THE RESPONSE OF *SALMONELLA ENTERICA* AND ENTEROHEMORRHAGIC *ESCHERICHIA COLI* IN LOW-MOISTURE ENVIRONMENTS

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MASTER OF SCIENCE

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ABSTRACT

The recent increase in outbreaks of low-moisture foods due to contamination with foodborne pathogens has led to an increase in interest of the response of these pathogens to low-moisture environments. In addition, knowledge of the response of foodborne pathogens to immediate desiccation stress is sparce. The first objective was to evaluate the long-term survival of enteric pathogens on wheat grain over the course of a year. Hard red spring wheat was inoculated with strains of *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) and their survival was monitored for a year. Strains of *Salmonella enterica* were detected over the course of 52 weeks, while all strains of EHEC passed below the limit of detection by 44 weeks. The second objective was to evaluate the tolerance of various enteric pathogens to sudden desiccations stress and wide variation was seen between species and among strains and growth conditions.

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DEDICATION

My dedication goes to my daughter, Amelia, becoming your mom has been one of the greatest joys of my life.

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1. INTRODUCTION

Foodborne pathogens are of major concern in the manufacturing of low-moisture food products. This concern has only recently been noted as low-moisture foods were previously thought to be low-risk for contamination with foodborne pathogens because they are incapable of supporting microbial growth. However, it is now known, that while they are not able to grow under these conditions, they are able to survive for extended periods of time in high enough levels to cause infection (*5,13*). Two of the foodborne pathogens that are of concern in low-moisture foods are *Salmonella* and *Escherichia coli*. One low-moisture food that has become increasingly linked to outbreaks of foodborne disease is wheat flour. Wheat flour has been connected to outbreaks of foodborne illnesses with increased frequency in recent years, specifically, outbreaks involving *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC). However, there is little information regarding the survival of these pathogens on wheat grain during long-term storage in a low moisture environment.

The long-term survival of foodborne pathogens is an important aspect of their persistence in a low-moisture environment, but there is more to it than just their survival. The response of the bacteria to immediate desiccation stress is also of interest. The main consequences of desiccation and the resulting loss of water includes the shrinkage of the cell and increase in intracellular salt and macromolecule concentration along with reduced fluidity of membrane lipids and damage to proteins and DNA (*9*). A pathogen's ability to tolerate desiccation stress and the resulting effects on the cell is thought to contribute to the persistence of pathogens in dry foods and food processing environments.

Our study aimed to obtain more information on the desiccation tolerance and long-term survival capabilities of *Salmonella enterica* and EHEC. The first objective was to observe the

fate of strains of different serovars of *Salmonella enterica* and EHEC serotypes on wheat grain over the course of one year, and the second objective was to observe the desiccation tolerance of various strains and serovars of *Salmonella enterica* and EHEC serotypes on a plastic surface.

2. LITERATURE REVIEW

2.1. Foodborne Disease

Foodborne diseases have a profound impact on both human health and the global economy. A case of foodborne disease occurs when a food product contaminated with a foodborne pathogen is consumed, and the ingested pathogen establishes itself in the human host or when a toxigenic pathogen establishes itself in a food product and produces a toxin which is then ingested by the human host (*6*). The former is classified as a foodborne infection, while the latter is classified as a foodborne intoxication. Foodborne infections generally have a longer time period between the time of ingestion and when symptoms occur, since an incubation period is required for the pathogen to establish itself. A foodborne disease outbreak is classified as two or more cases of similar illness resulting from the ingestion of a common food (*6*). There have been many foodborne diseases that have been identified worldwide causing the most severe cases in the very old, the very young, and the immunocompromised (*6*). In 2010, a global study showed that thirty-one foodborne hazards caused an estimated 600 million foodborne illnesses and 420,000 deaths, producing a global burden of 33 million Disability Adjusted Life Years (DALYs) (*42*). Annually, an estimated 300,000 hospitalizations and 5,000 deaths are related to foodborne disease in the United States, and just a single event of a foodborne disease outbreak can bring massive economic losses (*47*). Food safety incidents are estimated to cost the economy around $7 billion per year in the United States and foodborne illness has an estimated annual health related cost of $51.0 billion (*89*). This cost comes from notifying customers, removing all possibly contaminated product from retailors, and paying damages. Loss of consumer demand and lost markets are also represented in this cost (*47*). In the United States, there were 98 recalls of food and beverage products due to foodborne disease safety incidents in 2019 (*27*). This large

number of recalls is an indicator of how often food products can leave the manufacturer with the potential to cause serious disease. There are multiple factors that have led to the increase in number of food safety incidents, including globalized food trade, food production involving multiple manufacturing sites, and a complex supply chain (*47*). Foodborne pathogens are the biological agents that cause foodborne diseases, and it is estimated that each year, 31 pathogens cause 9.4 million cases of foodborne disease. Of these 9.4 million cases, an estimated 3.6 million are caused by bacteria (*88*).

2.2. Enteric Pathogens

Bacteria are a common cause of foodborne disease. Species of bacteria within the family Enterobacteriaceae are a big threat to food safety. Pathogenic bacteria of the family Enterobacteriaceae are known for causing enteric disease when ingested, more commonly known as gastroenteritis or food poisoning. Gastroenteritis occurs when there is an intestinal infection that is associated with disrupted intestinal absorptive and/or barrier function in addition to the passage of unformed stools (*58*). Enteric pathogens are defined as microbes that are able to cause enteric disease. Their pathogenesis can involve various factors including signals that trigger host inflammation, direct invasion, or secreted toxins that damage host cells. The intestinal epithelium, a heterogeneous mixture of cells located next to the microbiota and luminal contents, is where enteric infections occur (*66*). There is a barrier between the intestinal and epithelial cells and when it is breached by an enteric pathogen, leakage of luminal contents can occur which in turn disrupts the immune cells leading to an inflammatory response. This inflammatory response can lead to both diarrhea and disrupted absorptive function (*95*). There are many species of enteric pathogens that can cause enteric disease. *Salmonella* and *Escherichia* are two genera that contain species of enteric pathogens that pose the biggest threats to food safety.

