similar across all three conditions at both 1 and 22 h post treatment time points (Fig. 4 and 5) ($p>0.05$).

2.6. Discussion

Cross protection phenotypes have been demonstrated in *L. monocytogenes* when cells are subsequently exposed to multiple stresses. Studies have shown that pre-exposure to osmotic stress led to increased survival when treated with lethal levels of cell envelope stressors, such as bile salts and nisin (7, 16, 28). Exposure to osmotic stress leads to activation of a number of cellular responses, including compatible solute transport and the general stress response (31). Short-term exposure to 6% NaCl included increased transcript levels of *sigB* and *SigB*-regulated

genes, including *bsh* which encodes bile salt hydrolase (7). However, the potential for cross protection to sub-lethal levels of bile, or the potential for cell envelope stressors to provide cross-protection against osmotic stress has not been examined previously.

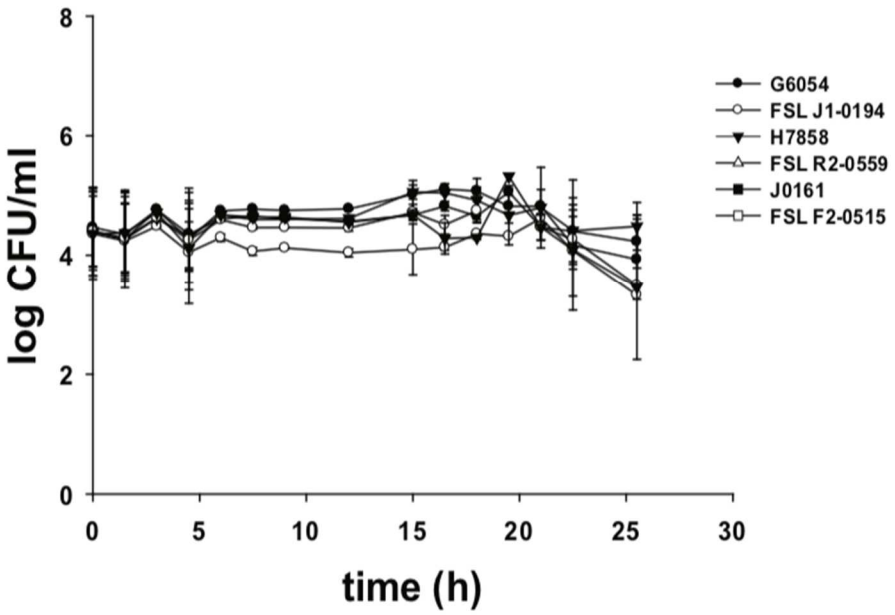

Figure 3. *Listeria monocytogenes* counts in 6% NaCl in BHI after pre-exposure to 1% bile in BHI. The error bars indicate standard deviation from three experimental replicates for each strain

Growth characteristics for six *L. monocytogenes* strains were measured in 6% NaCl to serve as a control before examining the potential for cross-protection from pre-exposure to salt and treatment with 1% bile. In this study, no significant differences were observed in growth characteristics (growth rate, doubling time or lag phases) between lineage I and II strains in BHI + 6% NaCl. A previous study by Bergholz et al., found that lineage I strains had significantly higher average growth rates than lineage II strains at 37°C in BHI + 6% NaCl (25). The collection of strains used in their study was large and hence they could show a more representative distinction between the two lineages. Recently, it has been observed that variation

in growth/survival characteristics may be more accurately differentiated when examining *L. monocytogenes* strains by clonal complexes and not by lineage types.

Supplementary Table 1. Differences in plate counts (BHIA – MOX) for all six strains at the conditions pre-exposure to 1% bile and treatment with 6% NaCl

	Strains					
time (h)	G6054	FSL J1-0194	H7858	FSL R2-0559	J0161	FSL F2-0515
0.0	0.08±0.15	0.10±0.18	0.03±0.08	-0.07±0.14	0.00±0.11	-0.05±0.07
1.5	0.03±0.08	-0.02±0.05	0.01±0.04	-0.05±0.11	0.03±0.02	-0.08±0.15
4.0	0.19±0.23	-0.07±0.04	-0.03±0.15	0.03±0.29	0.29±0.48	0.29±0.08
24.0	0.39±0.17	-0.19±0.07	0.13±0.20	0.04±0.08	0.17±0.92	0.39±0.08
25.5	0.04±0.01	0.07±0.68	-0.56±0.77	-0.22±0.03	-0.18±0.18	-0.02±0.19

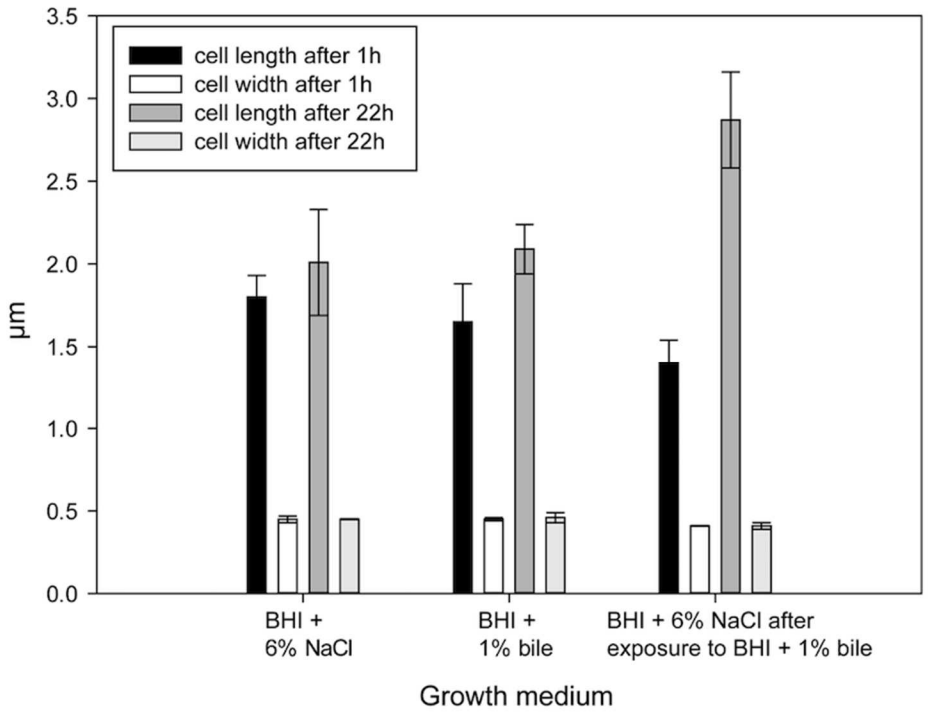

Figure 4. Measurements of *L. monocytogenes* (strain H7858) cell length and width from images obtained from Scanning Electron Microscopy. The error bars indicate standard deviation from a total of six images obtained from two experimental replicates.

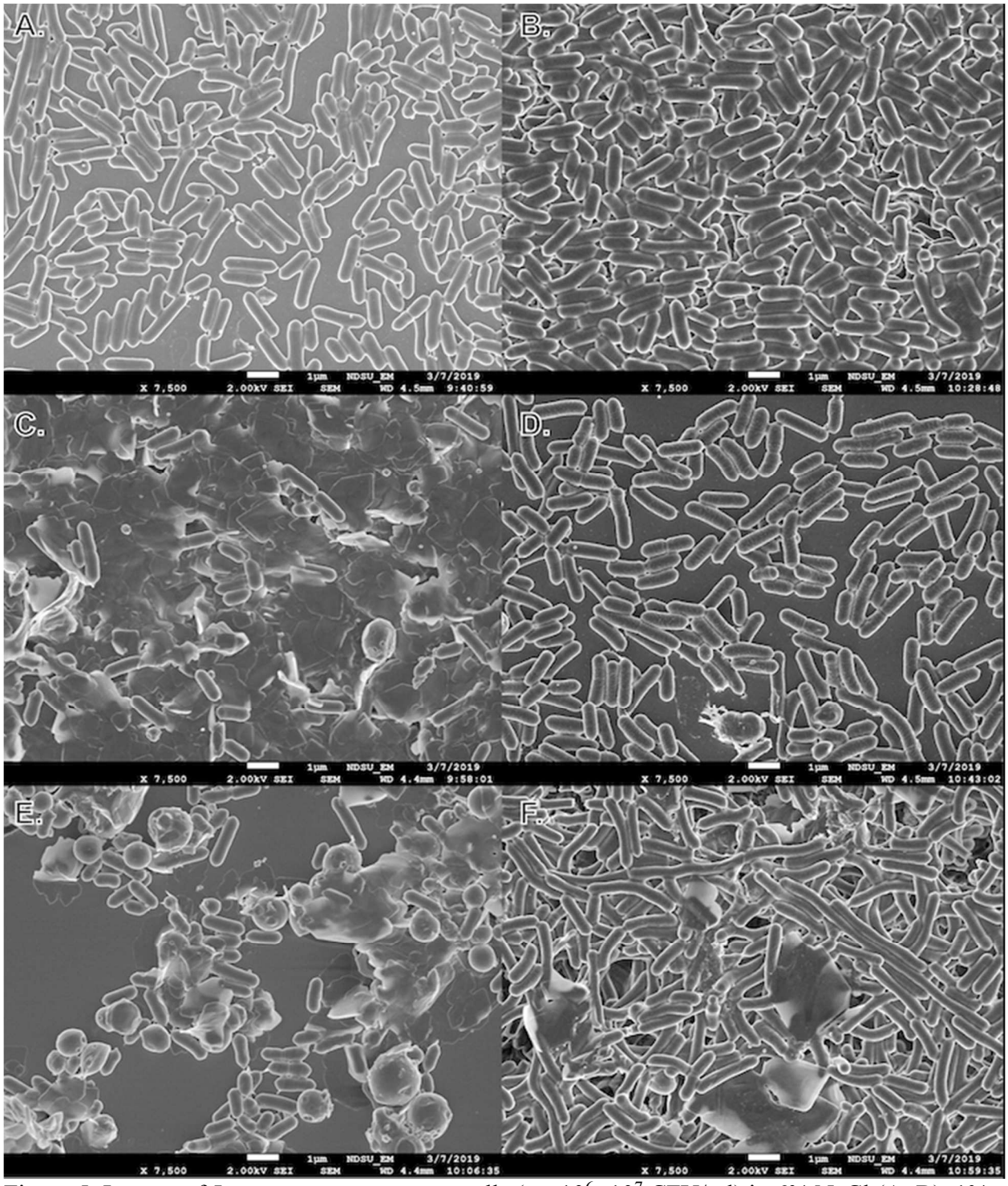

Figure 5. Images of *Listeria monocytogenes* cells (ca. 10^6 -10^7 CFU/ml) in 6% NaCl (A, B), 1% bile (C, D), and pre-exposure to 1% bile in BHI and treatment in 6% NaCl in BHI (E, F); images A, C and E are from 1 h post-treatment and images B, D, and F are from 22 h post-treatment. The scale bar indicates 1 μm, images are representative of two experimental replicates imaged at three locations from each replicate.

For example, Malekmohammadi et al., showed that pre-exposure to 6% NaCl led to increased survival during exposure to nisin (50 ppm) and that the stress tolerance capabilities varied among clonal complexes (29). Tang et al., demonstrated significant differences in growth rates in defined medium at 16°C among clonal complexes (32). Similarly, Hingston et al., observed significant differences in stress tolerance capabilities by clonal complexes for a large set of *L. monocytogenes* strains (8). It can be noted that significant differences in genetic and phenotypic characteristics may be better observed by clonal complexes than among lineage types. However, we did observe significantly higher cell densities and lower lag phases in BHI + 1% bile for lineage I strains compared to lineage II strains.

The majority of available data on *L. monocytogenes* exposed to bile stress are survival rates or overall log reductions of cells exposed to high concentrations of bile. Significant variation in log reductions have been observed between strains of *L. monocytogenes* and *L. innocua* when treated at high concentrations of bile (30% oxgall for 5 min) (16). Another study exposed 4 strains of *L. monocytogenes* (F2365, 10403S, EGDe, and HCC23) to 1% porcine bile in BHI broth for 6 h. (20). No apparent growth was observed within 6 h for all six strains at 1% porcine bile, and a decline in *L. monocytogenes* counts was observed at 5 and 10% bile for 3 of the 4 strains examined (20). We also used 1% bile, and monitored *L. monocytogenes* for 26 h where the observed lag phases under 1% bile were approximately 2 h for lineage I strains and ~4 for lineage II strains. While the concentration of bile in the duodenum is reported to be ~20 mM (on average 12.6 and 6.9 μmoles/ml for glycine and taurine conjugated bile acids, respectively) (33, 34), which is equivalent to ~1% bile, variations in concentrations of bile salts can occur by reabsorption through the intestinal epithelium potentially causing decreased/increased concentration of bile in the GI system (35, 36). In this study, we also investigated growth

parameters at 0.3% bile and in comparison to 1% bile, no significant differences in growth parameters were observed between both lineages in 0.3% bile.

We predicted that exposure to osmotic stress would increase subsequent resistance to bile, but found that pre-exposure to 6% NaCl followed by exposure to 1% porcine bile did not result in cross-protection, but rather had an additive effect of stresses as observed by significantly increased lag phases and decreased maximum cell densities. Previously, Begley et al., explored cross-protection phenotypes from pre-exposure to salt stress and treatment with bile (16). In their study, exponential phase cells were adapted to 5% NaCl, and then exposed to lethal concentrations of unconjugated bile salts (sodium cholate-sodium deoxycholate) which showed increased survival of *L. monocytogenes* strain LO28 by 4.13 logs. They also showed that exposure to 30% Oxgall led to increased death rates for *L. monocytogenes* lacking *bsh*, *pva*, and *btlB* genes, indicating these genes contribute to survival in the presence of bile (17).

However, studies investigating cross protection mechanisms from pre-exposure to cell envelope stress and treatment with osmotic stress are lacking. It is possible that cell envelope stresses, such as bile may form pores in the cell membrane and subsequent exposure to osmotic stress may lead to increased entry of Na^+ inside of the cells increasing the damage from subsequent salt exposure. In our study, pre-exposure to 1% bile and subsequent treatment with 6% NaCl led to no changes in *L. monocytogenes* counts for all six strains over 26 h. It was hypothesized that during this scenario, the cells may be forming filaments. Previously, Wright et al., conducted microscopic study on the morphological changes in *L. monocytogenes* and found significant changes in the lengths and width of all four strains when exposed to 5% porcine bile for 1 h under aerobic conditions (20). Similarly, *L. monocytogenes* ScottA and LO28 are known to form filaments when exposed to combinations of stresses such as at a low pH and in the

presence of 10% NaCl (37). Also, exposure of *L. monocytogenes* strains 10403S and FSL A1-254 to pH above 9.0 led to production of filaments (38). Filament formation for *L. monocytogenes* strain SLCC 5764 was also observed in TSB with salt concentrations above 1,000 mM and at pH greater than 9.0 (39). Similarly filament formation was observed in TSB stored at 3ºC and maintained at a pH of 6.0 and at 4% NaCl (40). Similar results were observed in our study showing formation of filaments or elongated cells exposed to 6% NaCl after pre-exposure to 1% bile. Formation of filaments in *L. monocytogenes* under exposure to such stresses may from a single colony forming unit on a media agar plate, but upon removal of the stress under a favorable condition, such filamentous cells can break into multiple cells providing a misleading information on lower number of cells.

In conclusion, here it was investigated that the survival and growth characteristics varied among *L. monocytogenes* lineages I and II strains indicating differences in stress tolerance capabilities among these strains. Differences in their ability to withstand such stresses may be attributed to their increased survival in the GI tract potentially leading to disease. Also, the study on *L. monocytogenes* pre-exposure to 1% bile for 30 minutes and subsequent treatment at 6% NaCl led to prolonged lag phase with no changes observed in *L. monocytogenes* counts over 26 h period. From this, it could be proposed that pre-exposure to a cell envelope stress and subsequent exposure to an osmotic stress may help control *L. monocytogenes* contamination in processing facilities. This may be further investigated by pre-exposure of *L. monocytogenes* to sanitizers commonly used in the food industry and treatment with salt for the control of *L. monocytogenes*.

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2.9. Conflict of Interest

The authors declare no conflict of interest.

2.10. References

1. Freitag NE, Port GC, Miner MD. 2009. *Listeria monocytogenes* - from saprophyte to intracellular pathogen. Nat Rev Microbiol 7:623-8.
2. de Noordhout CM, Devleesschauwer B, Angulo FJ, Verbeke G, Haagsma J, Kirk M, Havelaar A, Speybroeck N. 2014. The global burden of listeriosis: a systematic review and meta-analysis. Lancet Infect Dis 14:1073-1082.
3. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis 17:7-15.
4. Vázquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Domínguez-Bernal G, Goebel W, González-Zorn B, Wehland J, Kreft J. 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev 14:584-640.

5. Cole MB, Jones MV, Holyoak C. 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. J Appl Bacteriol 69:63-72.

6. Chan YC, Wiedmann M. 2009. Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. Crit Rev Food Sci Nutr 49:237-53.

7. Bergholz TM, Bowen B, Wiedmann M, Boor KJ. 2012. *Listeria monocytogenes* shows temperature-dependent and -independent responses to salt stress, including responses that induce cross-protection against other stresses. Appl Environ Microbiol 78:2602-12.

8. Hingston P, Chen J, Dhillon BK, Laing C, Bertelli C, Gannon V, Tasara T, Allen K, Brinkman FS, Truelstrup Hansen L, Wang S. 2017. Genotypes Associated with *Listeria monocytogenes* Isolates Displaying Impaired or Enhanced Tolerances to Cold, Salt, Acid, or Desiccation Stress. Front Microbiol 8:369.

9. Cornu M, Beaufort A, Rudelle S, Laloux L, Bergis H, Miconnet N, Serot T, Delignette-Muller M. 2006. Effect of temperature, water-phase salt and phenolic contents on *Listeria monocytogenes* growth rates on cold-smoked salmon and evaluation of secondary models. International Journal of Food Microbiology 106:159-168.