2.2.1. *Salmonella*

The genus *Salmonella* in the family Enterobacteriaceae contains species of pathogens that are some of the most common causes of enteric infections worldwide. *Salmonella* are rod shaped, flagellated, Gram-negative, facultative anaerobes. The genus *Salmonella* is divided into two species, *S. enterica* and *S. bongori*. These two species are classified based on differences in their 16S rRNA sequences. *S. enterica* is then further classified into six subspecies based on both biochemical properties and genomic relatedness (*83*). *S. enterica* subsp. *enterica* is the subspecies found predominantly in mammals and accounts for approximately 99% of *Salmonella* infections in humans, while the other five subspecies of *S. enterica* and *S. bongori* are primarily found in the environment (*19*). Within these subspecies, *Salmonella* can be further classified into serovars using the Kauffmann–White classification scheme. This classification is based on characteristic antigenic determinants including the lipopolysaccharide (O), capsular (K), and flagellar protein (H) antigens (*8*). The H antigen may occur in either or both of two forms referred to as phase 1 and phase 2 and the O antigens occur on the surface of the outer membrane and are determined by sugar sequences on the cell surface (*33*). The K antigens are heat-sensitive polysaccharides on the bacterial capsular surface and are the least common among the antigens found in the serotypes of *Salmonella* (*19*). More than 2,600 serovars have been classified using the Kauffmann–White scheme with over half of them belonging to *S. enterica* subsp. *enterica* and many of these serovars are capable of causing illness in both humans and animals (*49*). *S. enterica* serovars Typhimurium and Enteritidis are the leading causes of *Salmonella* infections worldwide (*80*). Human infections of *Salmonella* typically occur when contaminated food products are ingested, resulting in gastroenteritis.

The most common manifestation of *Salmonella* infection, or salmonellosis, is self-limited, uncomplicated gastroenteritis with symptoms presenting anywhere from 6 to 72 hours after ingestion (*33*). Worldwide, gastroenteritis caused by *Salmonella* infection accounts for 93.8 million cases and 155,000 deaths each year (*62*). The symptoms that accompany gastroenteritis are non-bloody diarrhea, nausea, vomiting, abdominal cramps, headaches, and muscle aches. Diarrhea, however, is the foremost indication of gastroenteritis. Occasionally, asymptomatic infections can occur. The duration of these symptoms may vary, but typically lasts 2 to 7 days (*45*). Cases of salmonellosis are one of the most common bacterial foodborne diseases across the globe (*2*).

2.2.1.1. Epidemiology of Salmonellosis

Salmonellosis is a disease that affects both humans and animals worldwide, and *Salmonella* spp. are the leading cause of bacterial foodborne disease in the United States (*6*). More than 95% of salmonellosis cases are foodborne and they account for approximately 30% of foodborne disease related deaths in the United States annually (*45*). The CDC estimates that each year over one million people in the United States become infected with salmonellosis leading to an average of 19,000 hospitalizations and 380 deaths (*88*). Salmonellosis is a major public health concern as the bacteria has a number of animal reservoirs, can be found naturally in the environment, and both human and animal carrier states exist. *Salmonella* spp. are widespread in the environment and commonly found in farm effluents, human sewage, and materials subject to fecal contamination (*74*). *S. enterica* serovars are able to adapt to a variety of hosts and can cause disease in both humans and animals. Animals that serve as the primary reservoirs for *Salmonella* include cows, chickens, pigs, and turkeys. *S. enterica* is able to persist in the intestinal tracts of animals creating chronic carriers that continuously shed the bacteria in their feces, serving as a

reservoir for future contamination (*49*). This also makes the fecal-oral route an important mode of transmission. Various pests including rodents and insects also play a role in the transmission of *Salmonella*. Rodents can acquire *Salmonella* from the feces of infected domestic or wild animals and are able to carry the bacteria in their intestinal tract asymptomatically, making them significant vectors and reservoirs for its transmission (*49*). Pests such as these have been associated with the contamination of animal feeds, water, and stored grains on farms (*3,100*). *Salmonella* is ubiquitous and non-fastidious because the bacteria are capable of growing and multiplying under a variety of environmental conditions outside of a host. For example, *Salmonella* spp. are known to be persistent in both dry environments and in water for time periods spanning several months (*49*).

Salmonellosis can be transmitted through various different modes including the fecal-oral route, consumption of contaminated food products, and occasionally person to person (*45*). The consumption of contaminated food products is the primary mode of transmission for *S. enterica* (*33*). This mode of transmission for salmonellosis can occur through the consumption of inadequately cooked raw meats or eggs, cross-contaminated foods, unpasteurized dairy products, contaminated fruit and vegetable products, and contaminated and inadequately treated drinking water (*43*). Improper storage and direct contact with raw ingredients also play an important role in the transmission of *Salmonella*. Salmonellosis has been identified and recognized in all countries, but it appears to be most prevalent in regions with concentrated animal husbandry (*80*). The severity of salmonellosis in humans can vary depending on the serovar involved and the health of the human host. The most serious infections are recognized predominantly in infants, the elderly, and immunocompromised persons (*19*). Despite global improvements in

sanitation and hygiene, the incidence of salmonellosis continues to increase creating both an economic and public health burden for industrialized and underdeveloped countries (*62*).

2.2.1.2. Pathogenesis of Salmonella

Nearly all strains of *Salmonella* are pathogenic because they are capable of invading, replicating, and surviving in human host cells which results in disease (*19*). Pathogenic *Salmonella* spp. utilize a variety of virulence factors to colonize host cells through attachment, invasion, and bypassing the hosts gastrointestinal defense mechanisms and immune responses. These virulence factors include flagella phase variation, a complete lipopolysaccharide coat, adhesion systems, type-III secretion systems (T3SS), plasmids, and the ability to produce toxins (*33,49*). These virulence factors allow pathogenic *Salmonella* ingested in food to survive the passage through the gastric acid barrier, invade the mucosa of the intestinal tract, and produce toxins. Promptly following ingestion, the bacteria colonize the ileum and the colon. From here they invade the epithelium starting with an initial attachment to specific receptors on the epithelial cell surface. Fimbriae are one of the most common adhesion systems and mediate adhesion of *Salmonella* to host cells, food, and different surfaces (*17*). The process of invasion for pathogenic *Salmonella* spp. begins with the induction of actin rearrangements on the surface of the host cells, which stimulates internalization of the bacteria. This process is induced when the bacteria inject effector molecules via a T3SS into the host cell that activates a signal transduction pathway which triggers the actin rearrangements, described as membranous ruffles (*50*). This is a remarkable characteristic of pathogenic *Salmonella*; the bacterium is capable of inducing its own phagocytosis in order to gain access to the host cell. The invasion causes the epithelial cells to release multiple proinflammatory cytokines, which induce an acute inflammatory response and can also be responsible for damage to the intestine (*22*). The

inflammatory response is the cause for most of the symptoms accompanying salmonellosis including fever, chills, abdominal pain, and diarrhea. After the engulfment of *Salmonella* into the host cell, the bacterium is encased in a vacuole composed of the host cell membrane. Effector proteins are also injected into the vacuole through a T3SS, thus bypassing the host cell immune response and allowing for the bacteria to survive and replicate in the host cell (*19*). After the invasion of the epithelium is complete, and the bacteria are able to multiply intracellularly, and some serovars are capable of elaborating cytotoxins that can inhibit host cell protein synthesis (*49*). The ability of *Salmonella* spp. to persist in the host cell is crucial for pathogenesis. *Salmonella* serovars that lack this ability are non-virulent.