10. Shabala L, Lee SH, Cannesson P, Ross T. 2008. Acid and NaCl limits to growth of *Listeria monocytogenes* and influence of sequence of inimical acid and NaCl levels on inactivation kinetics. Journal of Food Protection 71:1169-1177.

11. Dowd GC, Joyce SA, Hill C, Gahan CG. 2011. Investigation of the mechanisms by which *Listeria monocytogenes* grows in porcine gallbladder bile. Infect Immun 79:369-79.

12. Shen Q, Soni KA, Nannapaneni R. 2015. Stability of sublethal acid stress adaptation and induced cross protection against lauric arginate in *Listeria monocytogenes.* Int J Food Microbiol 203:49-54.

13. Kang J, Wiedmann M, Boor KJ, Bergholz TM. 2015. *VirR*-Mediated Resistance of *Listeria monocytogenes* against Food Antimicrobials and Cross-Protection Induced by Exposure to Organic Acid Salts. Appl Environ Microbiol 81:4553-62.

14. van Schaik W, Gahan CG, Hill C. 1999. Acid-adapted *Listeria monocytogenes* displays enhanced tolerance against the lantibiotics nisin and lacticin 3147. J Food Prot 62:536-9.

15. White SJ, McClung DM, Wilson JG, Roberts BN, Donaldson JR. 2015. Influence of pH on bile sensitivity amongst various strains of *Listeria monocytogenes* under aerobic and anaerobic conditions. J Med Microbiol 64:1287-96.

16. Begley M, Gahan CG, Hill C. 2002. Bile stress response in *Listeria monocytogenes* LO28: adaptation, cross-protection, and identification of genetic loci involved in bile resistance. Applied and environmental microbiology 68:6005-6012.

17. Begley M, Sleator RD, Gahan CG, Hill C. 2005. Contribution of three bile-associated loci, *bsh*, *pva*, and *btlB*, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. Infection and Immunity 73:894-904.

18. Zhang Q, Feng Y, Deng L, Feng F, Wang L, Zhou Q, Luo Q. 2011. *SigB* plays a major role in *Listeria monocytogenes* tolerance to bile stress. Int J Food Microbiol 145:238-43.

19. Sewell D, Allen SCh, Phillips CA. 2015. Oxygen limitation induces acid tolerance and impacts simulated gastro-intestinal transit in *Listeria monocytogenes* J0161. Gut Pathog 7:11.

20. Wright ML, Pendarvis K, Nanduri B, Edelmann MJ, Jenkins HN, Reddy JS, Wilson JG, Ding X, Broadway PR, Ammari MG, Paul O, Roberts B, Donaldson JR. 2016. The Effect of Oxygen on Bile Resistance in *Listeria monocytogenes*. J Proteomics Bioinform 9:107-119.

21. Zhang C, Zhang M, Ju J, Nietfeldt J, Wise J, Terry PM, Olson M, Kachman SD, Wiedmann M, Samadpour M, Benson AK. 2003. Genome diversification in phylogenetic lineages I and II of *Listeria monocytogenes*: identification of segments unique to lineage II populations. J Bacteriol 185:5573-84.

22. Orsi RH, den Bakker HC, Wiedmann M. 2011. *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. Int J Med Microbiol 301:79-96.

23. Haase JK, Didelot X, Lecuit M, Korkeala H, Achtman M, Group LmMS. 2014. The ubiquitous nature of *Listeria monocytogenes* clones: a large-scale Multilocus Sequence Typing study. Environ Microbiol 16:405-16.

24. Ward TJ, Ducey TF, Usgaard T, Dunn KA, Bielawski JP. 2008. Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria monocytogenes* isolates. Applied and environmental microbiology 74:7629-7642.

25. Bergholz TM, den Bakker HC, Fortes ED, Boor KJ, Wiedmann M. 2010. Salt stress phenotypes in *Listeria monocytogenes* vary by genetic lineage and temperature. Foodborne Pathog Dis 7:1537-49.

26. Ribeiro VB, Destro MT. 2014. *Listeria monocytogenes* serotype 1/2b and 4b isolates from human clinical cases and foods show differences in tolerance to refrigeration and salt stress. J Food Prot 77:1519-26.

27. Schmid B, Klumpp J, Raimann E, Loessner MJ, Stephan R, Tasara T. 2009. Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. Appl Environ Microbiol 75:1621-7.

28. Bergholz TM, Tang S, Wiedmann M, Boor KJ. 2013. Nisin resistance of *Listeria monocytogenes* is increased by exposure to salt stress and is mediated via *LiaR*. Applied and environmental microbiology 79:5682-5688.

29. Malekmohammadi S, Kodjovi KK, Sherwood J, Bergholz TM. 2017. Genetic and environmental factors influence *Listeria monocytogenes* nisin resistance. J Appl Microbiol.

30. Baranyi J, Roberts TA. 1994. A dynamic approach to predicting bacterial growth in food. Int J Food Microbiol 23:277-94.

31. Sleator RD, Gahan CG, Hill C. 2003. A postgenomic appraisal of osmotolerance in *Listeria monocytogenes*. Applied and Environmental Microbiology 69:1-9.

32. Tang S, Wiedmann M, Gardner AL, Brown AM, Boor KJ, Bergholz TM. 2015. Clonal Clustering Using 10-Gene Multilocus Sequence Typing Reveals an Association Between Genotype and *Listeria monocytogenes* Maximum Growth Rate in Defined Medium. Foodborne Pathog Dis 12:972-82.

33. Altman PL, Dittman DS. 1968. Metabolism, p 262. Federation of American Societies for Experimental Biology, Bethesda, Md.

34. Mallory A, Kern F, Smith J, Savage D. 1973. Patterns of bile acids and microflora in the human small intestine: I. Bile acids. Gastroenterology 64:26-33.

35. Philipp B. 2011. Bacterial degradation of bile salts. Appl Microbiol Biotechnol 89:903-15.

36. Davis ML, Ricke SC, Donaldson JR. 2019. Establishment of *Listeria monocytogenes* in the Gastrointestinal Tract. Microorganisms 7:75.

37. Bereksi N, Gavini F, Benezech T, Faille C. 2002. Growth, morphology and surface properties of *Listeria monocytogenes* Scott A and LO28 under saline and acid environments. Journal of applied microbiology 92:556-565.

38. Giotis ES, Blair IS, McDowell DA. 2007. Morphological changes in *Listeria monocytogenes* subjected to sublethal alkaline stress. International journal of food microbiology 120:250-258.

39. Isom LL, Khambatta ZS, Moluf JL, Akers DF, Martin SE. 1995. Filament formation in *Listeria monocytogenes*. Journal of Food Protection 58:1031-1033.

40. Vail KM, McMullen LM, Jones TH. 2012. Growth and filamentation of cold-adapted, log-phase *Listeria monocytogenes* exposed to salt, acid, or alkali stress at 3°C. J Food Prot 75:2142-50.

3. SURVIVAL AND GROWTH OF WILD-TYPE AND *RPOS*-DEFICIENT *SALMONELLA* NEWPORT STRAINS IN SOIL EXTRACTS PREPARED WITH HEAT-TREATED POULTRY PELLETS[1]

3.1. Abstract

Manure runoff can transfer pathogens to farmlands or to water sources leading to subsequent contamination of produce. Untreated biological soil amendments (BSA), like manure, can be contaminated with foodborne pathogens, such as *Salmonella* Newport which may lead to transfer of *Salmonella* to fruits or vegetables. Studies have reported the occurrence and survival of *Salmonella* in manure or manure slurries. However, data on the survival and growth of *S.* Newport is lacking in matrices simulating runoff. We quantified the survival/growth of wild type (WT) *S.* Newport and *rpoS*-deficient (Δ*rpoS*) strains in sterile and non-sterile soil extracts prepared with (amended) or without (unamended) heat-treated poultry pellets at 25°C. *S.* Newport WT and Δ*rpoS* populations reached a maximum cell density of 6-8 log CFU/ml in 24-30 h in amended and unamended soil extracts and remained in stationary phase for up to 4 days. *S.* Newport in amended soil extracts exhibited a decreased lag phase (λ, 2.87±1.01 h) and higher maximum cell densities (N_{max}, 6.84±1.25 CFU/ml) compared to λ (20.10±9.53 h) and N_{max} (5.22±0.82 CFU/ml) in unamended soil extracts. In amended soil extract, the Δ*rpoS* strain had no measurable λ, similar growth rates (μ_{max}) compared to WT, and a lower N_{max} compared to the WT strain. Unamended non-sterile soil extracts did not support the growth of *S.* Newport WT, and led to a decline in populations for the Δ*rpoS* strain.

[1] The material in this chapter was co-authored by Manoj K. Shah, Rhodel Bradshaw, Esmond Nyarko, Patricia D. Millner, Deborah Neher, Thomas Weicht, Teresa M. Bergholz, and Manan Sharma. Manoj K. Shah had primary responsibility in experiment design, data collection, and writing the manuscript. The co-authors provided guidance, supervision, and revision of the experimental design and the manuscript.

S. Newport had lower cell densities in non-sterile soil extracts (5.94±0.95 CFU/ml) than in sterile soil extracts (6.66±1.50 CFU/ml), potentially indicating competition for nutrients between indigenous microbes and *S.* Newport. The most favorable growth conditions were provided by amended sterile and non-sterile soil extracts, followed by sterile unamended soil extracts for both *S.* Newport strains. *S.* Newport may grow to greater densities in amended extracts, providing a route for increased *Salmonella* levels in pre-harvest produce growing environments.

3.2. Highlights

- Soil extracts prepared with HTPP supported growth of *S.* Newport by 4 to 5 log CFU/ml in 96 h
- Lack of *rpoS* led to diminished growth or decline in survival of *Salmonella* Newport
- Presence of indigenous microbes impaired *S.* Newport growth in soil extracts

3.3. Introduction

In recent years, foodborne disease outbreaks due to *Salmonella* spp. have been increasingly associated with the consumption of raw vegetables and leafy greens (1, 52). Each year, non-typhoidal *Salmonella* spp. are estimated to cause 1.2 million foodborne infections, 23,000 hospitalizations, and 450 deaths in the United States (153). *Salmonella* spp. can be introduced to the fields used to grow fruits and vegetables through various means, such as use of contaminated manure, irrigation water, wild animal scat, and bird droppings (78, 113, 114). *Salmonella* spp. from soil can eventually transfer to vegetables and leafy greens, potentially leading to foodborne disease outbreaks (95). *Salmonella enterica* subspecies *enterica* serovar Newport has been responsible for several outbreaks related to contaminated produce, such as

cucumbers and tomatoes. The possible sources of *S.* Newport associated with cucumbers in 2014, and with tomatoes in 2005, were attributed to environmental reservoirs, potentially including contaminated soils containing animal manure (6, 7).

Various BSAAO (Biological Soil Amendment of Animal Origin) are used in the organic cultivation of fruits and vegetables. The use of composted manure can minimize the transfer of pathogens from amended soils to crops, however, the use of raw or untreated livestock animal manure is still practiced, leading to increased risks of contamination of produce by enteric pathogens present in manure (105). While studies have shown that both composted and raw manure can harbor pathogens, raw animal manure is known to contain higher numbers of pathogens, such as *Salmonella* populations as high as 10^5-10^7 CFU/g (93-95). Leaching of manure runoff from animal farms to crop fields and eventually to water sources have been observed previously, which can lead to the spread of enteric pathogens on the farm or in the pre-harvest environment. For example, water wells which supplied drinking water were contaminated with cattle manure runoff containing *Escherichia coli* O157:H7 and *Campylobacter* spp. from nearby farms following rainfall in Walkerton, Canada in 2000 leading to hundreds of illnesses and multiple deaths (4). Similarly, lettuce contaminated with shiga toxin-producing *E. coli* in 2005 in Sweden that caused 135 illnesses with 11 cases of hemolytic uremic syndrome was retrospectively associated to a farm upstream of the irrigation point (114). Also, an outbreak of *E. coli* O157:H7 infections was associated with lettuce irrigated using water contaminated with cattle manure in the U.S. in 2006 (113).

Salmonella spp. and *E. coli* O157:H7 can survive for several months in manure-amended soils and manure slurries (116, 119, 120). Some BSAAO are heat-treated and pelletized to inactivate pathogens and stabilize nutrients, such as nitrogen. Heat treated poultry pellets (HTPP)

may not contain pathogens, but their nutrient levels, when amended to soils, may promote or affect survival of enteric pathogens already present in or introduced to soils through contaminated irrigation water or animal fecal deposits. Irrigation or rainfall events may result in growth of inoculated pathogens in soil (124, 154). This may be due to increased availability of nutrients by addition of adequate amount of water causing water-soluble nutrients to be readily available for bacterial growth. However, the extent of growth of *Salmonella* spp. in amended or unamended soil extracts is unknown. It is possible that nutrients from soil or manure dissolve in water, allowing this runoff to support not only survival but also growth of *Salmonella* spp. In addition, soil has a diverse population of indigenous microbes that can affect pathogen survival by either competing with *Salmonella* spp. for available nutrients or providing pathogens nutrients through metabolic activity. It is important to understand the roles of such microbes regarding the survival of harmful pathogens. Amended soils and runoff may pose environmental stresses to pathogens, where the general stress response regulator RpoS has been shown to contribute to prolonged survival of *Salmonella* and *E. coli* O157:H7 in manure-amended soil (130, 131).

Our objectives were to a) quantify the growth/survival characteristics of *S*. Newport in soil extract prepared with or without HTPP, b) understand the role of *rpoS* in the survival of *S.* Newport in soil extracts, and c) investigate the role of indigenous microorganisms in the survival/growth characteristics of *S.* Newport WT and *rpoS*-deficient strains. We have addressed these questions by quantifying growth/survival of wild type (WT) *Salmonella* Newport and *rpoS*-deficient (Δ*rpoS)* strains in soil extracts prepared from unamended and HTPP-amended soil in the presence and absence of indigenous microorganisms.

3.4. Materials and Methods

3.4.1. Soil extract preparation

Soil (fine, loamy, mesic Aquic Hapladults soils) were obtained from the USDA ARS Beltsville Agricultural Research Center (BARC) North farm (Beltsville Maryland). For preparation of unamended extract, 500 g of soil were added to 1 L of deionized water in a 2 L bottle. For amended extract, 30 g of heat-treated poultry pellets (HTPP), a commercial BSA (3-2-3, N-P-K) was added to 470 g of soil and then added to 1 L of deionized water into a 2- L bottle. The bottles were incubated at 25ºC shaking at 50 rpm for 24 h. This incubation temperature was selected to simulate stagnant water containing manure runoff, and has the potential for indigenous microorganisms to grow during the incubation period. After incubation, the resulting liquid extract was transferred to 250 ml centrifuge bottles. These extracts were centrifuged at 5000 x *g* for 22 min at 25ºC to remove heavy soil particles. After centrifugation, the supernatant was collected and used as non-sterile extracts. To obtain sterile extracts, supernatants were filtered using a 0.2 µm filter (Thermo Fisher Scientific, Waltham, MA). The extracts were stored at -20ºC for up to 72 h prior to use.

3.4.2. Microbial profiling of soil extracts

Microbial populations were quantified from 25 ml of prepared soil extracts after centrifugation. Quantification of total heterotrophs, fecal coliforms, *E. coli,* and fungi were performed using Tryptic Soy Agar (TSA), MacConkey agar, Tryptone Bile X-Glucuronide agar (TBX) (Neogen Corp., Lansing, MI), and Potato Dextrose agar (PDA) (Becton, Dickinson and Company, Franklin Lakes, NJ), respectively. Appropriate dilutions of soil extracts were spread plated in the volume of 0.1 ml on these agar plates and incubated at 37ºC for 48 h for total heterotrophs, 42ºC for 24 h for fecal coliforms, 37ºC for 24 h for *E. coli*, and at 25ºC for 5 days

for fungi (yeasts and molds). All colony forming units (CFU) were counted on TSA and PDA for total heterotrophs and fungi, respectively. Pink/red colonies on MacConkey Agar and blue and green colonies on TBX agar were counted as fecal coliforms and *E. coli*, respectively.

3.4.3. Chemical analyses of soil extract

Soil extracts were sent to University of Delaware Soil Testing Program to determine pH, and quantify other nutrients, such as total carbon (TC), total organic carbon (TOC), P_2O_5, NH_4-N, NO_3-N, and trace elements.

3.4.4. Strain preparation and inoculation of extracts

A rifampicin-resistant *Salmonella enterica* subsp. *enterica* serovar Newport WT strain was obtained from the USDA ARS Environmental Microbial and Food Safety Laboratory (EMFSL) and has been described previously (6). A *rpoS*-deficient (*ΔrpoS*) kanamycin-resistant *S.* Newport strain was constructed using the lambda red recombination method (155) as previously described (156). These strains were stored at -80ºC, and were streaked separately for isolation on XLT4 agar plates (Neogen Corp., Lansing, MI) containing 80 □g/ml rifampicin and 25 μg/ml kanamycin for *S.* Newport WT and *ΔrpoS* strains, respectively. An isolated colony of *S.* Newport WT and *ΔrpoS* strains were transferred to separate 30 ml of Tryptic Soy Broth supplemented with 80 □g/ml rifampicin and 25 μg/ml kanamycin, respectively, and incubated at 37ºC for 24 h. After incubation, the overnight bacterial cultures were serially diluted, and 3 ml of the diluted culture was added to 297 ml of both sterile and non-sterile amended and unamended soil extracts in sterile flasks for both WT and *ΔrpoS* strains to obtain an initial population of approximately 3 log CFU/ml. These flasks were incubated at 25ºC shaking at 125 rpm.

3.4.5. S. Newport populations and determination of growth curves

To obtain potential survival/growth curves for the different extracts, *S.* Newport populations were quantified at 0, 4, 8, 24, 30, 48, 72, and 96 h post inoculation. At these time points, 1 ml from the inoculated extracts was removed from the bottle, and appropriate serial dilutions were prepared in phosphate buffered saline (PBS), which were then plated on XLT4 agar plates containing 80 □g/ml rifampicin and 25 μg/ml kanamycin for WT and *ΔrpoS S.* Newport strains, respectively. These plates were incubated at 37ºC for 24 h and black-colored colonies were counted. Plate counts were used to determine populations (CFU/ml) which were log-transformed, and those values were used to generate survival curves of *S.* Newport in each extract type. Growth curves were fit using the Baranyi model in R version 3.4.2 using the 'nlsmicrobio' package (157) with the equation [y(t) = *ln x*(t)] where x(t) is the cell concentration (CFU/ml) at time (t), and y(t) is the growth rate.