The attachment to and subsequent invasion of host cells performed by *Salmonella* spp. are under precise genetic control and involves numerous genes located on several pathogenicity islands. *Salmonella* pathogenicity islands (SPIs) are gene clusters located in specific areas of the chromosomes or on plasmids and are responsible for encoding virulence factors (*24*). SPIs are also often characterized to be associated with transfer RNA and mobile genetic elements (*90*). Two key pathogenicity islands are *Salmonella* pathogenicity island 1 and 2 (SPI-1 and SPI-2) which encode two T3SS, which are multi-channel proteins that are used for the delivery of effector molecules into the host cells by injecting them across cell membranes and into the cytoplasm (*6*). SPI-1 is required for the invasion of host cells and induction of macrophage apoptosis and SPI-2 is required for replication within macrophages and systemic infections (*49*).

2.2.1.3. Health and Economic Impact of Salmonella

According to the World Health Organization, *Salmonella* is one of four key global causes of diarrheal diseases and is among pathogens that cause the greatest impact on the human population and is implicated with outbreaks and sporadic cases of human foodborne diseases

worldwide (*99*). Salmonellosis poses a global public health threat due to its significant morbidity and mortality rates, its high endemicity, and difficulty in adopting universal control measures. The global human health impact of salmonellosis is high. There are an estimated 93.8 million cases of illness of which an estimated 80.3 million are foodborne and cause 155,000 deaths each year around the world (*62*). *Salmonella* is also estimated to cause an annual global burden of over 4 million DALYs (*42*). In the USA, data provided by the Foodborne Diseases Active Surveillance Network (FoodNet) show that *Salmonella* are the largest contributor to death statistics among bacterial foodborne pathogens at 39% (*4*). The estimated annual number of cases of domestically acquired foodborne salmonellosis is 1,027,561 leading to an estimated 19,336 hospitalizations and 378 deaths (*88*). These annual cases of salmonellosis lead to an estimated economic and health related cost of 4.43 billion US dollars (*89*).

2.2.2. *Escherichia coli*

Escherichia coli (*E. coli)* forms a large and diverse group of ubiquitous bacteria that are Gram-negative, facultative anaerobic, non-spore forming rods, that may or may not be flagellated (*77*). The majority of the strains of *E. coli* are non-pathogenic and some are even beneficial to humans. Other strains of *E. coli*, however, have acquired characteristics (i.e., the production of toxins) which make them pathogenic to humans (*31*). Pathogenic variants of *E. coli* have both a high morbidity and mortality rate, leading them to be a major public health concern worldwide. Pathogenic *E. coli* are categorized into six groups based on their pathogenic mechanism. These groups are enterohemorrhagic, enteropathogenic, enterotoxigenic, enteroaggregative, enteroinvasive, and attaching and effacing *E. coli* (*14*). Enterohemorrhagic *E. coli* (EHEC, also known as Shiga toxin-producing *E. coli* [STEC]), are the biggest threat of the pathogenic *E. coli* to food safety in developed countries (*88*).

2.2.2.1. Enterohemorrhagic Escherichia coli

EHEC is a foodborne zoonotic agent that has been associated with an increasing number of outbreaks of foodborne disease worldwide and poses a serious public health concern. EHEC are a subset of larger group of Shiga-toxin producing *E. coli* (STEC). There are more than 200 serotypes of STEC. A subset of these serotypes is known to cause illness in humans, and EHEC are a further subset of these human-pathogenic STEC. Infections with EHEC cause episodes of mild to severe diarrhea and a small percentage of these infections developing into hemolytic uremic syndrome (HUS). HUS is a severe complication of an EHEC infection and can lead to kidney failure and death (*6*). The serotype of EHEC that poses the most significant public health threat currently, is O157:H7. Each year, EHEC O157:H7 is estimated to cause 63,000 cases of foodborne disease with 2,100 hospitalizations and 20 deaths in the United States (*88*). While O157:H7 is currently the predominant strain, accounting for approximately 75% of the EHEC infections worldwide, there are other non-O157 serotypes that have been identified as causes of foodborne illness (*26*). In the United States, a group of serotypes referred to as the “big six” accounts for the majority of non-O157 serotypes that are isolated from clinical infections. Theses serotypes are O111, O26, O121, O103, O145, and O45. O157 along with the “big six” are considered adulterants in the United States.

2.2.2.2. EHEC Epidemiology

EHEC was first recognized as a human pathogen in 1982, when an investigation by the CDC into two outbreaks of severe bloody diarrhea associated with the same fast food restaurant chain led to the EHEC serotype O157:H7 being identified as the cause (*98*). These outbreaks occurred in Michigan and Oregon and were transmitted by the same source of under cooked beef. Following these outbreaks, it was found that ruminants, especially cattle, are the primary

reservoir of EHEC and a wide variety of foods have since been found to serve as a vehicle for EHEC outbreaks (*78*). In 1983, the association of EHEC O157:H7 and other EHEC serotypes with cases of HUS was made (*53*). EHEC has also been found to have a low infectious dose of 10-100 colony-forming units (*77*). Epidemiological studies have been performed across all corners of the world and EHEC has been established as the major cause of bloody diarrhea and HUS (*97*). EHEC is capable of causing non-bloody diarrhea, bloody diarrhea, and HUS in all age groups with the young and the elderly being the most susceptible (*51*). Specifically, serotype O157:H7 can cause widespread outbreaks and serious morbidity making it one of the biggest threats to food safety (*52*). Hemorrhagic colitis caused by EHEC is characterized by symptoms including abdominal cramps, nausea, vomiting, and diarrhea that can become progressively bloody (*26).* Symptoms usually present three to four days after exposure with an average duration of eight days.

The principal routes of transmission for EHEC are through ingestion of contaminated food or water, person to person, and animal contact (*52*). There is a wide spectrum of animal reservoirs for EHEC primarily consisting of ruminants, with cattle being the most prominent. One primary reason cattle are a natural reservoir for EHEC is due to the fact that EHEC is not pathogenic for adult cattle, which allows the bacteria colonization in cattle to be asymptomatic (*77*). Cattle are able to transmit EHEC to humans through shedding the pathogen in their feces. Fecal shedding of the pathogen can be either brief or long term and the pathogens can survive for months in cattle manure (*84*). EHEC is capable of persisting in the environment for long periods of time making the pathogens more apt to transmission. Through contamination with fecal matter, EHEC can be found in many other reservoirs including water, soil, meant, fruits, and vegetables (*97*).