3.4.6. Statistical analysis

Growth experiments were arranged as a completely randomized design of a complete factorial combination of two strains (WT and *ΔrpoS S.* Newport) and four extract types: unamended non-sterile (UNS), unamended sterile (US), amended non-sterile (ANS), and amended sterile (AS). Treatments were replicated four times. The obtained parameters lag phase (λ), growth rate (μ_{max}), and maximum cell density (N_{max}) were treated as dependent variables whereas the strains and types of soil extract were treated as independent variables. Two-way analysis of variance (ANOVA) was conducted in SAS 9.4 (SAS Institute, Cary, NC) using proc GLIMMIX and corrections for multiple comparisons was done using 'tukey=adj' at an adjusted p-value of 0.05 for significant interactions.

3.5. Results

3.5.1. Microbial and chemical characteristics of soil extracts

Abundance of total heterotrophs, fecal coliforms, *E. coli* and fungi were similar among HTPP-amended and unamended non-sterile soil extracts, which shows that bacterial loads were not affected by addition of HTPP (Table 3). To study the impact of the presence of indigenous microorganisms on *S.* Newport survival, the non-sterile soil extracts were filter sterilized, leading to substantial decrease in the counts of total heterotrophs, fecal coliforms, *E. coli* and fungi in sterile soil extracts (Table 3). Chemical nutrients and pH values of unamended sterile and non-sterile extracts were similar (Table 4). The same trend was observed for amended extracts (Table 2). However, nutrient and pH levels differed substantially between unamended and amended soil extracts. The pH of both UNS (6.40) and US (6.50) soil extracts were lower than that of ANS (7.39) and AS (7.36) soil extract. The P_2O_5 in ANS (116.13 mg/L) and AS (118.97 mg/L) soil extracts were greater than in UNS (2.27 mg/L) and US (3.67 mg/L) soil extracts. Similarly, NH_4-N were greater in ANS (118.18 mg/L) and AS (64.30 mg/L) soil extracts as compared to UNS (0.43 mg/L) and US (0.42 mg/L) soil extracts. NO_3N levels were found to be greater in unamended soil extracts compared to amended soil extracts. However, all forms of carbon, such as total inorganic carbon (TIC), total carbon (TC) and total organic carbon (TOC) were present in greater concentrations in amended soil extracts than in unamended soil extracts (Table 4). Other all trace elements, such as Al, B, Ca, Cu, Fe, K, Mg, Mn, Na, P, S, and Zn were also found to be in higher concentrations in amended soil extracts than in unamended soil extracts. Observations for all nutrients were found to be similar between sterile and non-sterile soil extracts (Table 4).

Table 3. Microbial profile of soil extracts

Soil extract	Total heterotrophs[1]	Fecal coliforms	*E. coli*	Fungi
UNS	1.30×10^5	3.30×10^4	2.50×10^2	1.70×10^5
US	<10	<1	<1	2.50×10^2
ANS	3.55×10^5	7.30×10^4	6.50×10^1	1.50×10^5
AS	<10	<1	<1	3.50×10^2

[1]All counts are in CFU/ml. (UNS=Unamended Non-sterile, US=Unamended sterile, ANS=Amended Non-sterile, AS=Amended sterile)

Table 4. Chemical characteristics of soil extracts

	Unamended soil extract		Amended soil extract	
	Non-sterile	Sterile	Non-sterile	Sterile
pH	6.4	6.5	7.39	7.36
P2O5	2.27[1]	3.67	116.13	118.97
NH4-N	0.43	0.42	118.18	64.3
NO3-N	7.96	4.45	2.67	3.89
TIC	1.44	0.26	15.02	12.2
TC	33.15	40.04	1270.65	988.2
TOC	31.71	39.78	1255.63	976
Al	0.33	0.09	0.37	0.27
B	0.01	0.01	0.7	0.63
Ca	4.24	6.79	41.07	37.87
Cu	0.01	0.02	1.96	1.73
Fe	0.17	0.1	2.78	2.56
K	4.73	8.59	410.92	385.64
Mg	1.26	2.08	36.07	37.67
Mn	0.09	0.14	0.79	0.84
Na	0.52	0.8	162.89	151.05
P	0.99	1.6	50.71	51.95
S	0.93	1.34	202.5	182.86
Zn	0.02	0.02	0.61	0.56

[1]all concentrations are represented in mg/L.

3.5.2. Survival/Growth of S. Newport WT

Mean WT *S.* Newport populations at 0 h ranged from 3.07 to 3.23 log CFU/ml for UNS, US, ANS, and AS extracts (Figure 6). In UNS, an appropriate growth curve could not be fit with the Baranyi model, but a maximum population of 4.16±1.45 log CFU/mL was observed at 30 h in UNS. Significant ($p<0.05$) increases in cell densities (N_{max}) were observed in other soil extracts within 96 h (Table 3). The highest N_{max} was observed to be 8.40±0.31 log CFU/mL in AS extract, which was significantly greater than that observed in ANS (6.59±0.77 log CFU/mL) and US (6.19±0.97 log CFU/mL) soil extracts ($p<0.05$) (Table 5). Overall, significantly greater WT *S.* Newport populations (N_{max}) were observed in amended soil extract than in the unamended soil extract ($p<0.05$). In addition, sterile soil extracts supported a greater N_{max} than the non-sterile soil extract for WT *S.* Newport populations ($p<0.05$). Similarly, growth rates (μ_{max}) were estimated to be 0.60±0.17 h^{-1}, 0.48±0.12 h^{-1}, and 0.53±0.07 h^{-1} for US, ANS, and AS, respectively, which were not significantly different from each other ($p>0.05$). However, the lag phase observed in US extract was estimated to be 20.17 h, which was significantly greater than those observed for AS (2.86±1.39 h) and ANS (2.89±0.66 h) soil extracts ($p<0.05$) (Table 5).

3.5.3. *Survival/Growth of ΔrpoS* S. *Newport*

The mean *ΔrpoS S.* Newport population at 0 h ranged from 2.76 to 2.86 log CFU/ml for UNS, US, ANS, and AS extracts (Figure 7). Similar to the WT *S.* Newport strain, an appropriate growth curve could not be fit with the Baranyi model for UNS soil extract for *S.* Newport *ΔrpoS* strain, and a decrease in cell density was observed over time with a density of 1.59±0.41 log CFU/mL observed at 96 h for UNS soil extract. On the other hand, cell densities (N_{max}) observed in US, ANS, and AS soil extracts were significantly ($p<0.05$) greater than those observed in UNS.

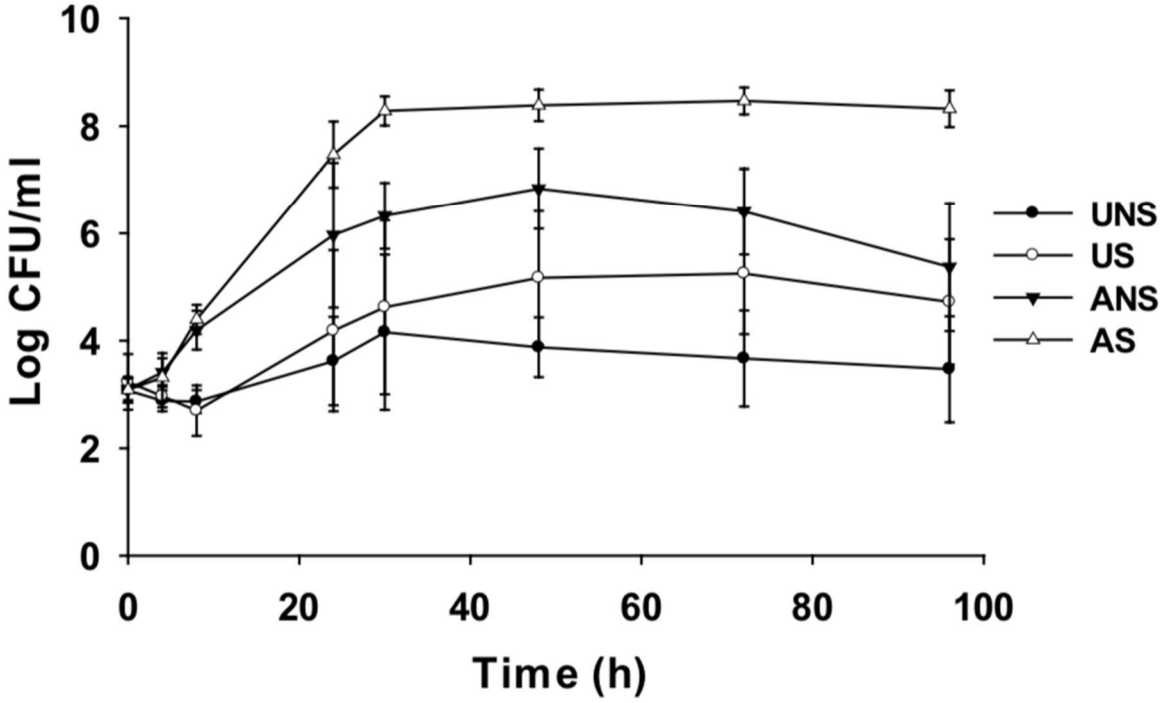

Figure 6**.** Growth/survival of *Salmonella* Newport WT in soil extracts, unamended non-sterile (UNS), unamended sterile (US), amended non-sterile (ANS) and amended sterile at 25ºC from four experimental replicates. Error bars indicates standard deviation.

The highest N_{max} was observed to be 7.09±0.16 log CFU/ml for AS extract which was significantly greater than that of ANS (5.29±0.63 log CFU/ml) and US (4.76±0.46 log CFU/ml) ($p<0.05$) (Table 5). The amended and sterile soil extracts had greater N_{max} than the unamended and non-sterile soil extracts ($p<0.05$). The maximum N_{max} for *S.* Newport *ΔrpoS* strain in each of these extracts were observed to be significantly lower than that observed for *S.* Newport WT ($p<0.05$) (Table 5). For the *ΔrpoS S.* Newport strain, the maximum growth rates (μ_{max}) were observed to be 0.65±0.09 h^{-1} and 0.61±0.19 h^{-1} in UNS and AS soil extracts, respectively, which were not significantly different from each other ($p>0.05$). The μ_{max} for *ΔrpoS S.* Newport strain in US soil extract was observed to be 0.29±0.11 h^{-1} which was significantly lower than in UNS

and AS ($p<0.05$) (Table 5). No significant differences were observed between WT and *ΔrpoS S.* Newport strains for μ_{max} ($p>0.05$).

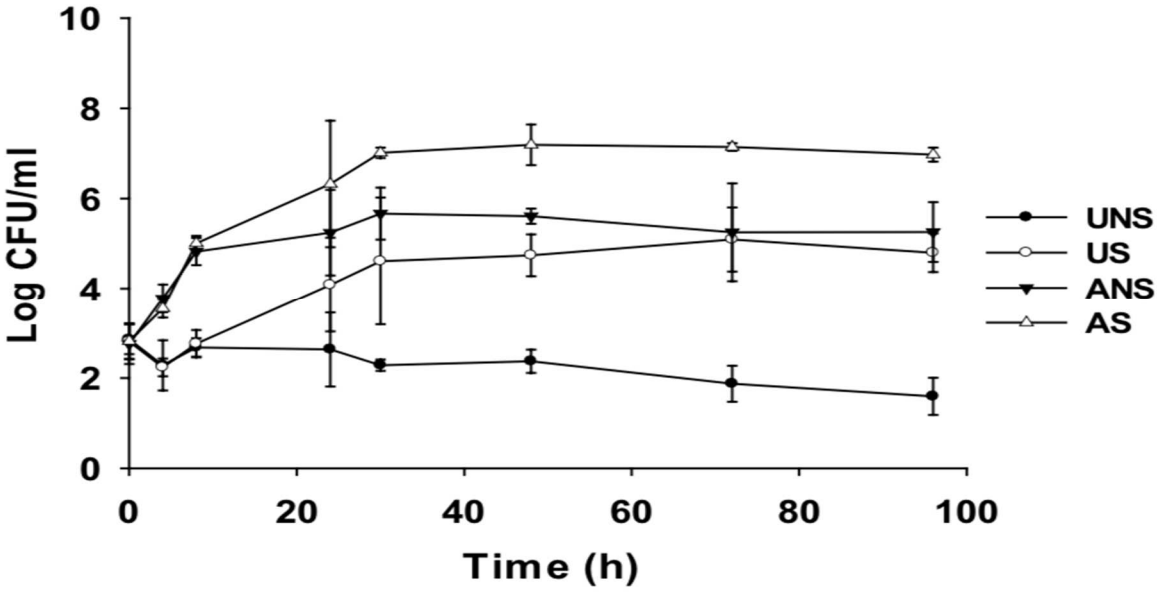

Figure 7. Growth/survival of *Salmonella* Newport *ΔrpoS* in soil extracts, unamended non-sterile (UNS), unamended sterile (US), amended non-sterile (ANS) and amended sterile at 25°C from four experimental replicates. Error bars indicates standard deviation.

Conversely, no lag phase (λ) was observed for the *ΔrpoS S.* Newport strain except in US soil extract. The observed λ for *S.* Newport *ΔrpoS* strain in US soil extract was measured to be 20.04±12.25 h, which was similar to the λ for *S.* Newport WT (20.17±8.79 h) in US soil extract ($p>0.05$).

3.6. Discussion

Manure runoff from animal farms has been implicated in previous outbreaks associated with produce commodities (4). Cattle manure and poultry litter can contain harmful pathogens, and *Salmonella* has been shown to survive for a prolonged period in these matrices (93, 94).

Table 5. Growth Parameters of WT and Δ*rpoS S.* Newport strains in soil extracts using the Baranyi model

Strain	Soil Extract	Lag phase (ʎ)	μmax (h^{-1})	Nmax (log CFU/mL)
WT	UNS	NA[1]	NA	NA
	US	20.17±8.79^{B2}	0.60±0.17^{AB}	6.19±0.97^{AC}
	ANS	2.89±0.66^{A}	0.48±0.12^{AB}	6.59±0.77^{A}
	AS	2.86±1.39^{A}	0.53±0.07^{AB}	8.40±0.31^{B}
ΔrpoS	UNS	NA	NA	NA
	US	20.04±12.25^{B}	0.29±0.11^{B}	4.76±0.46^{C}
	ANS	-[3]	0.65±0.09^{A}	5.29±0.63^{AC}
	AS	-[3]	0.61±0.19^{A}	7.09±0.16^{AB}

[1]NA' indicate model were unable to be generated using the Baranyi model.
[2]Within each column, different letters indicate significantly different values at $p < 0.05$.
[3]'- 'indicate no parameters were estimated

If manure runoff can support the growth of *Salmonella* spp., there is increased likelihood for longer environmental persistence leading to elevated risk of subsequent transfer to fresh fruits and vegetables. In this study, soil extracts amended with HTPP supported growth of both WT and Δ*rpoS S.* Newport strains with an increase in cell densities of 4-5 logs CFU/ml over 96 h. However, in unamended non-sterile soil extracts *S.* Newport WT populations remained similar over 96 h. The increased levels of nutrients due to the addition of HTPP promoted higher N_{max} compared to unamended extracts. Similarly, sterile amended soil extracts (AS) supported higher N_{max} than the non-sterile amended soil extracts (ANS), which may have been influenced by competition from indigenous microorganisms with *S.* Newport for available nutrients. *S.* Newport Δ*rpoS* strain in amended soil extracts (AS) showed no measurable ʎ and a similar μ_{max} as compared to the WT strain; however, a lower N_{max} for the Δ*rpoS* strain as compared to WT strain was observed. In unamended non-sterile soil extracts (UNS) *S.* Newport Δ*rpoS* populations declined by 1 log CFU/ml over 96 h whereas no decrease in *S.* Newport WT population was observed. This indicates that a functional *rpoS* may aid growth of *S.* Newport in

amended soil extract. Importantly, populations of the Δ*rpoS* strain declined over 96 h in unamended soil extracts whereas WT *S.* Newport strain did not decline and survived during this period, showing that RpoS improved *Salmonella* Newport survival under these non-host conditions. RpoS supports the survival of *Salmonella* spp. and *E. coli* O157:H7 in non-host environments, including in aged broiler litter under desiccation (130). Furthermore, *E. coli* O157:H7 strains with a mutation in *rpoS* had shorter survival durations compared to the WT strains in manure amended soil (131). Taken together, these studies and our data indicate that RpoS has an important role in improved survival of *S.* Newport in soil extracts.

In our study, growth characteristics for both *S.* Newport WT and *rpoS*-deficient strains were greater in sterile than in non-sterile soil extracts. Similar observations were made for *Salmonella* spp. in sterile and non-sterile biosolids (109). In the latter study, a lower N_{max} and reduced μ_{max} was observed in non-sterile biosolids compared to that in sterile biosolids, which indicated that competition from indigenous microorganisms for available nutrients limited the growth of *S.* Newport. Other studies investigated growth of *Salmonella* and *E. coli* O157:H7 in amended soil extracts. *S.* Enteritidis PT 30 populations increased by up to 3-log CFU in in soil prepared with almond hull extract (123). *Salmonella* spp. populations in various composts types (biosolids, manure and yard wastes) increased by 1.5 log CFU during storage at 25ºC over 3 days (124). Growth of *Salmonella* spp. in autoclaved composts with 40 and 50% moisture content supported up to a 4-log increase in *Salmonella* counts within 3 days of storage (125). These studies show that *Salmonella* spp. may grow in biological soil amendments, or amended soil extracts given sufficient nutrients and a lack of microbial competition.