After the bacteria are shed into the environment, humans acquire EHEC primarily through consuming contaminated food products. One major route EHEC enters the food chain is through the contamination of meat with cattle fecal matter during slaughter or meat processing. This is why the consumption of raw or undercooked foods with cattle origins is the most common mode of transmission for EHEC O157:H7 (*52*). Vegetables and fruits, most likely contaminated with cattle manure during harvest or processing, have also been implicated in the transmission of EHEC (*1*, *11*). The ability of the bacteria to persist in unfavorable conditions in the environment also enables EHEC to survive in food products previously considered safe from food-borne pathogens (*5*).

2.2.2.3. EHEC Pathogenesis

The pathogenesis of EHEC involves the interaction of multiple bacterial virulence factors and specific pathogen-associated molecular patterns with host cells (*54*). The fundamental features of EHEC pathogenesis include binding to epithelial cells, colonization of the gut, and toxin production. In order to colonize and infect the host, EHEC bacteria must overcome multiple obstacles including acid stress in the stomach, bile secretion in the small intestine, passage through the mucus layer, and the intestinal microflora (*46*). The key virulence factors of EHEC include fimbriae and adhesins, acid-resistance, T3SS, and Shiga-toxin production.

After ingestion, EHEC serotypes survive the acidic environment of the stomach through the expression of acid-resistant systems (*46*). EHEC has an elaborate acid-resistance (AR) system which enables it to survive the acidic environment of the stomach and small intestine. The pathogens response to the stress from the acidic environment not only allows the bacteria to survive but was also found to activate properties linked to motility and cell adhesion (*77*). After passage through the stomach and small intestine, the initial binding of the pathogen to the

epithelium occurs, this is assumed to take place at the follicle associated epithelium of Peyer's Patches in the small intestine (*12*). The bacteria form attaching and effacing (A/E) lesions allowing for colonization (*77*). EHEC utilizes a T3SS that injects the Tir (translocated intimin receptor) into the cytoplasm of target cells. Once in the host cytoplasm, Tir is directed to the host cytoplasmic membrane where it is inserted as a hairpin structure. The central domain of Tir then binds to intimin, an outer membrane protein on the pathogen, forming a tight attachment of the bacteria to the host cell (*56*). The bacteria can also be taken up by intestinal microfold cells and transferred to underlying macrophages where they are able to survive and produce Shiga-toxin (Stx) which is released into the intestinal lumen (*20*).

Production of Stx is the primary virulence factor of EHEC. Stx is a phage encoded toxin that consists of one A subunit and five identical B subunits (*51*). The B subunits form a pentamer that binds to a cell surface glycolipid receptor called globotriaosylceramide-3 (Gb3) (*77*). This binding specificity of the toxin determines where the bacteria can cause disease in the host. Once the toxin has entered the host cells, the A subunit is able to inhibit host cell protein synthesis by exhibiting RNA N-glycosidase activity against the 28S rRNA inducing apoptosis and an inflammatory response in cells promoting damage to the host (*54*). Stx released by the pathogen can also bind to endothelial cells that express the Gb3 receptor allowing for toxin absorption into the bloodstream and dissemination to other organs, thus toxin production mediates both local and systemic disease (*97*).

The virulence factors required for EHEC pathogenesis have been study primarily in EHEC O157:H7. The genes required for virulence can be found in multiple locations, within the chromosomal pathogenicity island LEE (locus for enterocyte effacement), the chromosomally integrated lambdoid prophage genome, and the large plasmid pO157. LEE has five major

operons that encode for a T3SS, regulators, chaperones, and effector proteins (*77*). The chromosomally integrated lambdoid prophage genome is where the *stx* genes are located that encode for Stx production. The plasmid pO157 is a 90kb plasmid that has been found to be present in nearly all EHEC O157 clinical isolates (*97*).

2.2.2.4. Health and Economic Impact of EHEC

EHEC has a large impact both on human health and the economy globally. There are an estimated 2.8 million cases of illness from EHEC infection resulting in 3,890 cases of HUS and 230 deaths annually, worldwide (*63*). The estimated annual global burden of EHEC is 13,000 DALYs (*42*). There are approximately 63,000 cases of EHEC O157:H7 foodborne infection annually in the United States and an additional 112,000 cases of non-O157 EHEC foodborne infections. From these cases, there are 2,100 hospitalizations and 20 deaths from O157 infection and 270 hospitalizations and no deaths from non-O157 infection (*88*). These annual cases of EHEC infection in the United States lead to an estimated economic and health related cost of 708 million U.S. dollars. (*89*). Despite the fewer annual cases and deaths from EHEC than other major foodborne pathogens, it is still important that this pathogen be prioritized concerning control measures for foodborne pathogens due to the pathogens high risk for causing long term, severe health issues. Additionally, there is currently no treatment available for EHEC infections. Antibiotics and antidiarrheal medications are not recommended for use in the case of an EHEC infection as they promote the production of Stx and may increase the risk of HUS (*35*). Thus, EHEC is a major threat to both public health and food safety.

2.3. Low Moisture Foods

Low moisture foods (LMFs) have been defined as food products that have a water activity (a_w) of less than 0.85 and are considered less susceptible to microbial spoilage (*87*). A

wide range of food products are characterized as LMFs and they form an integral part of the human diet. These products include cereals, dried fruits and vegetables, flour, herbs, honey, powdered infant formula, peanut butter, nuts, and pastas. These food products are either naturally low in moisture or are high moisture products that have been subjected to a drying process. These food products have a long shelf life and are usually stable for years. The characteristic low a_w of LMFs is a benefit as it is a barrier to growth for many pathogens including both *Salmonella* and EHEC (*81*).

Water activity was originally applied by the food industry as a quantitative measure that is used in the determination of the shelf life of a product (*23*). The a_w value is the ratio of the vapor pressure of water in a food matrix to that of pure water at the same temperature, it is dependent on both storage temperature and the composition of the product (*69*). The minimum a_w required for bacterial growth and toxin formation is 0.94 for *Salmonella spp.* and 0.95 for EHEC (*5*). While low a_w does not support the growth of bacteria, it does not prevent contamination or survival of many pathogens (*40*).

2.3.1. Foodborne Pathogens and Low Moisture Foods

LMFs may not support microbial growth due to their low a_w, however, many of these food products have been implicated in outbreaks of foodborne pathogens. During the winter of 2000-2001 an outbreak of *Salmonella enteritidis* PT30 was detected in Canada, and the association between raw whole almond consumption and infection was made. *Salmonella enteritidis* PT30 was detected in raw whole natural almonds collected from case homes, retail, distribution, warehouse sources, and from environmental swabs taken from processing equipment and associated orchards. The identification of almonds as the source of a foodborne outbreak had been previously unheard of, and this opened the door to the consideration of LMFs

as vehicles for foodborne pathogens (*48*). Since the 2000's LMFs have been implicated in a large number of foodborne disease outbreaks and recalls with *Salmonella spp.*, EHEC O157 and the "big-six" non-O157 EHEC serotypes being the principal pathogens involved (*87*).