Our study shows that *S.* Newport growth in soil extracts is affected by the nutrients present, and that nutrients from HTPP resulted in increased populations of both wild-type and

rpoS-deficient *S.* Newport strains. The *rpoS* status of *S.* Newport also affected the maximum population attained, indicating that a functional *rpoS* gene may promote higher population levels of *S.* Newport in soil extracts and amended soil runoff. Examination of other strains, genetic factors, microbial competition factors, and nutrient factors in non-host environments is required to fully characterize the survival of *S.* Newport in soil and soils containing BSAAOs. Our study demonstrates that *S.* Newport can not only survive but grow in unamended or amended soil extracts to high populations which can lead to potential contamination of soils, water sources, and produce commodities.

3.7. Acknowledgements

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3.9. References

1. Angelo, K. M., A. Chu, M. Anand, T. A. Nguyen, L. Bottichio, M. Wise, I. Williams, S. Seelman, R. Bell, M. Fatica, S. Lance, D. Baldwin, K. Shannon, H. Lee, E. Trees, E. Strain, L. Gieraltowski, and C. f. D. C. a. P. (CDC). 2015. Outbreak of *Salmonella*

Newport infections linked to cucumbers--United States, 2014. *MMWR Morb Mortal Wkly Rep*. 64:144-147.

2. Auld, H., D. MacIver, and J. Klaassen. 2004. Heavy rainfall and waterborne disease outbreaks: the Walkerton example. *J Toxicol Environ Health A*. 67:1879-1887.
3. Baranyi, J., and T. A. Roberts. 1994. A dynamic approach to predicting bacterial growth in food. *Int J Food Microbiol*. 23:277-294.
4. CDC. 2018, S*almonella*. Available at: https://www.cdc.gov/salmonella/index.html. Accessed, November 2018.
5. Chen, Z., and X. Jiang. 2017. Thermal Resistance and Gene Expression of both Desiccation-Adapted and Rehydrated *Salmonella enterica* Serovar Typhimurium Cells in Aged Broiler Litter. *Appl Environ Microbiol*. 83:e00367-17.
6. Chinivasagam, H. N., M. Redding, G. Runge, and P. J. Blackall. 2010. Presence and incidence of food-borne pathogens in Australian chicken litter. *Br Poult Sci*. 51:311-318.
7. Danyluk, M. D., M. Nozawa-Inoue, K. R. Hristova, K. M. Scow, B. Lampinen, and L. J. Harris. 2008. Survival and growth of *Salmonella* Enteritidis PT 30 in almond orchard soils. *J Appl Microbiol*. 104:1391-1399.
8. Fukushima, H., and R. Seki. 2004. High numbers of Shiga toxin-producing *Escherichia coli* found in bovine feces collected at slaughter in Japan. *FEMS Microbiol Lett*. 238:189-197.
9. Greene, S. K., E. R. Daly, E. A. Talbot, L. J. Demma, S. Holzbauer, N. J. Patel, T. A. Hill, M. O. Walderhaug, R. M. Hoekstra, M. F. Lynch, and J. A. Painter. 2008. Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005. *Epidemiol Infect*. 136:157-165.

10. Harrison, J. A., J. W. Gaskin, M. A. Harrison, J. L. Cannon, R. R. Boyer, and G. W. Zehnder. 2013. Survey of food safety practices on small to medium-sized farms and in farmers markets. *J Food Prot*. 76:1989-1993.

11. Kim, J., and X. Jiang. 2010. The growth potential of *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* in dairy manure-based compost in a greenhouse setting under different seasons. *Journal of Applied Microbiology*. 109:2095-2104.

12. Laidler, M. R., M. Tourdjman, G. L. Buser, T. Hostetler, K. K. Repp, R. Leman, M. Samadpour, and W. E. Keene. 2013. *Escherichia coli* O157:H7 infections associated with consumption of locally grown strawberries contaminated by deer. *Clin Infect Dis*. 57:1129-1134.

13. Mukherjee, A., D. Speh, E. Dyck, and F. Diez-Gonzalez. 2004. Preharvest evaluation of coliforms, *Escherichia coli*, *Salmonella*, and *Escherichia coli* O157:H7 in organic and conventional produce grown by Minnesota farmers. *J Food Prot*. 67:894-900.

14. Murphy, K. C., and K. G. Campellone. 2003. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli. BMC Mol Biol*. 4:11.

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

16. Reynnells, R., D. T. Ingram, C. Roberts, R. Stonebraker, E. T. Handy, G. Felton, B. T. Vinyard, P. D. Millner, and M. Sharma. 2014. Comparison of U.S. Environmental Protection Agency and U.S. Composting Council microbial detection methods in finished

compost and regrowth potential of *Salmonella* spp. and *Escherichia coli* O157:H7 in finished compost. *Foodborne Pathog Dis*. 11:555-567.

17. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis*. 17:7-15.

18. Semenov, A. V., L. van Overbeek, A. J. Termorshuizen, and A. H. van Bruggen. 2011. Influence of aerobic and anaerobic conditions on survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in Luria-Bertani broth, farm-yard manure and slurry. *J Environ Manage*. 92:780-787.

19. Shah, M., R. Bradshaw, E.T. Handy, C.L. East, T. Bergholz, and M. Sharma. 2018. Factors affecting *Salmonella* Newport survival in soil and subsequent transfer to spinach plants. *In Preparation*.

20. Sharma, M., P. D. Millner, F. Hashem, M. Camp, C. Whyte, L. Graham, and C. P. Cotton. 2016. Survival and Persistence of Nonpathogenic *Escherichia coli* and Attenuated *Escherichia coli* O157:H7 in Soils Amended with Animal Manure in a Greenhouse Environment. *J Food Prot*. 79:913-921.

21. Sharma, M., and R. Reynnells. 2016. Importance of Soil Amendments: Survival of Bacterial Pathogens in Manure and Compost Used as Organic Fertilizers. *Microbiol Spectr*. 4:159-175.

22. Sidhu, J., R. A. Gibbs, G. E. Ho, and I. Unkovich. 2001. The role of indigenous microorganisms in suppression of *Salmonella* regrowth in composted biosolids. *Water Res*. 35:913-920.

23. Sodha, S. V., M. Lynch, K. Wannemuehler, M. Leeper, M. Malavet, J. Schaffzin, T. Chen, A. Langer, M. Glenshaw, D. Hoefer, N. Dumas, L. Lind, M. Iwamoto, T. Ayers, T. Nguyen, M. Biggerstaff, C. Olson, A. Sheth, and C. Braden. 2011. Multistate outbreak of *Escherichia coli* O157:H7 infections associated with a national fast-food chain, 2006: a study incorporating epidemiological and food source traceback results. *Epidemiol Infect*. 139:309-16.

24. Söderström, A., P. Osterberg, A. Lindqvist, B. Jönsson, A. Lindberg, S. Blide Ulander, C. Welinder-Olsson, S. Löfdahl, B. Kaijser, B. De Jong, S. Kühlmann-Berenzon, S. Boqvist, E. Eriksson, E. Szanto, S. Andersson, G. Allestam, I. Hedenström, L. Ledet Muller, and Y. Andersson. 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. *Foodborne Pathog Dis*. 5:339-349.

25. van Hoek, A. H., H. J. Aarts, E. Bouw, W. M. van Overbeek, and E. Franz. 2013. The role of *rpoS* in *Escherichia coli* O157 manure-amended soil survival and distribution of allelic variations among bovine, food and clinical isolates. *FEMS Microbiol Lett*. 338:18-23.

26. Vimont, A., C. Vernozy-Rozand, M. P. Montet, C. Lazizzera, C. Bavai, and M. L. Delignette-Muller. 2006. Modeling and predicting the simultaneous growth of *Escherichia coli* O157:H7 and ground beef background microflora for various enrichment protocols. *Appl Environ Microbiol*. 72:261-268.

27. You, Y., S. C. Rankin, H. W. Aceto, C. E. Benson, J. D. Toth, and Z. Dou. 2006. Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Appl Environ Microbiol*. 72:5777-5783.

4. *SALMONELLA* NEWPORT IN SOIL AMENDED WITH HEAT-TREATED POULTRY PELLETS SURVIVED LONGER AND MORE READILY TRANSFERRED TO AND PERSISTED ON SPINACH[1]

4.1. Abstract

Biological soil amendments of animal origin (BSAAO) like untreated poultry litter are commonly used as biological fertilizers, but can harbor bacterial foodborne pathogens like *Salmonella enterica* leading to potential transfer of pathogens from soils to fruits and vegetables intended for human consumption. Heat-treated poultry pellets (HTPP) can provide produce growers a slow release fertilizer with a minimized risk of pathogen contamination. However, little is known about the impact of HTTP-amended soil on survival of *Salmonella enterica*. The contributions of RpoS and potential formation of viable but non-culturable cells to *Salmonella* survival in soils are also inadaquately understood. We quantified the survival of *S*. Newport wild-type (WT) and *rpoS*-deficient (*ΔrpoS*) strains in HTPP-amended and unamended soil with or without spinach plants over 91 days using culture and quantitative PCR methods with Propidium Monoazide (PMA-qPCR). A simulated 'splash' transfer of *S.* Newport from contaminated soil to spinach leaves was evaluated at 35 and 63 days post-inoculation (dpi). *S.* Newport WT and *ΔrpoS* reached the limit of detection - 1.0 log CFU/gdw (gram dry weight) in unamended soil after 35 days, whereas 2-4 log CFU/gdw was observed for both WT and *ΔrpoS* strains at 91 dpi in HTPP-amended soil.

Cell densities in soils determined by PMA-qPCR and plate counts methods were similar

[1] The material in this chapter was co-authored by Manoj K. Shah, Rhodel Bradshaw, Esmond Nyarko, Eric T. Handy, Cheryl East, Patricia D. Millner, Teresa M. Bergholz, and Manan Sharma. Manoj K. Shah had primary responsibility in experiment design, data collection, and writing the manuscript. The co-authors provided guidance, supervision, and revision of the experimental design and the manuscript.

($p>0.05$). Higher levels of *S.* Newport from HTTP-amended soil transferred to and survived on spinach leaves for longer periods of time compared to *S.* Newport from unamended soil ($p<0.05$). *Salmonella* Newport introduced to HTTP-amended soils survived for longer periods and was more likely to transfer to and persist on spinach plants compared to unamended soils.

4.2. Importance

Heat-treated poultry pellets (HTPP) are a slow-release fertilizer suitable for use by organic growers of fruit and vegetables. However, subsequent contamination of soil on farms can occur via several routes, such as use of contaminated irrigation water or scat from wild animals. Upon contamination, the presence of HTPP in soil prolongs the survival of *S.* Newport. Here we show that HTPP amendment led to increased *S.* Newport survival in soil and led to greater likelihood of *S*. Newport transfer to and survival on spinach plants. There were no significant differences in survival durations of WT and *ΔrpoS S.* Newport isolates. The statistically similar populations recovered by plate count and estimated by PMA-qPCR for both strains in amended and unamended soil indicate that all viable populations of *S.* Newport in soils were culturable in this study.

4.3. Keywords

Biological soil amendments of animal origin (BSAAO), Heat-treated poultry pellets, *Salmonella* Newport, *rpoS*, soil, transfer, spinach, PMA-qPCR

4.4. Introduction

Leafy green vegetables and fruits, such as spinach, lettuce, and tomatoes, have frequently been associated with foodborne disease outbreaks due to *Salmonella enterica* and *Escherichia coli* O157:H7 (1-3).From 2004 to 2012 in the United States, 313 foodborne disease outbreaks in fresh vegetables and fruits (for example, salad, leafy greens, tomato, sprouts, berries, melons)

have occurred due to viruses and bacteria, with the second-most (71) and third-most (46) number of outbreaks attributed to *Salmonella enterica* and *E. coli*, respectively (4). Fresh produce may become contaminated at various stages in the farm-to-fork continuum, with pathogens surviving on raw fruits and vegetables for periods long enough to cause human illness. Untreated biological soil amendments of animal origin (BSAAO), such as animal manure and poultry litter, or treated BSAAO like composted or heat-treated manure using validated processes, are added to soil to provide essential nutrients for growth of vegetables (5-7). However, untreated BSAAO can be contaminated with various pathogens, such as *Salmonell*a *enterica*, enterohemorrhagic *E. coli*, and *Campylobacter* (8-11). *Salmonella enterica* subspecies *enterica* serovar Newport has been responsible for outbreaks associated with contaminated tomatoes (2005) and cucumbers (2014) originating from the Mid-Atlantic United States (2, 12). Although not specifically identified, the sources of *S.* Newport for these outbreaks were attributed to environmental reservoirs and potentially contaminated soil (2, 12).

In the U.S, fruit and vegetable growers complying with the United States Department of Agriculture (USDA) National Organic Program (NOP) are required to apply untreated BSAAO to soils at least 90 or 120 days before the harvest of the crop to minimize pathogenic contamination. Organic farmers rely on BSAAOs to provide nutrients to crops and a nationwide survey showed that 58% of the organic farmers used untreated manure and 48.6% of the untreated manure was from poultry (13). In the same study, 24.7% of the farmers used untreated manure in fresh produce production and 5.7% of those were not found to comply with the NOP guidelines. Use of poultry litter which has been heat-treated and pelletized (heat treated poultry pellets, HTPP) as a BSAAO may provide sufficient nutrients to leafy green vegetables while minimizing the risk of enteric bacterial contamination associated with untreated manure in the

pre-harvest environment. However, introduction of pathogens through the use of contaminated irrigation water (14, 15), scat from wild animals (16), bird droppings (17, 18), and runoff from nearby livestock operations (19-21) are all potential routes that may introduce bacterial pathogens to soils in crop fields, and the presence of HTPP in soil may aid survival as evidenced by increased growth in soil extracts prepared with HTPP amended soil (22).

Salmonella enterica survival in soil is dependent on several factors, such as soil and amendment types, moisture, irrigation, temperature, seasons, and geographic locations (23-25). Other agricultural factors that may affect the persistence of *Salmonella enterica* in soils are unknown, such as the presence or absence of plants and irrigation events. Plants may affect many soil physicochemical properties, and the presence of a rhizosphere may alter the microbial community of soils affecting *Salmonella* survival. Similarly, irrigation events may increase the moisture content of the soil as well as increase the solubility of carbon compounds in soils containing biological amendments, affecting levels of *Salmonella* spp. Kim et al. showed up to a 4-log increase in *Salmonella* spp. from initial levels in dairy composts with moisture content of 40 and 50% (26). Similarly, little is known about the mechanisms employed by *S.* Newport during survival in soil. RpoS, which codes for sigma factor 38, is involved in the general stress response and has been shown to play an important role in the survival of *S.* Typhimurium and *E. coli* O157:H7 in soils (27, 28). The extended survival of wild-type *rpoS* strains compared to *rpoS*-dysfunctional or deficient cells in soils indicates that enteric pathogen cells undergo physiological stress in non-host environments. Under these conditions, quantification of *Salmonella* spp. may be underestimated by traditional culture or direct plating methods as the recovery of physiologically stressed cells may be inhibited by the presence of selective agents in the media or aggregation of cells on a agar plate may lead to reduced cell counts. Quantitative

polymerase chain reaction (qPCR) may recover higher numbers of viable *Salmonella enterica* cells than traditional agar count method from soil (24, 29). While differences in *Salmonella enterica* cell counts in soil have been observed between traditional agar count methods and PMA-qPCR (propidium monoazide-qPCR), the effect of BSAAOs on physiological stress or differences in recovery of *Salmonella* by culture and culture-independent methods have not been fully described.

Salmonella spp. may be transferred from the soil to the leaves of spinach or lettuce during rain or irrigation splash events, and can subsequently persist for several days on leaves (30). Additionally, splash events can potentially lead to transfer of nutrients from soil to the leaves, which could increase the duration of *Salmonella* spp. survival. Studies about survival and transfer of *Salmonella* from soil to leafy greens are lacking. However, transfer of manure dust particles containing *Salmonella enterica*. to leaves of leafy green plants have shown varying degree of survival up to 14 days (31). During commercial spinach production, leaves may be harvested twice from the same plants but the likelihood of *Salmonella* transfer from soil to leaves at these different harvest times are lacking. Transfer of soil-adapted *Salmonella* from contaminated soil to carrots and radishes has been studied (32, 33); transfer of *Salmonella* spp. from soil to spinach foliar surfaces is not well understood. However, field experiments document that splash transfer of *E. coli* O157:H7 from animal scat to Romaine lettuce is affected by the distance between lettuce and scat, and the age of the scat before irrigation at a Salinas, CA site (34).

In this study, survival of wild-type (WT) and *rpoS*-deficient (*ΔrpoS*) *Salmonella* Newport in unamended soil and soil amended with HTPP in the presence and absence of spinach plants was determined. Population changes after irrigation events were quantified at selected weeks

during the longitudinal study. Quantification of viable cells was determined using traditional culture and PMA-qPCR methods in soil with spinach cultivation. In addition, moisture content and chemical characteristics of soil were measured to study their impact on *S.* Newport persistence. Simulated splash events preceding multiple spinach harvests were conducted to determine the transfer of *S. Newport* from amended and unamended soil to spinach leaves, and its survival on leaves was also assessed.

4.5. Materials and Methods

4.5.1. Source of S. Newport and rpoS mutant construction

A rifampicin-resistant *Salmonella enterica* subsp. *enterica* Serovar Newport wild type (WT) strain was obtained from the USDA ARS Environmental Microbial and Food Safety Laboratory and has been described previously (2). A *rpoS*-deficient (*ΔrpoS*) kanamycin-resistant *S.* Newport strain was constructed using the lambda red recombination method (47). Plasmids pKD4 and pKM208 were obtained from Addgene (Addgene, Cambridge, MA). Homologous regions of 50 bp length upstream (5' TGCTAGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTTATGAGTCAG 3') and downstream (5'GCGCTCATTCATGGGAACAGTTTTTTTCCGGTCAGCTGTCTGACCGGA 3') of the *S.* Newport *rpoS* gene were identified and paired with priming regions 20 bp upstream (5' GTGTAGGCTGGAGCTGCTTC 3') and downstream (5' CATATGAATATCCTCCTTAG 3') of the kanamycin cassette on the pKD4 plasmid. These primers were used for amplification of the kanamycin cassette (KanR) on pKD4 by PCR. Next, pKM208 was transformed into rifampicin resistant *S.* Newport by electroporation using a MicroPulser^{TM} (Bio-rad, Hercules, CA) at 1.8Kv Rifampicin-resistant *S.* Newport + pKM208 was cultured in Luria Bertani Broth (LB) containing 100 μg/ml ampicillin for 5 h at 30°C to obtain an optical density of 0.5,

followed by induction of red recombinase by addition of IPTG (Isopropyl-β-D-1-thiogalactopyranoside) at a final concentration of 1mM for 20 min prior to making cells electrocompetent as described by Murphy et al. (48). The amplified region of pkD4 with attached homologous regions upstream and downstream of *rpoS* was electroporated into the above prepared electrocompetent rifampicin-resistant *S.* Newport + pKM208 cells using the MicroPulser^{TM} (Bio-rad, Hercules, CA) at 2.5 Kv. The electroporated cells were incubated shaking at 30^{o}C for 90 min and then spread plated on LB agar containing 20 μg/ml kanamycin and incubated at 37^{o}C overnight. Kanamycin-resistant colonies were screened for the presence of the kanamycin cassette using the homology primers described above and kanamycin cassette primers (upstream: 'k1' - 5' GGGCACAACAGACAATCGGC 3' and downstream: 'kt' - 5' GCGGTCCGCCACACCCAGCC 3'). Loss of function of *rpoS* was confirmed by the lack of catalase activity and glycogen production (49).

4.5.2. Preparation of soil

Soil (fine, loamy, mesic Aquic Hapladults soils) was obtained from the USDA-ARS Beltsville Agricultural Research Center (BARC) North farm (Beltsville Maryland). Soil (10 kg) was evenly distributed into each of 28 window-box planters (Missry Associates, Inc., Dunellen, NJ) measuring 61 cm x 17.8 cm x 15.6 cm before placing in a growth chamber. Sterile deionized water was added to bring the soil in each planter to field capacity as observed by outflow of water at the bottom trough. After 24 h, commercial heat-treated poultry pellets (HTPP), with a nutrient content of 3-2-3 (N-P-K), were distributed evenly on top of the soil of 14 planters at the equivalent rate of 5 tons per acre (106.5 g per planter), and manually incorporated to a depth of 5 cm.

4.5.3. Growth chamber conditions

The growth chamber conditions reassembled a spring season in the Mid-Atlantic region (Maryland) with day (13 h, at 22-24 ^{o}C) and night (11 h, at 15-18^{o}C) settings. Similarly, the relative humidity was set at 40-60 % during the day and 30-60 % during the night and the photosynthetic photon flux density was measured to be similar across the growth chamber.

4.5.4. Soil inoculation

S. Newport WT and *ΔrpoS* strain were inoculated into separate planters. The rifampicin-resistant WT *S.* Newport strain and both kanamycin and rifampicin-resistant *ΔrpoS* strain were isolated onto XLT4 agar plates (Neogen Corp., Lansing, MI) with rifampicin (80 µg/ml) and kanamycin (25 µg/ml), respectively, and incubated at 37°C for 24 h. Isolated colonies of WT and *ΔrpoS* strain were separately inoculated into 30 ml of tryptic soy broth (TSB) with rifampicin (80 µg/ml) and kanamycin (25 µg/ml), respectively, and incubated at 37°C for 24 h. Cultures (100 µl) for each strain were transferred to 12 flasks of 100 ml TSB with rifampicin (80 µg/ml) and kanamycin (25 µg/ml), respectively, for WT and *ΔrpoS* strains and incubated at 37°C for 24 h. After incubation, cells were harvested by centrifugation at 10,000 x *g* for 5 min and washed in phosphate buffer solution (PBS) by centrifugation at 10,000 x *g* for 5 min. Washed cell pellets were suspended in 200 ml of PBS resulting in 2,400 ml of total cell suspensions for each strain. Cell suspensions were dispensed into a battery-powered backpack sprayer (H.D. Hudson Manufacturing Company, Chicago, IL). An additional 800 ml PBS was used to dilute the bacterial cultures to a create an inoculum with populations of 8.81 and 8.48 log CFU/ml for WT and *ΔrpoS S.* Newport strains, respectively. Surfaces of soil in 12 planters were each spray-inoculated with 175 ml of either the WT or *ΔrpoS S.* Newport inoculum in a biological safety

cabinet and returned to the growth chamber. Spray inoculation resulted in approximately 7 log CFU/gdw (gram dry weight) soil for both WT and *ΔrpoS* strains on day 0.

4.5.5. Spinach seeding and soil irrigation

Spinach seeds of 'Baby greens spinach'- *Spinacea oleracea* hybrid (Botanical Interest, Inc., Broomfield, CO) were soaked in 2 % bleach for 10 min, rinsed with sterile deionized water, and placed in the dark at 4°C for 48 h to stimulate germination. Seeds were planted 24 h post-inoculation of *S.* Newport to soil. Seeds were sown 4-5 cm apart in rows of two into 12 of the 24 planters: 3 planters each containing WT or *ΔrpoS S.* Newport strains with HTPP (amended) or without HTPP (unamended). Spinach seeds were sown in two additional planters (one amended and one unamended) not containing *S.* Newport to conduct chemical analyses on soil containing spinach plants. Seeds were sown approximately 1.27 cm deep and 5 cm apart in two rows along the length of each planter. Each planter was irrigated with 500 ml of sterile deionized water on days 7, 14, 28, 49 and 50 after inoculation.

4.5.6. Soil sampling and S. Newport enumeration

Soils were sampled on days 0, 7, 8, 14, 21, 28, 49, 50, 51, 63, 77, and 91. On each sampling day, a core soil sampler (LaMotte Company, Chestertown, MD) was used to collect soil 3-5 cm deep from two locations in each planter, which were then composited in a sterile whirl-pak bag (Nasco, Fort Atkinson, WI). The location where each soil sample was taken was marked with a plastic plant tag to avoid future sampling of soil from the same location within the planter. The composite soil sample (30 g) was diluted using sterile deionized water and further diluted as appropriate in PBS for *S.* Newport enumeration. Diluted samples were spread plated manually or using a WASP2 spiral plater (Microbiology International, Frederick, MD) on modified tryptic soy agar (TSA) (Neogen Corp., Lansing, MI) containing rifampicin (80 □g/ml),

ammonium ferric citrate (0.2 %), sodium thiosulfate (6 %), and cycloheximide (5 %) to distinguish *S.* Newport cells as black colony- forming units (CFU). Plates were incubated at 37°C for 24-48 h and colonies were counted manually or using an automated Protocol colony counter (Synbiosis, Cambridge, UK). Colony-counts were converted to log CFU/gram dry weight (gdw) soil. The limit of detection for the plate count method was 1.0 log CFU/gdw. When *S.* Newport counts were below the limit of detection, presence/absence of *S.* Newport WT and *ΔrpoS* was performed by enrichment method. Briefly, 30 g of soil samples were added to 270 ml of Buffered Peptone Water (Neogen Corp., Lansing, MI) supplemented with rifampicin (80 mg/ml) for 24 h at 37°C followed by streaking on XLT4 agar containing rifampicin (80 µg/ml) and incubation at 37°C for 24 h. Appearance of typical black colonies were considered presumptive for *S.* Newport.

4.5.7. Chemical analyses of soil

Soil samples for chemical analyses (Table S1) were analyzed throughout the study from uninoculated planters, but representing the four experimental conditions of HTPP-amended and unamended soils with or without spinach plants. Composite core soil samples as described above were collected and sent for chemical analyses through the University of Delaware Soil Testing Program, Newark, DE. Moisture content was determined on each soil sample (5 g) using 'TrueDry CV9' (Decagon Device, Pullman, WA).

4.5.8. Determination of S. Newport count by qPCR

Quantification of viable *S.* Newport were conducted on inoculated samples collected from planters containing spinach plants only. Homogenates of inoculated soils and PBS were used to extract DNA for qPCR. Briefly, 30 ml of 1:10 diluted homogenate for each sample was centrifuged at 3,000 x *g* for 2 min to sediment soil particles to avoid interference with PMA

(propidium-monoazide) dye. Preliminary experiments showed no loss of *S.* Newport cells in diluent before and after centrifugation. After centrifugation, 30 ml of supernatant was transferred to a sterile centrifuge tube and re-centrifuged at 12,000 x *g* for 10 min. The resulting supernatant was discarded and the cell pellet was homogenized in the remaining 1 ml diluent before transferto a 1-ml tube and centrifuging at 12,000 x *g* for 2 min. This cell pellet was suspended in 400 µl PBS for use in PMA staining to quantify viable *S.* Newport populations. In addition, qPCR without PMA dye was conducted on 0, 21, 35, 63, and 91 days to estmate total *Salmonella* count.

For staining, 100 µl of PMA enhancer (Biotium, Fremont, CA) was added to each 400 µl sample followed by PMA (Biotium, Fremont, CA) addition at a final PMA concentration of 75 µM. Samples were stored in dark for 10 min and then placed in a LED photolysis device PMA-Lite (Biotium, Fremont, CA) for 30 min. Total *S.* Newport cells without PMA staining were also quantified as explained above, but without the addition of PMA on days 0, 21, 35, 63, and 91. After light exposure, DNA was extracted from each sample using 'DNeasy PowerSoil kit' (Qiagen, Frederick, MD), following the manufacturer's protocol, but with increased bead-beating time of 20 min. After DNA extraction, qPCR was performed using the protocol described by Kawasaki et al. (50). This protocol used primers (Forward TS-11: GTCACGGAAGAAGAGAAATCCGTACG and Reverse TS-5: GGGAGTCCAGGTTGACGGAAAATTT) that targeted a 1.8-kb HindIII DNA fragment specific to *Salmonella* species, and the probe (5'FAM-ACAAGAAGCCCTGAGCGCCGCTGTGAT-BHQ13'). The qPCR reaction mixture consisted of 20 µl total volume with the final concentrations of TS11 and TS-5 at 0.12 µM, and S-FAM at 0.025 µM. The thermal cycle was set up as follows: 1 cycle for 10 min at 95°C, 40 cycles each

for 20 s at 95°C, 30 s at 64°C and 30 s at 72°C, and 1 cycle for 7 min at 72°C in a Mx3005P real time PCR unit (Agilent, Santa Clara, CA). For each sample, qPCR was conducted in duplicate and the observed cycle threshold values (C_t) values were recorded. Obtained C_t values were converted to log CFU/gdw based on a standard curve. For generation of the standard curve, soil samples were inoculated with WT or *rpoS*-deficient *S.* Newport cells ranging from 3 to 8.5 log CFU/gdw in duplicate. PMA-qPCR was conducted as described above and the obtained C_t values were plotted against the log CFU/gdw to obtain a standard equation (y=-3.446x + 45.398). Based on the standard curve, the detection range of *S.* Newport qPCR quantification was determined to be 3.5 to 8.5 log CFU/gdw.

4.5.9. Transfer and survival of S. Newport on spinach leaves

Transfer of *S.* Newport WT and *rpoS*-deficient strains from soil to leaves was carried out at day 28 or 35 dpi (days post inoculation) (Harvest I) and 63 dpi (Harvest II). For the Harvest I, transfer from unamended soil to leaves was conducted at 28 dpi and at 35 dpi for HTPP-amended soil. The transfer event at Harvest II was conducted at 63 dpi only from HTPP-amended soil to leaves pre-wet with water. At the above specified dpi for both harvests, inoculated soil samples, collected for *S.* Newport enumeration were diluted 1:2 in sterile deionized water and mixed. Homogenates (200 µl) of the diluted soil samples were inoculated onto multiple spots of 5 – 10 µl each on two to three leaves of nine spinach plants in each planter. Estimation of most probable number (MPN) of *S.* Newport was conducted on 0 (2 h post inoculation), 1, and 2 days post transfer using a 3-dilution (9-tube) MPN with three replicates in a 48-well block (VWR, Radnor, PA). On each of these days, leaves were collected from three separate plants from each planter, providing a total of nine plants per condition each day. Inoculated leaves were trimmed with sterile scissors above soil and placed in a small sterile whirl-pak bag (Nasco, Fort Atkinson, WI).

Six milliliters (enough volume to submerge the leaves) of BPW with rifampicin (80 μg/ml) were added to these bags and hand-massaged for 1 min and incubated for 5 min at room temperature. Bags were then hand-massaged again for 1 min and 1 ml of diluent was transferred to each of 3 wells of a 48-well block, then serially diluted as appropriate (0.1, 0.01) using BPW with rifampicin (80 μg/ml) for MPN determination. Blocks were incubated for 24 h at 37°C and after incubation, 1 μl from each well was streaked onto XLT4 agar with rifampicin (80 μg/ml). After incubation at 37°C for 24 h XLT4 agar plates were examined for typical *Salmonella* colony appearance; MPN estimates (MPN calculator, Build 23; http://members.ync.net/mcuriale/mpn/index.hmtl) were determined from the number of wells in each dilution that were positive for the presence of *S.* Newport.

4.5.10. Estimation of inactivation rates

The log CFU/gdw values from plate count at each condition were used to estimate death rates of *S.* Newport WT and *ΔrpoS* strains using Microsoft Excel addin GInaFiT version 1.6 (51). A log linear death model was used with the following equation where N is the final population, N_0 is the population on day 0, k_{max} is the death rate, and t is time in days.

$$N = N_0 * exp(-k_{max} * t)$$

4.5.11. Statistical analyses

Each experimental condition consisted a total of three planters, each serving as an individual replicate. Triplicate plate count (log CFU/gdw), qPCR counts, k_{max}, and moisture content obtained at each condition were used as the dependent variable whereas the strain type, amendment type, presence/absence of spinach plant, and day were used as the independent variables. Statistical analyses were conducted using ANOVA in SAS 9.4 (SAS Institute, Cary, NC) using 'GLIMMIX' procedure and corrections for multiple comparisons was done using

'Tukey' (p=0.05) for significant interactions. Correlation statistics were generated between chemical characteristics measured on day 0 and k_{max} using a stepwise correlation discrimination procedure 'proc stepdisc' in SAS 9.4. The number of plants from which *S.* Newport was recovered was estimated as significantly different by calculating odds ratio using Fisher's exact test in SAS 9.4 using t-test procedure with harvest, day, and amendment as dependent variables and the result 'positive' as the outcome variable, with p=0.05 considered for significant differences.

4.6. Results

4.6.1 S. Newport survival was enhanced in HTPP-amended soil

After inoculation, *S.* Newport WT levels in both HTPP-amended and unamended soil on day 0 were similar (p>0.05), measured as 7.35±0.26 and 7.40±0.14 log CFU/gdw (gram dry weight), respectively (Fig. 8). Similar populations were observed for *S.* Newport *ΔrpoS* counts on day 0 in amended and unamended soil (Fig. 8) (p>0.05). At day 7, both WT (5.66±0.29 log CFU/gdw) and *ΔrpoS* (5.21±0.19 log CFU/gdw) counts in unamended soil were observed to be significantly lower than WT (6.87±0.16 log CFU/gdw) and *ΔrpoS* (6.93±0.26 log CFU/gdw) counts in amended soil (p<0.05). No significant differences were observed between *S.* Newport WT and *ΔrpoS* populations during week 1 (p>0.05) in unamended soils. *S.* Newport in unamended soil declined rapidly by day 35, with levels of WT (2.23±0.81 log CFU/gdw) and *ΔrpoS* (1.30±0.28 log CFU/gdw) significantly lower than WT (5.45±0.33 log CFU/gdw) and *ΔrpoS* (5.15±0.0.20 log CFU/gdw) levels in amended soil (p<0.05). *S.* Newport counts in unamened soil were below the limit of detection (LOD) of 1 log CFU/gdw from day 49 onwards. However, unamended soil contained both *S.* Newport strains as determined by enrichment until day 91. Counts above LOD were observed for *S.* Newport WT (3.42±0.85 log CFU/gdw) and

ΔrpoS (2.85±0.18 log CFU/gdw) in amended soil until 91 days (Fig. 8). The only significant differences between WT and *ΔrpoS* counts were observed on days 49 and 50 in amended soil, and on day 35 in unamended soil ($p<0.05$).

Maximum inactivation rates (k_{max} in log CFU/gdw/day) of both *S.* Newport strains were significantly ($p<0.05$) different in HTPP-amended and unamended soils (Table 6). The average k_{max} values for *S.* Newport WT (0.12±0.02 log CFU/gdw/day) and *ΔrpoS* (0.13±0.01 log CFU/gdw/day) in HTPP-amended soil were significantly lower than that in unamended soil for *S.* Newport WT (0.33±0.04 log CFU/gdw/day)) and *ΔrpoS* (0.36±0.03 log CFU/gdw/day)), respectively ($p<0.05$). No significant differences in k_{max} were observed between *S.* Newport WT and *ΔrpoS* strains in either HTTP-amended or unamended soils (Table 6) ($p>0.05$).