It is evident that LMFs remain susceptible to microbial contamination and pose a risk to consumers. It is expected that both *Salmonella* and EHEC may be present on any raw food materials due to the ubiquitous nature of both of these pathogens (*81*). The raw materials and ingredients in LMF products can come from a wide range of items from agricultural products coming directly from harvest to other highly processed materials. This allows for the possibility for cross-contamination to occur from a variety of sources. After the contamination is introduced, the conditions of the LMF may not allow for the growth of pathogenic bacteria, but the frequency of outbreaks and recalls suggests that the pathogens are able to survive at high enough levels to cause infection.

The long-term survival of these pathogens has since been well documented on various LMFs (*5*, *81*). While there is no growth, and bacterial metabolism is significantly reduced, vegetative cells of both *Salmonella spp.* and EHEC can remain viable for months to years in low moisture conditions (*23,44*). It is important to perform these pathogen behavior analyses on samples of every LMF that is of concern as the survival of *Salmonella* and EHEC can vary by both bacterial strain and food product composition. Both *Salmonella* and EHEC have a low infective dose of 10-100 CFU and thus even the presence of low numbers of these pathogens in LMFs poses a risk to consumers. Low levels of these foodborne pathogens in LMFs have the possibility of serving as an inoculum in higher moisture foods if reconstituted leading to a potential for growth of the pathogens and further contamination (*40*).

2.3.2. Desiccation Tolerance of Enteric Pathogens

The increase in associations between outbreaks of foodborne pathogens and LMFs has led to an interest in the desiccation tolerance of enteric pathogens. It is known that enteric pathogens like EHEC and *Salmonella* can easily adapt to extreme environmental conditions including low and high temperatures, pH, or desiccation (*81*). A study by Hiramatsu et al. showed that desiccated cells of different *Salmonella* and EHEC strains inoculated and dried on paper disks were able to survive 22-24 months when stored at 4°C (*44*). Another study was conducted observing the effect of desiccation on a plastic surface on *Salmonella enterica* to other stressors (*38*). This study indicated that desiccation induces cross-tolerance of *Salmonella enterica* to other stressors. It is evident that further research needs to be conducted to gain further insight into how various serotypes of enteric pathogens react to desiccation.

2.4. Flour Outbreaks and Recalls

One LMF that has been increasingly implicated in outbreaks of foodborne pathogens within the last couple of years is flour. Wheat flour is a raw, minimally processed product and contamination can potentially occur at any point in the process chain from harvest in the field to flour milling. Flour is intended to be mixed with other ingredients and cooked prior to consumption. Both flour and flour-based mixes have been implicated as the source of foodborne *Salmonella* and EHEC outbreaks. The subsequent investigations and recalls of contaminated products can be very costly for the food industry (*87*). The financial impact of recalls analyzed by the cumulative abnormal return (CAR) over a recall event time period is estimated in a loss in corporate value of $243 million (*36*).

In 2008, an outbreak of *Salmonella* Typhimurium phage type 42 was associated with the consumption of raw flour (*71*). The initial investigations indicated that consumption of uncooked

batter was associated with illness and the environmental investigation included testing flour and other baking ingredients from case homes, unopened flour from retail stores, and the inspection of an implicated flour mill. This strain of *Salmonella* was recovered from flour from the case homes, unopened packs from retail stores, and packs from recalled product. However, *Salmonella* was not detected in environmental samples taken from the implicated flour mill (*71*).

A multistate outbreak of EHEC O157:H7 occurred in 2009 in the United States. It resulted in 77 illnesses, 35 hospitalizations, and 10 cases of HUS that were linked to the consumption of uncooked, commercial prepackaged ready to bake cookie dough that contained contaminated raw flour (*76*). The flour in the cookie dough was suspected as it was the only ingredient not to undergo a pathogen kill step. In 2016, infections of EHEC serotypes O121:H19 and O26:H11 were linked to contaminated flour from a large domestic producer (*13*). There was a total of 63 cases in 24 states with 17 hospitalizations and 1 case of HUS (*55*). Trace back investigation identified a common flour production facility (*13*). Along with this, there were two other flour-related outbreaks of EHEC O121 that occurred in Canada in 2016 and 2017. These outbreaks linked to flour and flour products lead to 30 illnesses in 6 provinces (*73*).

In these outbreaks, inspectors did not identify a source of contamination at the implicated facilities. This suggests that the ingredients were contaminated earlier in the production chain. Wheat is the ingredient in flour that is most likely to be contaminated as early as in the field prior to harvest (*13*). Another piece of evidence supporting the hypothesis of wheat grain contamination prior to milling is that cereals and grains were ranked as the highest concern from a microbiological food safety perspective compared to other LMFs in 2014 (*25*).

2.5. Wheat Grain

Wheat is the third most produced crop in the world and is a key export commodity for the United States (*16*). Wheat flour is a central component of many people's diets as it is an important ingredient in a wide range of food products including breads, cakes, cookies, and pastas. In the US, there are 6 recognized classes of wheat, categorized by their hardness, color, and growing season. These classes include durum, hard white, soft white, hard red winter, soft red winter, and hard red spring. Hard red spring wheat (HRS) is made into flour that is primarily used for bread baking and is primarily grown in the northern plains of the US. The seed of the wheat plant is of primary interest as it can be milled into flour. Looking into the manufacturing process of flour can give an insight into where the contamination may be introduced.

2.5.1. Wheat Processing

The wheat milling process begins with the harvest of the wheat grain at the farm. Harvesting practices, irrigation, manure, and the post-harvest environment have all been suggested as possible sources of *Salmonella* and EHEC contamination for wheat grain (*55*). It was recently discovered that EHEC serotype O157:H7 can internalize in the tissues of wheat seedlings from contaminated soil or irrigation water and survive on the flowering wheat heads (*68*). Harvested wheat grain then can be stored in various spots including on the farm, in a country elevator, or in a terminal elevator (*79*). The wheat grain is then delivered to flour mills by covered trucks and hopper rail cars. When the wheat arrives at the facility, it undergoes inspection testing moisture content, test weight, unsound kernels, and foreign material (*79*). After passing inspection, the wheat grain is moved via conveyors and bucket elevators into grain bins or silos where specific heat, moisture, and air conditions must be kept. A fumigation process may also be used to eliminate pests.

Now the milling process begins, with the wheat grain moving through equipment that separates the grain from other unwanted materials that may be present. The grain then goes through the tempering process to be conditioned for milling. Small amounts of moisture are added in precise amounts in order to toughen the outer bran of the wheat grain. This makes it easier to separate the parts of the wheat kernel during milling. The grain is then ground, separating the parts of the wheat grain which are then sifted leaving a fine flour product (*79*). Whole grain flour uses the entire grain including the outer bran layer which is more likely to become contaminated (*55*). In contrast, white flour only uses the innermost endosperm, but contamination could still occur after or during the removal of the bran layer. It is important to note that in the production of flour, while there are steps to remove pests or other foreign objects present on the incoming wheat grain, there is no sterilization or pathogen kill step.

2.5.2. Pathogen Detection on Wheat Grain

A baseline study was conducted to determine the prevalence and levels of microbial pathogens in wheat grain samples taken prior from milling (*75*). These samples were taken from the wheat grain being brought in on rail cars to the production facility for milling. A total of 3,891 samples were tested for the presence of EHEC and *Salmonella* spp. and the positive samples where assayed for most probable number (MPN) counts. *Salmonella* was detected in 1.23% of the samples with an average of 0.110 MPN/g and EHEC was detected in 0.44% of samples with an average of 0.039 MPN/g. The results of this study support the hypothesis that the contamination of wheat flour can occur prior to the milling process.

2.6. Research Questions

The presence and persistence of enteric pathogens in low-moisture environments has been identified and continues to be a threat to food safety. There is little information available on

the survival of enteric pathogens on wheat grain, even though wheat grain to be used for milling was found to be contaminated with *Salmonella* and EHEC and there are multiple foodborne disease outbreaks linked to raw flour consumption (*75*). There is also little information on the tolerance of these pathogens to the immediate stress of desiccation. The first part of our study aimed to determine the fate of strains of various serovars of *Salmonella enterica* and EHEC serotypes on wheat grain over the course of a year and the second part of our study aimed to quantify the desiccation tolerance of various strains and serotypes of *Salmonella* and EHEC over the short-term desiccation stress.

3. FATE OF *SALMONELLA ENTERICA* AND ENTEROHEMORRHAGIC *ESCHERICHIA COLI* ON WHEAT GRAIN

3.1. Abstract

Wheat flour has been connected to outbreaks of foodborne illnesses with increased frequency in recent years, specifically, outbreaks involving *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC). However, there is limited information regarding the survival of these pathogens on wheat grain during long-term storage in a low moisture environment. This study aims to evaluate the long-term survival of these enteric pathogens on wheat grain over the course of a year. Hard red spring wheat was inoculated with strains of four serovars of *Salmonella enterica* (Enteritidis*,* Agona, Tennessee, and Montevideo) and six serotypes of EHEC (O157:H7, O26:H11, O121:H19, O45:NM, O111:H8, and O103:H2) in triplicate, sealed in Mylar bags to maintain the water activity, and stored at room temperature (22 ± 1°C). The survival of each pathogen was evaluated by plating onto differential media*.* Viable counts of strains from all four serovars of *Salmonella* (Enteritidis*,* Agona, Tennessee, and Montevideo) were detected on wheat grain stored at room temperature (22 ± 1°C) for the duration of the study (52 weeks). Viable counts of strains from EHEC serotypes O45:NM, O111:H8, and O26:H11 were only detected for 44 weeks and strains from serotypes O157:H7, O121:H19, and O103:H2 were only detected for 40 weeks until they passed below the limit of detection (2.0 log CFU/g). *D*-values were found to be significantly different between *Salmonella* and EHEC (adj. $p \leq 0.05$) with *Salmonella D*-values ranging from 22.9 ± 2.2 to 25.2 ± 1.0 weeks and EHEC *D*-values ranging from 11.4 ± 0.6 to 13.1 ± 1.8 weeks. There were no significant differences amongst the four *Salmonella* strains or amongst the six EHEC strains (adj. $p > 0.05$). These observations highlight the wide range of survival capabilities of enteric pathogens in a low

moisture environment and confirm these pathogens are a food safety concern when considering the long shelf life of wheat grain and its products.

3.2. Introduction

In recent years, low-moisture foods and food ingredients have been implicated as vehicles for foodborne pathogens with increased frequency. Previously, low-moisture foods were not generally a concern in transmitting foodborne illnesses because of their low water activity (a_w). Low a_w does not support the growth of pathogens (*87*). It is now known, however, that even though pathogens cannot grow in these conditions, they are able to survive for extended periods of time at concentrations high enough to cause infection (*5*). This has especially become apparent in the increase in frequency of foodborne disease outbreaks and recalls linked to wheat flour. For example, in 2019, there were 14 recalls of flour due to contamination with *Escherichia coli* and *Salmonella* (*27*). An outbreak of *E. coli* O26 led to the nationwide recall of four different flour products (*28*). There was a total of 21 confirmed infections across 9 states with 3 hospitalizations (*10*). This most recent outbreak, along with a number of other recent outbreaks of *Salmonella* and *E. coli* have brought to question the safety of both wheat flour and wheat flour products (*41*).

The heightened awareness of foodborne disease outbreaks linked to low-moisture foods has led to an increase in available information and studies regarding the presence and survival of foodborne pathogens on low-moisture food products. The minimum a_w required for the growth of most pathogenic bacteria is 0.87. Low moisture foods are defined as those with an $a_w < 0.85$, and these food products are either naturally low in moisture or they are produced from foods with a higher a_w that have been intentionally dried (*5*). Numerous studies have shown that *Salmonella* and various serogroups of enterohemorrhagic *Escherichia coli* (EHEC) can survive

prolonged storage on low moisture foods (*7, 57*). *Salmonella* spp., *E. coli* O157:H7, and other non-O157 EHEC serogroups are some of the primary pathogens that have been involved in outbreaks with low moisture foods, and specifically, with wheat flour. The survival of *Salmonella* and EHEC serogroups O45, O121, O145, O26, O103, O111, and O157 have been evaluated on wheat flour (*29,30*). All EHEC serogroups survived above the limit of detection of 2 LOG CFU/g for 12 weeks. The *Salmonella* strain cocktail containing *S.* Typhimurium, *S.* Agona, *S.* Enteritidis, and *S.* Anatum survived above the limit of detection for 16 weeks. Similar to other studies conducted on various low-moisture foods, the pathogens were found to be able to survive for extended periods of time (*29,30*). The survival of only a few cells of these pathogens can be enough to cause disease (*13*).