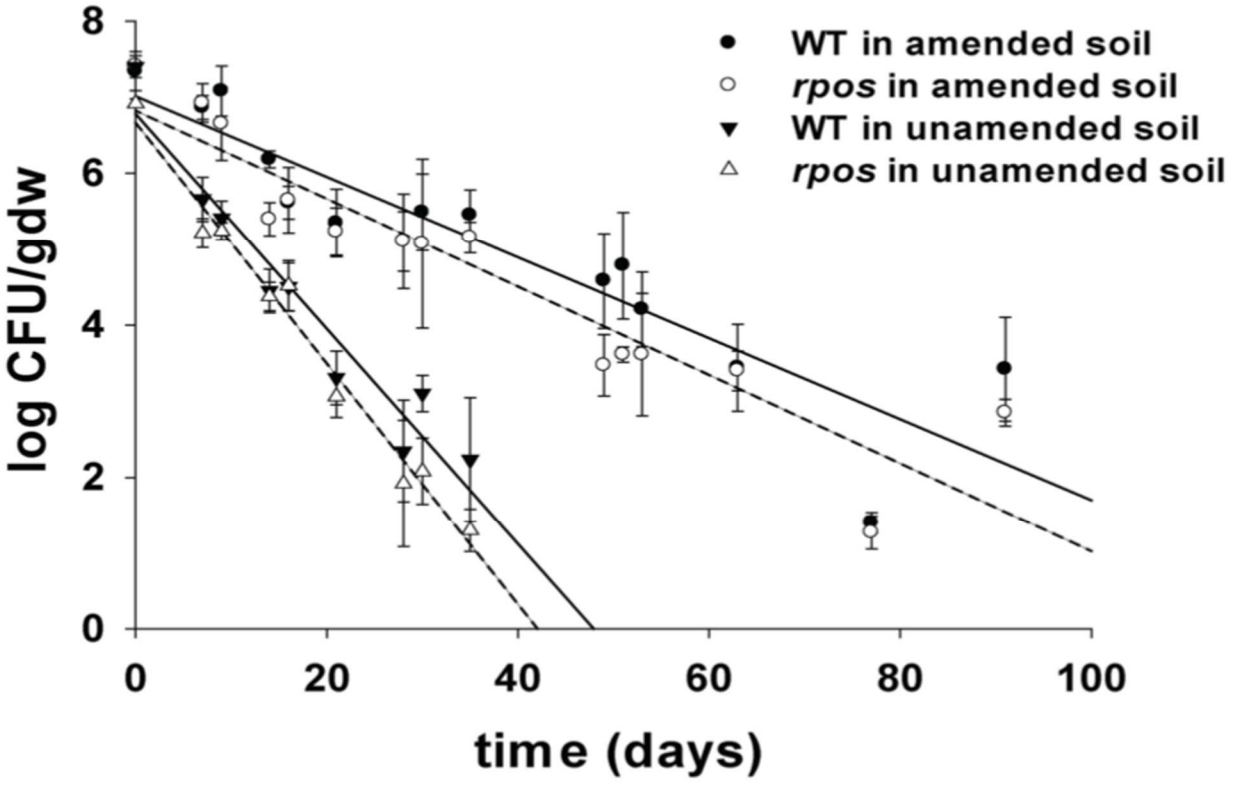


Figure 8. *S.* Newport WT (solid line) and *ΔrpoS* (dashed lines) survival in HTPP-amended and unamended soil (n=3 experimental replicates) with regression lines. Error bars indicate standard deviation and arrows show days of irrigation.

4.6.2. S. Newport survival in soil with or without spinach cultivation was similar

Soil containing spinach plants showed no significant differences in supporting the survival of both *S.* Newport WT and *ΔrpoS* strains as compared to survival in soil without spinach (Fig. 9) ($p>0.05$). Levels of *S.* Newport WT and *ΔrpoS* strains in both unamended and amended soil with or without spinach were similar on all days (Fig. 9a and 9b) ($p>0.05$). Also, the k_{max} for *S.* Newport was statistically similar between soils with or without spinach plants (Table 6) ($p>0.05$). The average k_{max} for *S.* Newport WT (0.10±0.01 log CFU/ gdw/day) and *ΔrpoS* (0.10±0.02 log CFU/gdw/day) in amended soil containing spinach plants were similar to k_{max} observed for *S.* Newport WT (0.12±0.02 log CFU gdw/day) and *ΔrpoS* (0.13±0.01 log CFU gdw/day) in soil without spinach plants ($p>0.05$). Similarly, no significant diferences in k_{max} for *S.* Newport were observed by the presence and absence of spinach plants in unamended soil ($p>0.05$).

Table 6. Inactivation rates (k_{max}) of *S.* Newport WT and *ΔrpoS* strains in HTPP amended and unamend soil planted with or without spinach.

strains	amendment	Spinach	k_{max} *(log CFU gdw^{-1} day^{-1})*
WT	yes	yes	0.10±0.01^{A}
		no	0.12±0.02^{A}
	no	yes	0.36±0.03^{B}
		no	0.33±0.04^{B}
ΔrpoS	yes	yes	0.10±0.02^{A}
		no	0.13±0.01^{A}
	no	yes	0.38±0.03^{B}
		no	0.36±0.03^{B}

Different superscript letters indicate significantly different values for k_{max} at p=0.05.

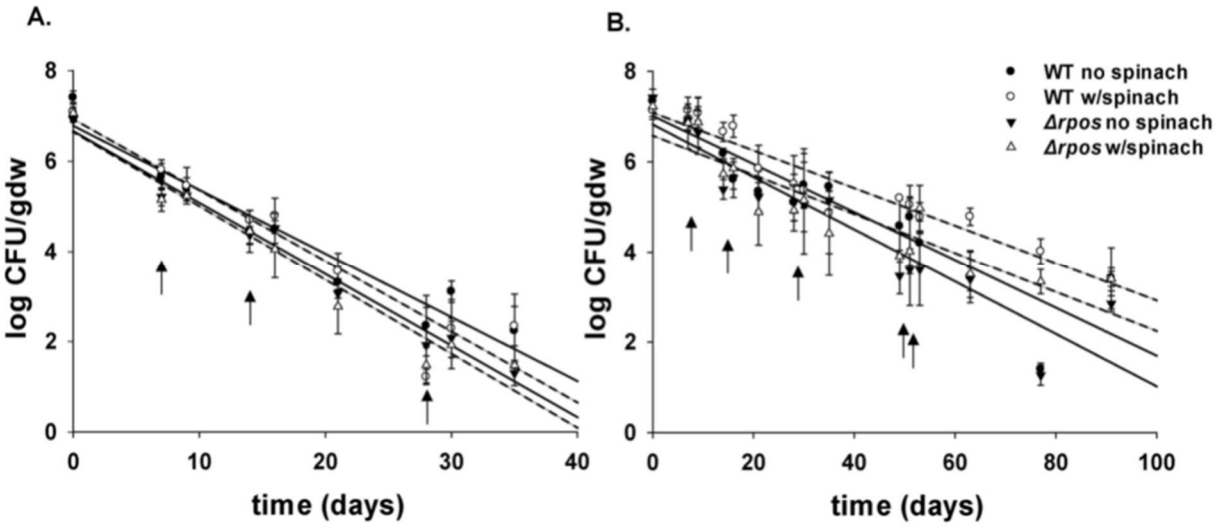


Figure 9. *S.* Newport WT and *ΔrpoS* survival in unamended (A) or HTPP-amended (B) soil without spinach (solid lines) or with spinach (dashed lines) from three experimental replicates with regression lines. Error bars indicate standard deviation and arrows show days of irrigation.

4.6.3. Quantification of S. Newport by PMA-qPCR and culture recovery were similar

S. Newport WT and *ΔrpoS* strains were quantified using PMA-qPCR in soil containing spinach plants. *S.* Newport WT levels on day 0 were quantified to be 7.47±0.32 and 7.48±0.26 log CFU/gdw using PMA-qPCR in amended and unamended soil, respectively, which were not significantly different from plate count of 7.14±0.20 and 7.09±0.21 log CFU/gdw in amended and unamended soil, respectively (Fig. 10) ($p>0.05$). *S.* Newport populations before and after irrigation events were also found to be similar between quantification methods ($p>0.05$). On day 77, PMA-qPCR counts of 3.98±0.83 and 3.98±0.20 log CFU/gdw for *S.* Newport WT and *ΔrpoS* strains, respectively, in amended soil, were similar to plate counts ($p>0.05$) with levels reaching the LOD of 3.5 log CFU/gdw for PMA- qPCR after 77 days (Fig. 10).

4.6.4. Irrigation led to increase in soil moisture without significant effects on S. *Newport counts*

In all treatments**,** soil was irrigated on days 7, 14, and 28, and significant increases in moisture percentages were observed (Fig. S1a) ($p<0.05$). The average soil moisture before

irrigation under all conditions were 17.5±2.2%, 13.5±4.0%, and 5.7±3.9% on days 7, 14, and 28, respectively, which significantly increased to 20.2±2.8%, 17.5±2.9%, and 11.5±4.2% after irrigation events on days 8, 15, and 29, respectively (Fig. S1a).

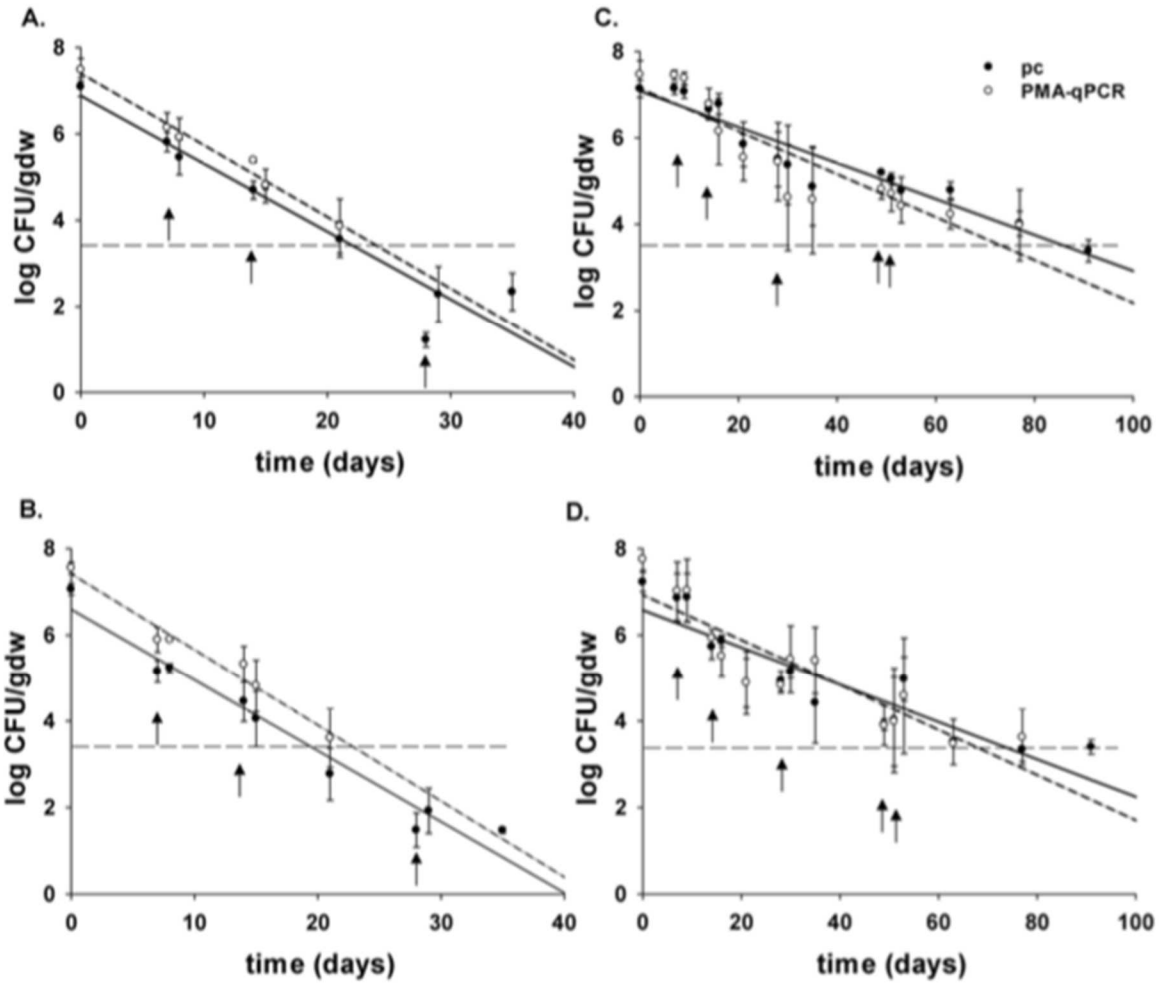


Figure 10. *S.* Newport WT (A) and *ΔrpoS* (B) in unamended soil and *S.* Newport WT (C) and *ΔrpoS* (D) in HTPP-amended soil as determined by plate count (pc, solid line), and PMA-qPCR (viable cells, dashed lines)), (n=3 experimental replicates) with regression lines. Error bars indicate standard deviation and arrows show days of irrigation. Dashed line shows the limit of detection (3.5 log CFU/gdw) for PMA-qPCR.

In contrast, significant changes in *S.* Newport WT and *ΔrpoS* populations were not observed before and after any irrigation events (Fig. 1 and 3) ($p>0.05$). Amended soils were irrigated on consecutive days 49 and 50 with significant increases in soil moisture on days 50

(11.0±2.2%) and 51 (13.6±1.7%) as compared to day 49 (3.9±2.8%) ($p<0.05$). However, irrigation on two consecutive days 49 and 50 did not appear to significantly affect *S.* Newport populations (Fig. 8 and 9) ($p>0.05$). Average soil moisture was observed to be the greatest in amended soil without spinach (11.9±6.5%) than amended soil with spinach (9.6±7.2%) ($p<0.05$). The presence or absence of spinach plants, and whether or not soils were amended or unamended, did not appear to have a significant effect on *S.* Newport populations after irrigation ($p>0.05$). Initial moisture content in unamended and amended soil were correlated with k_{max}, which showed that higher initial moisture content was significantly correlated with lower k_{max} (Table 7) ($p<0.05$).

4.6.5. Initial levels of total nitrogen and water soluble carbon were significantly correlated with increased S. Newport survival

The chemical characteristics of soil evaluated by measuring potassium (K), phosphorus (P_2O_5-P), nitrogen (NH_4-N + NO_3-N), total water extractable carbon (water soluble carbon), and ratio of total carbon and nitrogen (C:N) were observed to be substantially higher in HTPP-amended soil than in unamended soil on day 0 (Fig. S1b-f). No differences in these characteristics were observed in soil with or without spinach cultivation. Levels of these nutrients did not change substantially over the 91-day period, although a slight decrease in nitrogen content was observed. Therefore, the initial concentration of nutrients measured at day 0 was used to determine associations between soil nutrient characteristics and *S.* Newport survival. Total nitrogen measured as c.a. 33.44 mg/kg (NH_4-N+NO_3-N) (r^2:0.9991, $p<0.001$), and total water extractable carbon measured as 400.83 mg/kg (water soluble carbon) (r^2:1.0, $p<0.001$) in HTPP-amended soil were both significantly ($p<0.05$) correlated with increased *S.* Newport survival (lower k_{max}) (Table 7).

Table 7. Correlation statistics between *S.* Newport decline rate (kmax log CFU/day) and initial soil characteristics observed on day

	r^2	p value
moisture	0.8479	<0.0001
NH_4N+NO_3N	0.9991	<0.0001
TOC*	1.0000	<0.0001

*TOC is total water extractable carbon

4.6.6. Transfer and survival of S. Newport on spinach leaves

Spinach plants grown in soil with or without amendments containing *S.* Newport WT and *ΔrpoS* strains were harvested 35 days after sowing. Prior to transfer of *S.* Newport, nine plants were harvested to determine if any *S.* Newport was present on the plants. Two of the nine harvested spinach plants from amended soils contained low levels of *S.* Newport WT (average MPN of 1.33±1.37 / plant (Table 8). When HTPP-amended and unamended soil slurries were transferred to leaves of growing spinach plants, 4/9 plants (199.15±199.35 MPN/plant) and 5/9 plants (0.58±0.36 MPN/plant) were positive for *S.* Newport WT, respectively, when analyzed on the same day as the transfer event. Similarly, 4/9 (89.90±107.18 MPN/plant) and 3/9 (4.37±4.29 MPN/plant) plants were observed to be positive for *S.* Newport *ΔrpoS* when amended and unamended soil slurries were applied, respectively, after transfer. For plants receiving HTTP-amended soil slurries, 6/9 plants (15.84±17.31 MPN/plant) were positive for *S.* Newport WT and 4/9 (0.48±0.29 MPN/plant) plants for *S.* Newport *ΔrpoS* at 1 d post-transfer. For plants receiving unamended soil slurries, only 1/9 plants (0.36 MPN/plant) were positive for *S.* Newport *ΔrpoS* at 1 d post-transfer. At 2 d post-transfer, 2/9 plants (0.36±0.00 MPN/plant) which received HTPP-amended slurries were positive for *S.* Newport WT (Table 8).

After the first harvest (Harvest I), leaves on spinach plants in HTTP-amended soils were allowed to regrow and plants were harvested a second time (Harvest II) on day 63 (Table 8).