Wheat flour plays a predominant role in the diets of many people, as it is a fundamental ingredient in a variety of food products including breads, pastas, cakes, and cookies. Even though wheat flour is an essential part of many people's diets, linking outbreaks to the consumption of raw or undercooked flour is difficult as it is not included on most routine state and national foodborne disease questionnaires (*13*). Between 2016 and 2017, there were two major outbreaks of foodborne illnesses linked to flour, one in the United States and one in Canada. Both of these outbreaks had 30 or more confirmed cases over widespread geographical regions and in both cases the contaminated flour was linked back to a single flour milling facility, one facility in the U.S. and one in Canada (*13, 34*). Even though the epidemiologic data confirmed the source of each outbreak was flour produced in a single facility, the source of contamination within the facility could not be identified. Based on the fact that current milling practices do not apply any methods to reduce microbial populations on the wheat grain prior to milling, it is hypothesized that the wheat grain is where the contamination is being introduced

(*93*). It is also hypothesized that this contamination is introduced to wheat grain prior to its arrival at the flour mill, and possibly even prior to harvest. This hypothesis was supported when samples of wheat grain were taken from incoming rail cars, bringing the grain to the milling facility, and the samples were tested for the presence of various foodborne pathogens (*75*). A total of 3,891 grain samples were tested for the presence of *Salmonella* and EHEC, 0.44% where positive for EHEC and 1.23% were positive for *Salmonella.* This study confirms that foodborne pathogens can be present on wheat grain prior to being obtained for milling, but there is little to no information on the long-term survival capabilities of these pathogens on wheat grain.

Prior to being transported to the milling facility and after harvest, wheat grain can be stored for up to and sometimes over a year either in grain bins on the farm or in a county grain elevator (*18*). Evidence shows that pathogens can survive long-term storage in low moisture environments similar to this, but *Salmonella* and EHEC's survival capabilities on wheat grain are unknown. The objective of this study was to evaluate the survival of multiple strains of *Salmonella* and enterohemorrhagic *Escherichia coli* on wheat grain over the course of a year.

3.3. Materials and Methods

3.3.1. Strain Selection

Four *Salmonella* strains and six enterohemorrhagic *Escherichia coli* (EHEC) were evaluated in this study. *Salmonella* strains were selected to include serovars commonly involved in outbreaks associated with low-moisture foods (Table 1). These strains were obtained from the food safety lab at Cornell University, with the exception of *S.* Enteritidis which is from the American Type Culture Collection. EHEC strains were selected to include serotype O157:H7 and non-O157 serotypes commonly implicated in human illnesses (Table 1). All these strains are from the Thomas S. Whittam STEC Center at Michigan State University, with the exception of

the 0121:H19 strain, which was obtained from the Michigan Department of Health and Human Services.

Table 1. *Salmonella* and *Escherichia coli* strains used in the survival study.

Species	Serovar/Serotype	Strain	Isolation Source
Salmonella enterica	Enteritidis	ATCC BAA-1045	Food
Salmonella enterica	Agona	FSL S9-0322	Food
Salmonella enterica	Tennessee	FSL R6-0494	Human clinical
Salmonella enterica	Montevideo	FSL R8-3881	Human clinical
Escherichia coli	O157:H7	RIMD 0509952	Human clinical
Escherichia coli	O26:H11	TW16501	Human clinical
Escherichia coli	O121:H19	PNUSAE002568	Human clinical
Escherichia coli	O45:NM	TW07947	Human clinical
Escherichia coli	O111:H8	TW7926	Human clinical
Escherichia coli	O103:H2	TW08101	Human clinical

3.3.2. Inoculation of Wheat Grain

The inoculation protocol previously described by Snelling et al. was used with minor modifications (*92)*. Each strain was streaked onto LB agar plates from culture stocks stored at -80°C and incubated for 20 h at 37°C. A single colony was selected from this plate and transferred to 5mL LB broth and incubated for 16 h at 37°C while shaking at 200 RPM. From the LB broth culture, 250 µL was spread uniformly onto an LB agar plate (100 mm x 15 mm) using a sterile spreader (Fisher Scientific Inc, Waltham, MA). These plates were incubated for 24 h at 37°C to achieve confluent growth over the entire plate. Each strain was independently grown and inoculated onto wheat at three separate time points, and these were used as three independent replicates.

A blend of hard red spring (HRS) wheat varieties grown in 2017 were used for this experiment: 29.5% Linkert, 29.5% Glenn, 15.2% SY Soren, 9.8% Elgin-ND, 9.5% ND VitPro, and 6.5% SY Ingmar. The mixture was homogenized with a homogenizer (FPB-005, American

Process Systems, Gurnee, IL) and cleaned for processing and milling on a dockage tester (Carter Day International, Minneapolis, MN) with a number 8 riddle. The wheat grain was stored at 4°C until use. Prior to inoculation, the wheat grain was brought to room temperature (22 ± 1°C) and the a_w was measured using the Aqualab 4 TE a_w meter (METER Group, Inc. USA). The inoculation was performed in a bio-safety cabinet. For inoculation of one replicate, a 1000 g aliquot of wheat grain was measured into a 25 cm × 38 cm Whirl-Pak bag (Nasco Inc, Fort Atkinson, WI). To achieve 6 log CFU/g, the bacterial lawn from half of a plate was collected with a sterile spreader and resuspended in 2.5 mL of water in a sterile beaker. The bacterial suspension was poured over 1000 g of wheat grain in the Whirl-Pak bag and mixed by hand for approximately 5 minutes to obtain a homogeneous distribution of the bacterial suspension on the wheat grain. The inoculated wheat grain was then transferred into a sterilized stainless-steel tray (30.5 by 23 cm).

3.3.3. Evaluating the Homogeneity of Inoculated Wheat Grain

In order to evaluate the homogeneity of the bacteria inoculated onto the wheat grain, six 25 g samples were randomly taken from the 1000 g of wheat grain in the stainless-steel tray for one *Salmonella* and one *E. coli* strain. These samples were plated at the time of inoculation (0h), 24 h, 48 h, 72 h, and 7 days. Each sample was weighed in a Whirl-Pak bag, diluted with Butterfield dilution buffer, and homogenized in a masticator (IUL instruments, Spain) for 90 seconds. Serial dilutions were plated in duplicate onto Tryptic Soy agar (TSA) supplemented with 0.1% ferric ammonium citrate (J.T. Baker Inc., Phillipsburg, NJ) and 0.06% sodium thiosulfate (VWR Inc, Radnor, PA) for the *Salmonella* strains and MacConkey Agar (EMD Millipore Corporation, Billerica, MA) for the *E. coli* strains, with the exception of 0157:H7 which was plated on sorbitol MacConkey agar (HiMedia Laboratories Pvt. Ltd. India). The

plates were incubated at 37°C for 24 h and black colonies indicative of *Salmonella*, pink colonies indicative of *E. coli,* and translucent colonies indicative of 0157:H7 were enumerated using a Q-count (Advanced Instruments Inc., Norwood, MA). In addition to this initial homogeneity test, four randomly selected 11 g samples from the 1000 g of inoculated wheat grain were enumerated at the time of inoculation for each strain to confirm homogeneity. A standard deviation of < 0.5 log CFU/g was deemed to be an acceptable indication of homogeneity of the inoculum for each inoculated batch of wheat (*64*).