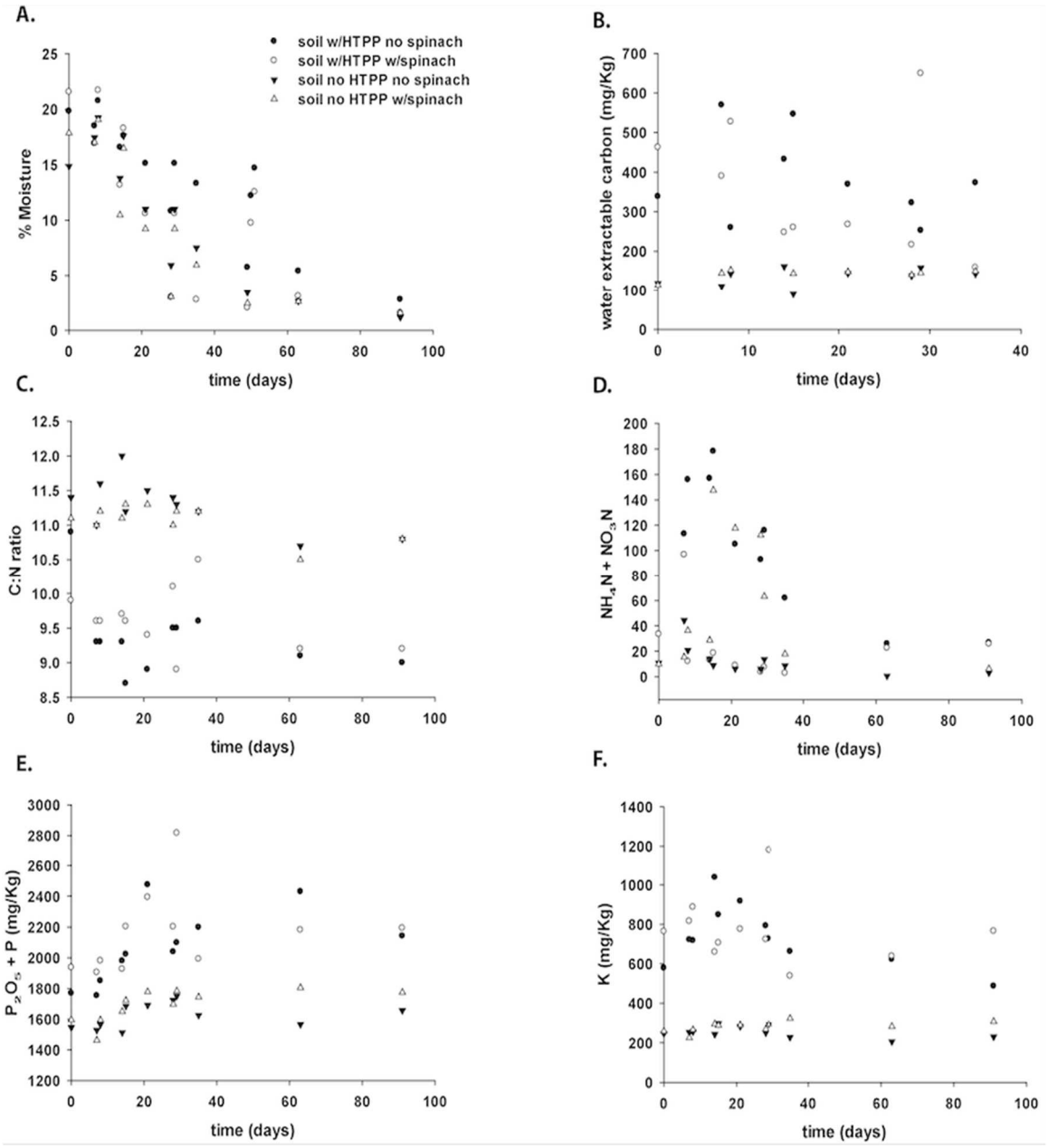
Unlike for the first harvest, leaves were misted with water to increase moisture on the leaves to mimic rain/irrigation events before manual transfer for the second harvest in HTPP-amended soil. On day 63, before transfer of *S.* Newport to spinach leaves, 3/9 and 2/9 plants were observed to be positive for *S.* Newport WT (2.88±4.29 MPN/plant) and *ΔrpoS* (2.20±0.14 MPN/plant), respectively, in HTPP-amended soil (Table 3). On day 0 after HTTP-amended soil slurries were applied to plants, 9/9 plants were positive for *S.* Newport WT (633.29±459.87 MPN/plant) and 9/9 plants were positive for *S.* Newport *ΔrpoS* (126.02±156.01 MPN/per plant). One day post-transfer, 9/9 and 6/9 plants were observed to be positive for *S.* Newport WT (28.89±47.46 MPN/plant) and *ΔrpoS* (3.32±2.03 MPN/plant), respectively. Two days post-transfer, 9/9 and 7/9 plants were observed to be positive for *S.* Newport WT (11.57±14.82 MPN/plant) and *ΔrpoS* (4.72±9.48 MPN/plant), respectively (Table 8).

Spinach plants grown in HTTP-amended soil containing *S.* Newport were significantly more likely to be positive for *S.* Newport than plants grown in unamended soil (odds ratio: 3.571, $p<0.05$). Also, significantly lower number of spinach plants were positive for *S.* Newport *ΔrpoS* strain as compared to *S.* Newport WT strain (odds ratio: 0.442, $p<0.05$). Increase in moisture on the leaves on day 63 at the second harvest event led to significantly greater number of plants positive for *S.* Newport than on the first harvest event on day 35 (odds ratio: 23.669) ($p<0.05$).

4.7. Discussion

4.7.1. HTPP-amended soil supported S. Newport survival for at least 91 days

S. Newport levels decreased by 3-5 logs over a 91-day period in HTPP-amended soil, while *S.* Newport in unamended soil reached the LOD (1.0 CFU/gdw) by day 35; however, unamended soil supported survival of *S.* Newport (detection by culture enrichment) until day 91.



Supplementary Figure 1. Chemical characteristics of soil over time.

Table 8. Number of spinach plants contaminated during transfer events with their MPN values.

	Harvest I (Day 35)				Harvest II (Day 63)	
	Unamended		Amended		Amended	
	S. Newport WT	*S.* Newport *Δrpos*	*S.* Newport WT	*S.* Newport *Δrpos*	*S.* Newport WT	*S.* Newport *Δrpos*
Before transfer	2/9 (1.33±1.37)*	0/9	0/9	0/9	3/9 (2.88±4.29)*	2/9 (2.20±0.14)*
Post-transfer						
2 h	5/9 (0.58±0.36)*	3/9 (4.37±4.29)*	4/9 (199.15±199.35)*	4/9 (89.90±107.18)*	9/9 (633.29±459.87)*	9/9 (126.02±156.01)*
24 h	0/9	1/9 (0.36±0.0)*	6/9 (15.84±17.31)*	4/9 (0.48±0.29)*	9/9 (28.89±47.46)*	6/9 (3.32±2.03)*
48 h	0/9	0/9	2/9 (0.36±0)*	0/9	9/9 (11.57±14.82)*	7/9 (4.72±9.48)*

*the values in parentheses are average MPN values observed for the positive spinach plants with their standard deviation

The prolonged survival (lower inactivation rate, k_{max}) of *S.* Newport in HTPP-amended soil can be correlated to the high levels of total nitrogen and total soluble carbon compared to levels in unamended soil. Similar observations have been made in other studies that used different BSAAO to study *Salmonella* spp. survival in soils. *S.* Newport survived for 107 days in dairy manure-amended soil stored at 25°C until it reached a LOD of 2 log CFU/g (120). *S.* Typhimurium inoculated via contaminated irrigation water into several types of manure-based composts exhibited survival for up to 231 days in soil (121). Survival of a three strain-inoculum of *Salmonella enterica* (Enteritidis, Heidelberg, and Typhimurium) in poultry compost and heat-treated poultry compost found that the strains survived for 77 and 14 days, respectively, when stored at 22°C (122). That study showed that the two compost types had very similar nutrient levels but higher concentrations of heavy metals were present in the heat-treated compost, which may have increased the inactivation rate of *Salmonella enterica*. Previous work has also shown that the same isolate of *S.* Newport used in the current study grew to higher popuations in simulated soil runoff containing HTPP as compared to unamended soil runoff (165). Overall, these studies demonstrate that pathogens can survive for long periods in BSAAO and their use as an amendment may lead to increased pathogen survival in soil.

In our study, the moisture content (4.2 – 21.4%) maintained to grow spinach plants via irrigation over 63 days in the growth chamber may have slowed the decline of *S.* Newport levels; the relatively high levels of water soluble carbon and nitrogen provided by the HTPP to the soil may have provided sufficient nutrients for *S.* Newport cells not to experience sudden physiological shock when introduced to amended soils. Similar to our study, Holley et al., found that higher moisture content in clay soil maintained at 80% field capacity showed increased *Salmonella* survival than that at 60% field capacity (110). Also, previous work has shown that

poultry litter provided higher nitrogen levels and potentially promoted greater survival of *E. coli* and *E. coli* O157:H7 in a greenhouse study (16). Moisture content, water extractable carbon (TOC), and nitrogen levels had a significant positive correlation with *S.* Newport survival. HTPP-amended soil retained greater levels of these characteristics promoting increased *S.* Newport survival compared to that in unamended soil. In the current study, initial moisture values of 20.7±2.9% in amended soil were associated with a slower inactivation rate. It should also be noted that soil moisture values decreased over time with no overall significant differences in moisture content between unamended or amended soil. This shows that the differences in k_{max} for *S.* Newport between unamended and amended soil may be attributed to differences in nutrient levels but not necessarily moisture levels. In a previous field study survival of non-pathogenic and O157-*E. coli* in untreated BSAAO-amended soils over multiple seasons, seasons in which the initial moisture content of soils was between 11.2 – 12.1% supported longer durations of *E. coli* survival, while those which had higher moisture contents (13.5 – 33%) supported shorter durations of survival (166). It is possible that *E. coli* and *S.* Newport respond differently to initial moisture content levels in BSAAO-amended soils. Also, irrigation events did not significantly affect *S.* Newport populations in HTPP-amended soils (soils containing a treated BSAAO). These findings contrast to previous work which showed that irrigation events in soils containing untreated BSAAOs affected *E. coli* populations over 56 days (34). However, that study was conducted with different pathogens (*E. coli*), soil types, untreated BSAAOs and in a different greenhouse environment.

4.7.2. Spinach cultivation and loss of rpoS function did not significantly impact S. Newport survival

In our study, cultivation of spinach plants did not affect the duration or level of *Salmonella* survival in unamended or HTPP amended soil. Similar levels of moisture and other nutrient characteristics in planters with or without spinach cultivation led to no significant differences in *S.* Newport survival under these conditions. RpoS has been shown to contribute to the survival of *Salmonella* in stressful conditions. Previous studies have shown the importance of *rpoS* for *Salmonella* Typhimurium survival during starvation in refrigerated (4.5°C), and high osmolarity environments (6 % NaCl) (129, 165). Similarly, Van Hoeak et al. reported that numerous *E. coli* O157 strains that survived for the longest number of days (>200 days) in manure amended soil had no mutations in the *rpoS* gene, whereas mutations were observed in strains surviving for fewer number of days (<155 days) (131), showing that *rpoS* may be functional during long term survival in manure-amended soil. In our study, no significant differences were observed between the survival of *Salmonella* Newport WT and *ΔrpoS* strains possibly because of favorable conditions nutrient and moisture conditions in the unamended and amended soils. In addition, *Salmonella enterica* is known to have many other stress response mechanisms that may contribute to survival other than expression of the *rpoS*-indiced general stress response.

4.7.3. S. Newport counts were similar between qPCR and plate count methods

PMA-qPCR has been used to quantify viable population of cells for several bacterial pathogens and have provided different levels of recovery compared to plate count methods under several physiological stressful conditions (140-142). PMA-qPCR detection of *E. coli* O157:H7 inoculated on lettuce leaves grown in a growth chamber maintained at 30 % relative humidity led

to a 4 log CFU increase in recovery of the pathogen when compared to plate count methods (140). Similarly, PMA-qPCR quantification of *L. monocytogenes* inoculated in pig manure and stored at 8 and 20°C recovered significantly greater populations compared to plate count methods (141). Differences in recovery of *S.* Typhimurium by *invA* gene qPCR and plate count methods without the use of PMA was studied in a microcosm experiment setup in glass vials using dairy cow manure (117). In their study, qPCR counts showed significantly greater levels of recovery of *Salmonella* Typhimurium compared to the plate count method in both manure-amended and unamended soil at 5, 15 and 25°C. However, the number of qPCR quantified cells without PMA count include DNA from dead *S.* Typhimurium cells resulting in greater counts. In our study, no significant differences were observed for *S.* Newport populations determined by PMA-qPCR and plate count methods. Mutations in *rpoS* has been attributed to rapid induction of viable but not culturable state for *Salmonella* Dublin, Oranienburg, and Typhimurium LT2 strains when incubated in 7 % NaCl (132). However, no differences between viable and culturable populations were observed for *S.* Newport WT and *ΔrpoS* strains in either amended or unamended soil. As referred to earlier, the water extractable carbon, nitrogen content, and moisture levels in HTPP-amended soils may have mitigated the physiological stress placed on inoculated *S.* Newport, which may account for the relative lack of differences in populations recovered by PMA-qPCR and plate count methods.

4.7.4. Prolonged S. Newport survival in amended soil led to increased S. Newport transfer to spinach plants

There are substantial gaps in the knowledge about the transfer of pathogens from soil to plants after an irrigation or rainfall event and their survival on the leaves until harvest. In this study, before manual transfer of contaminated soil to leaves, spinach plants were found to be

positive for *S.* Newport, which shows that farming activities can lead to potential produce contamination from contaminated soil. Manual transfer of contaminated soil to spinach plants, mimicking splash events, showed greater transfer of *S.* Newport from amended soil to spinach leaves, perhaps due to higher levels of *S.* Newport in HTTP-amended soils compared to unamended soils (Table 3). Nutrients in HTPP-amended soils diluted in water may have sustained *S.* Newport on spinach leaves after transfer leading to longer persistence of *S.* Newport on days 1 and 2 then when compared unamended soil. While studies of transfer of soil-adapted pathogens to produce have been lacking other studies have investigated survival of pathogens on leafy greens. Markland et al. showed that inoculation of *E. coli* O157:H7 at 5.7 log CFU/g on spinach leaves declined by approximately 4 log CFU/g in 24 h (84). In the same study, no *E. coli* O157:H7 were recovered on spinach leaves after 3 days. Other studies have investigated the transfer and survival of *Salmonella enterica* on produce from dried contaminated manure or irrigation water. Dried turkey manure dust inoculated with three *S.* enterica strains (Typhimurium CVM-98 and LT-2 and Enteritidis KPL 13076) was applied to both adaxial and abaxial leaves of spinach leaves and showed approximately 2-log reduction from an initial inoculation 3.5-4.0 log CFU/g in 14 days (82). In the same study, inoculation of spinach leaves with contaminated water at 6.5 – 7.0 log CFU/g showed approximately 5-log reduction in 14 days with substantial reduction observed on the adaxial surfaces of spinach leaves. The dried turkey manure protected *S. enterica* from the ultraviolet (UV) light disinfection, which led to longer survival durations on spinach leaves compared to *S. enterica* in water on spinach leaves. This is in agreement with results from the 1st harvest of our study, which showed that persistence of *S.* Newport WT in HTTP-amended soils on spinach was greater than in unamended soils. Our results from the 2nd harvest showed that persistence of *S.* Newport in HTTP-amended soils on

spinach leaves was enhanced by applying soil slurries to wet leaf surfaces. Similary, greater relative humidity have been shown to aid in increased survival of *Salmonella* spp., on cantaloupe rinds (83).

4.8. Conclusion

S. Newport survived for longer durations in HTPP-amended soils compared to in unamended soils due to higher nutrient (nitrogen, water extractable carbon) levels. Prolonged survival in HTPP-amended soils increased transfer of *S.* Newport to spinach plants. Increase in moisture on spinach leaves prior to transfer of *S.* Newport was associated with more frequent transfer, and promoted persistence of *S.* Newport on spinach plants. Similar levels of recovery were achieved by both plate count and qPCR methods, indicating that most *S.* Newport cells were viable and culturable in HTPP-amended soils under the observed conditions. The absence of the *rpoS* gene did not affect survival durations or inactivation rates of *S.* Newport in HTPP-amended or unamended soils. The relative availability of nutrients (nitrogen and water extractable carbon) in HTTP-amended soils compared to unamended soils, coupled with a high moisture content and relative humidity in the growth chamber, may have mitigated the physiological stress placed on *S.* Newport cells. Utilizing treated BSAAO to add nutrients for plant growth to soils may still require appropriate mitigation to minimize *Salmonella* Newport contamination of leafy greens in the pre-harvest environment.

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4.10. References

1. Hilborn ED, Mermin JH, Mshar PA, Hadler JL, Voetsch A, Wojtkunski C, Swartz M, Mshar R, Lambert-Fair MA, Farrar JA, Glynn MK, Slutsker L. 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of Mesclun lettuce. Archives of Internal Medicine 159:1758-1764.
2. Greene SK, Daly ER, Talbot EA, Demma LJ, Holzbauer S, Patel NJ, Hill TA, Walderhaug MO, Hoekstra RM, Lynch MF, Painter JA. 2008. Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005. Epidemiol Infect 136:157-165.
3. Wendel AM, Johnson DH, Sharapov U, Grant J, Archer JR, Monson T, Koschmann C, Davis JP. 2009. Multistate outbreak of *Escherichia coli* O157:H7 infection associated with consumption of packaged spinach, August-September 2006: the Wisconsin investigation. Clin Infect Dis 48:1079-1086.
4. Callejón RM, Rodríguez-Naranjo MI, Ubeda C, Hornedo-Ortega R, Garcia-Parrilla MC, Troncoso AM. 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. Foodborne Pathog Dis 12:32-38.
5. Tewolde H, McLaughlin MR, Way TR, Jenkins JN. 2016. Optimum Poultry Litter Rates for Maximum Profit versus Yield in Cotton Production. Crop Science 56:3307-3317.
6. Tewolde H, Sistani KR, McLaughlin MR. 2016. Residual Effect of Poultry Litter Applications on No-Till Cotton Lint Yield. Agronomy Journal 108:1405-1414.

7. Flavel TC, Murphy DV. 2006. Carbon and nitrogen mineralization rates after application of organic amendments to soil. J Environ Qual 35:183-193.

8. Létourneau V, Duchaine C, Côté C, Letellier A, Topp E, Massé D. 2010. Presence of zoonotic pathogens in physico-chemically characterized manures from hog finishing houses using different production systems. Bioresour Technol 101:4048-4055.

9. Chinivasagam HN, Redding M, Runge G, Blackall PJ. 2010. Presence and incidence of food-borne pathogens in Australian chicken litter. Br Poult Sci 51:311-318.

10. Volkova VV, Bailey RH, Wills RW. 2009. Salmonella in broiler litter and properties of soil at farm location. PLoS One 4:e6403.

11. Shepherd MW, Liang P, Jiang X, Doyle MP, Erickson MC. 2010. Microbiological analysis of composts produced on South Carolina poultry farms. J Appl Microbiol 108:2067-76.

12. Angelo KM, Chu A, Anand M, Nguyen TA, Bottichio L, Wise M, Williams I, Seelman S, Bell R, Fatica M, Lance S, Baldwin D, Shannon K, Lee H, Trees E, Strain E, Gieraltowski L, (CDC) CfDCaP. 2015. Outbreak of *Salmonella* Newport infections linked to cucumbers--United States, 2014. MMWR Morb Mortal Wkly Rep 64:144-147.

13. Pires AF, Millner PD, Baron J, Jay-Russell MT. 2018. Assessment of Current Practices of Organic Farmers Regarding Biological Soil Amendments of Animal Origin in a Multi-regional US Study. Food Protection Trends 38:347-362.

14. Steele M, Odumeru J. 2004. Irrigation water as source of foodborne pathogens on fruit and vegetables. J Food Prot 67:2839-2849.

15. Jones LA, Worobo RW, Smart CD. 2014. Plant-pathogenic oomycetes, *Escherichia coli* strains, and *Salmonella spp.* Frequently found in surface water used for irrigation of fruit and vegetable crops in New York State. Appl Environ Microbiol 80:4814-4820.

16. Laidler MR, Tourdjman M, Buser GL, Hostetler T, Repp KK, Leman R, Samadpour M, Keene WE. 2013. *Escherichia coli* O157:H7 infections associated with consumption of locally grown strawberries contaminated by deer. Clin Infect Dis 57:1129-1134.

17. Pedersen K, Clark L, Andelt WF, Salman MD. 2006. Prevalence of shiga toxin-producing *Escherichia coli* and *Salmonella enterica* in rock pigeons captured in Fort Collins, Colorado. J Wildl Dis 42:46-55.

18. Hellström S, Kiviniemi K, Autio T, Korkeala H. 2008. *Listeria monocytogenes* is common in wild birds in Helsinki region and genotypes are frequently similar with those found along the food chain. Journal of applied microbiology 104:883-888.

19. Santiago P, Jiménez-Belenguer A, García-Hernández J, Estellés RM, Hernández Pérez M, Castillo López MA, Ferrús MA, Moreno Y. 2018. High prevalence of *Salmonella* spp. in wastewater reused for irrigation assessed by molecular methods. Int J Hyg Environ Health 221:95-101.

20. Auld H, MacIver D, Klaassen J. 2004. Heavy rainfall and waterborne disease outbreaks: the Walkerton example. J Toxicol Environ Health A 67:1879-1887.

21. Söderström A, Osterberg P, Lindqvist A, Jönsson B, Lindberg A, Blide Ulander S, Welinder-Olsson C, Löfdahl S, Kaijser B, De Jong B, Kühlmann-Berenzon S, Boqvist S, Eriksson E, Szanto E, Andersson S, Allestam G, Hedenström I, Ledet Muller L, Andersson Y. 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. Foodborne Pathog Dis 5:339-349.

22. Shah MK, Bradshaw R, Nyarko E, Millner PD, Neher D, Weicht T, Bergholz T, Sharma M. 2019. Survival and growth of wild-type and *rpoS*-deficient *Salmonella* Newport strains in soil extracts prepared with heat-treated poultry pellets. J Food Prot. 82:501-506.

23. Sharma M, Millner PD, Hashem F, Camp M, Whyte C, Graham L, Cotton CP. 2016. Survival and Persistence of Nonpathogenic *Escherichia coli* and Attenuated *Escherichia coli* O157:H7 in Soils Amended with Animal Manure in a Greenhouse Environment. J Food Prot 79:913-921.

24. García R, Baelum J, Fredslund L, Santorum P, Jacobsen CS. 2010. Influence of temperature and predation on survival of *Salmonella enterica* serovar Typhimurium and expression of *invA* in soil and manure-amended soil. Appl Environ Microbiol 76:5025-5031.

25. Palacios MP, Lupiola P, Tejedor MT, Del-Nero E, Pardo A, Pita L. 2001. Climatic effects on *Salmonella* survival in plant and soil irrigated with artificially inoculated wastewater: preliminary results. Water Sci Technol 43:103-108.

26. Kim J, Jiang X. 2010. The growth potential of *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* in dairy manure-based compost in a greenhouse setting under different seasons. Journal of Applied Microbiology 109:2095-2104.

27. Chen Z, Jiang X. 2017. Thermal Resistance and Gene Expression of both Desiccation-Adapted and Rehydrated *Salmonella enterica* Serovar Typhimurium Cells in Aged Broiler Litter. Appl Environ Microbiol 83.

28. van Hoek AH, Aarts HJ, Bouw E, van Overbeek WM, Franz E. 2013. The role of *rpoS* in *Escherichia coli* O157 manure-amended soil survival and distribution of allelic variations among bovine, food and clinical isolates. FEMS Microbiol Lett 338:18-23.

29. Bech TB, Johnsen K, Dalsgaard A, Laegdsmand M, Jacobsen OH, Jacobsen CS. 2010. Transport and distribution of *Salmonella enterica* serovar Typhimurium in loamy and sandy soil monoliths with applied liquid manure. Appl Environ Microbiol 76:710-714.

30. Waitt JA, Kuhn DD, Welbaum GE, Ponder MA. 2014. Postharvest transfer and survival of *Salmonella enterica* serotype enteritidis on living lettuce. Lett Appl Microbiol 58:95-101.

31. Oni RA, Sharma M, Buchanan RL. 2015. Survival of *Salmonella enterica* in dried turkey manure and persistence on spinach leaves. J Food Prot 78:1791-1799.

32. Islam M, Doyle MP, Phatak SC, Millner P, Jiang X. 2004. Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. J Food Prot 67:1365-1370.

33. Natvig EE, Ingham SC, Ingham BH, Cooperband LR, Roper TR. 2002. *Salmonella enterica* serovar Typhimurium and *Escherichia coli* contamination of root and leaf vegetables grown in soils with incorporated bovine manure. Appl Environ Microbiol 68:2737-2744.

34. Atwill ER, Chase JA, Oryang D, Bond RF, Koike ST, Cahn MD, Anderson M, Mokhtari A, Dennis S. 2015. Transfer of *Escherichia coli* O157: H7 from simulated wildlife scat onto romaine lettuce during foliar irrigation. Journal of food protection 78:240-247.

35. You Y, Rankin SC, Aceto HW, Benson CE, Toth JD, Dou Z. 2006. Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. Appl Environ Microbiol 72:5777-783.

36. Islam M, Morgan J, Doyle MP, Phatak SC, Millner P, Jiang X. 2004. Persistence of *Salmonella enterica* serovar typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water. Foodborne Pathog Dis 1:27-35.

37. Chen Z, Kim J, Jiang X. 2018. Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* in animal waste-based composts as influenced by compost type, storage condition and inoculum level. J Appl Microbiol 124:1311-1323.

38. Holley RA, Arrus KM, Ominski KH, Tenuta M, Blank G. 2006. *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure. J Environ Qual 35:1170-1180.

39. Sharma M, Millner PD, Hashem F, Vinyard BT, East CL, Handy ET, White K, Stonebraker R, Cotton CP. 2019. *E. coli* survival duration in manure-amended soils is affected by spatiotemporal, agricultural, and weather factors: a multi-season, multi-site field study in the Mid-Atlantic US. Appl Environ Microbiol. 85: e02392-18.

40. McMeechan A, Roberts M, Cogan TA, Jørgensen F, Stevenson A, Lewis C, Rowley G, Humphrey TJ. 2007. Role of the alternative sigma factors sigmaE and sigmaS in survival of *Salmonella enterica* serovar Typhimurium during starvation, refrigeration and osmotic shock. Microbiology 153:263-269.

41. Moyne AL, Harris LJ, Marco ML. 2013. Assessments of total and viable *Escherichia coli* O157:H7 on field and laboratory grown lettuce. PLoS One 8:e70643.

42. Desneux J, Biscuit A, Picard S, Pourcher AM. 2016. Fate of Viable but Non-culturable *Listeria monocytogenes* in Pig Manure Microcosms. Front Microbiol 7:245.

43. Zhao X, Zhong J, Wei C, Lin CW, Ding T. 2017. Current Perspectives on Viable but Non-culturable State in Foodborne Pathogens. Front Microbiol 8:580.

44. Kusumoto A, Asakura H, Kawamoto K. 2012. General stress sigma factor RpoS influences time required to enter the viable but non-culturable state in *Salmonella enterica*. Microbiol Immunol 56:228-237.

45. Markland SM, Shortlidge KL, Hoover DG, Yaron S, Patel J, Singh A, Sharma M, Kniel KE. 2013. Survival of pathogenic *Escherichia coli* on basil, lettuce, and spinach. Zoonoses Public Health 60:563-571.

46. Stine SW, Song I, Choi CY, Gerba CP. 2005. Effect of relative humidity on preharvest survival of bacterial and viral pathogens on the surface of cantaloupe, lettuce, and bell peppers. J Food Prot 68:1352-1358.

47. Murphy KC, Campellone KG. 2003. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli*. BMC Mol Biol 4:11.

48. Murphy KC, Campellone KG, Poteete AR. 2000. PCR-mediated gene replacement in *Escherichia coli*. Gene 246:321-330.

49. Bhagwat AA, Tan J, Sharma M, Kothary M, Low S, Tall BD, Bhagwat M. 2006. Functional heterogeneity of RpoS in stress tolerance of enterohemorrhagic *Escherichia coli* strains. Appl Environ Microbiol 72:4978-4986.

50. Kawasaki S, Fratamico PM, Horikoshi N, Okada Y, Takeshita K, Sameshima T, Kawamoto S. 2010. Multiplex Real-Time Polymerase Chain Reaction Assay for Simultaneous Detection and Quantification of *Salmonella* Species, *Listeria*

monocytogenes, and *Escherichia coli* O157:H7 in Ground Pork Samples. Foodborne Pathogens and Disease 7:549-554.

51. Geeraerd AH, Valdramidis VP, Van Impe JF. 2005. GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. Int J Food Microbiol 102:95-105.

5. CONCLUSIONS

Salmonella enterica and *L. monocytogenes* are two major foodborne pathogens with one of the highest burden of illnesses, hospitalization, and deaths in humans. Both pathogens are able to adapt well in their respective environments leading to their increased survival. It is their enhanced stress adaptive capabilities to survive in various stressful environments, such as increased survival in bile and salt for *L. monocytogenes* leading to perhaps increased dissemination into our body causing listeriosis. Similarly, increased survival of *Salmonella* Newport in soil and manure offs for prolonged time periods leading to contamination of crops during cultivation, which causes illnesses in humans upon consumption of contaminated foods.

It is evident that significant differences in survival characteristics exist among strains of *L. monocytogenes* under 1% bile stress, showing increased stress adaptability of some strains (lineage I) that eventually may help in enhanced virulence/pathogenesis. Also, pre-exposure to salt and subsequent treatment with bile showed a cumulative impact on the survival for all six strains, which shows that no cross-protection phenomena was observed but rather an additive effect of both stresses were observed under my experimental conditions. It is probable that changes in experimental conditions, such as exposure to salt and bile stresses under anaerobic conditions or at higher concentrations of bile may provide differing results for growth/survival assays. On the other hand, it was interesting to see constant populations of *L. monocytogenes* cells under salt stress after pre-treatment with 1% bile. The fact that pre-exposure to a cell envelope stress and treatment with osmotic stress leads to bacteriostatic populations of *L. monocytogenes* cells can be exploited to our advantage to kill harmful pathogens utilizing a combination of both stresses. It also provides additional information on survival abilities of *L. monocytogenes* in the GI system pertaining to its virulence and pathogenesis. The formation of

filaments under this stressful condition also show that *L. monocytogenes* can adapt to the condition while dividing into multiple cells under favorable conditions. Further research needs to be conducted on this subject matter to elucidate the mechanisms involved in filament formation under such stressful conditions.

Salmonella survival and growth in soil extracts mimicking manure runoff was investigated to understand the produce contamination problem under pre-harvest conditions. *Salmonella* Newport increased in cell densities by 4 to 5 logs CFU/ml in HTPP amended soil extracts within 24 h and remained in stationary phase for 96 h. This indicates that contaminated manure runoff water that are rich in nutrients may lead to increase in bacterial populations in irrigation canals or farms. Use of such contaminated irrigation water can eventually contaminate soil and crops on the farms. On the other hand, it was observed that soil extracts prepared without HTPP showed survival of *S.* Newport for 96 h without significant increase in *S.* Newport counts that is because these matrices lacked essential nutrient for their growth. It was also evident that the presence of indigenous microbes and mutation in *rpoS* showed decreased survival or growth of *S.* Newport, which indicates that survival in such matrices are not favorable for *S.* Newport. This also shows that *rpoS* has an important role in increased survival under such conditions.

Similarly*, Salmonella* Newport survival in soil amended with HTPP was prolonged by at least 2 months, which shows that the presence of HTPP greatly increased *Salmonella* survival in soil. Presence of spinach and regular irrigation events did not show significant impact in *S.* Newport survival. This may be because, the rhizosphere of spinach may have minimal effect on *Salmonella* population and that irrigation events were not observed to increase the solubility of nutrients in HTPP. Unlike other studies that suggests formation of viable but non-culturable cells

under stressful conditions, this study showed no such *S.* Newport populations in soil with or without HTPP. Similarly, no significant differences were observed between wild-type and *rpoS*-deficient strains. This may be because the relative humidity and amount of moisture maintained in the soil needed to grow spinach plants may not have been as stressful for *S.* Newport. Also, it was observed that greater number of *S.* Newport cells transferred and survived to spinach plants from HTPP amended soil than from unamended soil. In addition, increased moisture on spinach leaves showed increased survival on spinach leaves, which indicates that transfer of pathogens to spinach leaves after splash events following rain events may lead to increased produce contamination. These results indicate several factors needs to be considered to minimize contamination of fresh produce on farms. It is not only important to use treated manure but also use proper irrigation water. Also, produce fields should be inspected for splash events after rain or irrigation occurrences to verify any potential transfer of soil debris to leafy greens or vegetables causing probable *Salmonella* transfer from the contaminated soil. Proper farming practices and robust guidelines based on strong scientific research, such as this should help mitigate produce contamination problems during cultivation on farms.

6. FUTURE WORK

In this study, differences in growth parameters were observed among *L. monocytogenes* strains under 1% bile. Although the selection of strains included only three strains belonging to each lineage I and II, it suggests that significant differences in survival phenotypes occur among strains with varying genotypes. As discussed earlier, survival under several stresses, such as cold temperature and salt has been explored for various clonal complexes and show significant phenotypic differences among them. It may be hypothesized that growth and survival characteristics differ among *L. monocytogenes* strains in bile stress, which may be investigated further among serotypes and most importantly among clonal complexes. Also, such differences in survival maybe due to genetic characteristics, such as presence/absence of genes or single nucleotide polymorphisms, which can be further studied. While variation in growth characteristic among *L. monocytogenes* were observed in 1% bile, it is unknown if similar observations can be made under the conditions representing the GI system, such as under anaerobic and acidic conditions. Also, in this study, no cross-protection phenomenon was observed for *L. monocytogenes* in contrast to that noted in previous studies that was conducted at higher concentrations of bile. Cross-protection may be observed between salt and bile stresses with pre-exposure of cells at higher concentration of bile for survival studies rather than for growth characteristics, which needs to be further investigated. In addition, formation of filaments has been observed under stressful conditions, however, little is known about the formation of filaments in *L. monocytogenes*. Also, very little is known about *L. monocytogenes* filament upon resuscitation to a favorable environment. Observation from other studies suggests that resuscitation of *L. monocytogenes* cells will lead to division of elongated cells into multiple cells

increasing the counts of *L. monocytogenes* under favorable conditions, which can also be further investigated.

This study showed increased growth of *S.* Newport in soil extract prepared with HTPP. It can be hypothesized that soil extracts prepared using other biological soil amendments of animal origin (BSAAOs) will show similar growth characteristics. Similarly, it can be hypothesized that *Salmonella enterica* can survive for longer periods and to a greater concentration in bodies of water in irrigation canals and farms containing manure runoffs if these niches contain some level of nutrients enriched from sources, such as manure runoff. *E. coli* O15:H7 and *L. monocytogenes* are other two pathogens that are also known to be found in contaminated BSAAO. It can be hypothesized that soil extracts containing BSAAOs will cause increased survival and growth of *L. monocytogenes* and *E. coli* O157:H7 as observed for *Salmonella enterica*.

This study also investigated the survival of *Salmonella enterica* inoculated at ~7.5 log CFU/gdw in soil with no increase in cells counts observed after irrigation events. Lower levels (3-4 log CFU/g or lower) of *Salmonella* and *E. coli* has been observed in contaminated manure and soil. It may be hypothesized that when soils are inoculated at lower levels, growth of cells may be observed after irrigation events as observed in previous studies for *E. coli*. Also, in this study, no formation of viable and culturable cells were observed in amended or unamended soil, which may be because of increased relative humidity and availability of nutrients. It may be hypothesized that decreased relative humidity and low availability of nutrients has a role in the formation of viable and non-culturable *Salmonella* cells in soils. Also, transfer of soil adapted *Salmonella* to leafy greens and their survival on such plants are inadequately studied. The results in this study suggest that increased moisture levels show increased *Salmonella* survival on spinach leaves. It may be hypothesized that there is prolonged survival of *Salmonella* on leafy

greens after rain events or in conditions with greater relative humidity. Such survival studies would provide additional information about *Salmonella* persistence on produce. In addition, this study was conducted in a growth chamber, which had a very strict set of conditions that may differ from conditions observed on a farm environment. Although similar results may be expected under farm conditions, it is equally important to be investigated. Such studies would provide more stringent results that can be used for drafting of appropriate and robust guidelines for farmers and all stakeholders for the mitigation of produce contamination problems during cultivation.

APPENDIX. PUBLICATIONS

1. Shah, Manoj K., Rhodel Bradshaw, Esmond Nyarko, Patricia D. Millner, Deborah Neher, Thomas Weicht, Teresa M. Bergholz, and Manan Sharma. "Survival and Growth of Wild-Type and *rpoS*-Deficient *Salmonella* Newport Strains in Soil Extracts Prepared with Heat-Treated Poultry Pellets." *Journal of food protection* 82, no. 3 (2019): 501-506.
2. Shah, Manoj K., Rhodel Bradshaw, Esmond Nyarko, Eric T. Handy, Cheryl East, Patricia D. Millner, Teresa M. Bergholz, and Manan Sharma. "*Salmonella* Newport in soils amended with heat-treated poultry pellets survived longer and more readily transferred to and persisted on spinach." *Appl. Environ. Microbiol.* (2019): AEM-00334.