3.3.4. A_w Adjustment and Long-Term Storage of Inoculated Wheat Grain

The a_w of the wheat grain was measured in duplicate both before and after inoculation. After inoculation, lithium chloride (anhydrous, 99%, 20 mesh; Alfa Aesar, Ward Hill, MA) was used to adjust the a_w back to its pre-inoculation level. The stainless-steel tray containing the 1000g of inoculated wheat grain was placed in a closed chamber (Coleman cooler 60.96 cm × 40.64 cm, Coleman Company, Inc., Kingfisher, OK) with 25g of lithium chloride that had been weighed and saturated with water in plastic trays (Fisher Scientific Inc,). The plastic tray of saturated lithium chloride was placed adjacent to the stainless-steel trays in the closed chamber in order to reduce the a_w. The a_w of the inoculated wheat grain was adjusted to its pre-inoculation level within 48 h. After the a_w had been adjusted, the wheat grain was prepared for long term storage. The 1000g of wheat grain were divided into 50g portions to be used to monitor bacterial survival over 52 weeks and packaged into Whirl-Pak bags. The Whirl-Pak bags were then sealed in Mylar bags to keep the a_w stable during changes in the ambient relative humidity. The Mylar bags were stored at 22 ± 1 °C.

3.3.5. Enumerating Bacterial Survival on Wheat Grain Over Time

Over the 52 weeks of storage, bacterial survival was first enumerated at 48 h post-inoculation, following a_w adjustment. This first enumeration was time point 0 (T0). After T0, bacterial survival was enumerated every 2 weeks for the first 12 weeks and then every 4 weeks for the subsequent 40 weeks. At each time point, one 50 g sample bag was removed from the Mylar bag and split into two 25 g samples. Each 25 g sample was diluted with Butterfield dilution buffer and homogenized in a masticator for 90 s. Serial dilutions of the *Salmonella* strains were plated in duplicate on TSA supplemented with ferric ammonium citrate and sodium thiosulfate and serial dilutions of the *E. coli* strains were plated in duplicate on MacConkey agar, with the exception of 0157:H7 which was plated in duplicate on sorbitol MacConkey agar. The plates were incubated for 24 h at 37 °C. Black colonies indicative of *Salmonella*, pink colonies indicative of *E. coli,* and translucent colonies indicative of 0157:H7 were enumerated with a Q-count. At each time point, two 25 g samples from the same inoculated batch of wheat grain were tested for each of three biological replicates.

3.3.6. Data Analysis

The survivor curves were generated using a linear model and only time points where the bacterial population was above the limit of detection (2.0 log CFU/g) were used.

$$N = N_0 + M(t)$$

N is the log-transformed bacterial population (CFU/g) at a given time (t) in weeks, N_0 is the log-transformed bacterial population (CFU/g) at t=0, and M is the slope of the survival curve. *D*-values were then calculated by taking the negative reciprocal of the M value. The total log reduction was calculated at t=40 weeks, when the first strains of EHEC reached the limit of detection, using the following equation.

$$L = -\log_{10}(\frac{n_{40}}{n_0})$$

Where L is the total log reduction, n_{40} is the bacterial population (CFU/g) at t=40 weeks and n_0 is the bacterial population at t=0. Each survival experiment was performed in triplicate and the results are reported as mean values with standard deviations. The *D*-values and total log reductions were compared by one-way analysis of variance (ANOVA) and the Tukey honest significant difference method was used in R version 4.0.3. Differences were considered significant at $p<0.05$.

3.4. Results and Discussion

3.4.1. Long-Term Survival on Wheat Grain Differs Between *Salmonella* and EHEC

After inoculation, the average initial levels of all strains of *Salmonella* and EHEC were 6.56 ± 0.17 log CFU/g. Prior to inoculation, the average a_w of the wheat grain was 0.52 ± 0.03 and rose to an average of 0.62 ± 0.03 immediately following inoculation. Within 48hr of inoculation, the a_w was adjusted to 0.47 ± 0.03 to match the pre-inoculation conditions. Throughout the 52-week storage period, the a_w remained constant at 0.46 ± 0.03. Viable counts of the strains from all four serovars of *Salmonella* (Enteritidis, Agona, Tennessee, and Montevideo) were detected on wheat grain stored at room temperature (22 ± 1°C) for the duration of the study (52 weeks), while viable counts of the strains from serotypes O45:NM, O111:H8, and O26:H11 were detected for 44 weeks and serotypes O157:H7, O121:H19, and O103:H2 were detected for 40 weeks (Fig. 1). After these time points, they passed below the limit of detection (2.0 log CFU/g). A linear decrease in the populations of *Salmonella* and EHEC was observed over long-term storage. The total log reduction seen over 40 weeks, the first timepoint when EHEC strains passed below the limit of detection, was significantly different between *Salmonella* and EHEC (adj. $p \leq 0.05$). The total log reduction seen amongst *Salmonella* strains ranged from 1.77 ± 0.33

to 2.03 ± 0.26 and the total log reduction seen amongst EHEC strains ranged from 3.21 ± 0.13 to 4.19 ± 0.19. There were no significant differences in the total log reductions of the four *Salmonella* strains (adj. $p > 0.05$). However, there were significant differences in the total log reductions of several EHEC serotypes (adj. $p \leq 0.05$). EHEC O103:H2 had a significantly higher total log reduction than O45:NM and O111:H8 (Fig. 2).

These total log reduction results follow similar trends as seen in other studies of the survival of *Salmonella* and EHEC on low-moisture foods. Malekmohammadi et al. observed average decreases of 3.5 to 3.7 log CFU/g over 24 weeks on flaxseed for *Salmonella* serovars Enteritidis, Montevideo, Tennessee, and Agona, with a non-linear reduction (*64*). Uesugi et al. reported a reduction of 3.4 log CFU/g of *S.* Enteritidis on almond kernels after 68 weeks of storage following a linear reduction, and Limcharoenchat et al. observed population declines of 2.3 log CFU/g of *S.* Enteritidis on almond kernels after 68 weeks of storage with the reduction fitting a log-linear model (*60, 96*). Each of these studies had starting inoculation densities of approximately 8.0 log CFU/g and the long-term storage was performed at room temperature, (22 ± 1 °C). The long-term survival of a *Salmonella* strain cocktail and EHEC serogroups O26, O103, O111, O157, O45, O121, and O145 was observed in wheat flour (*29,30*). In these studies, EHEC serogroups passed below the limit of detection between time points 12 and 16 weeks and *Salmonella* passed below the limit of detection between time points 16 and 20 weeks with starting inoculation densities between 8 and 9 log CFU/g.

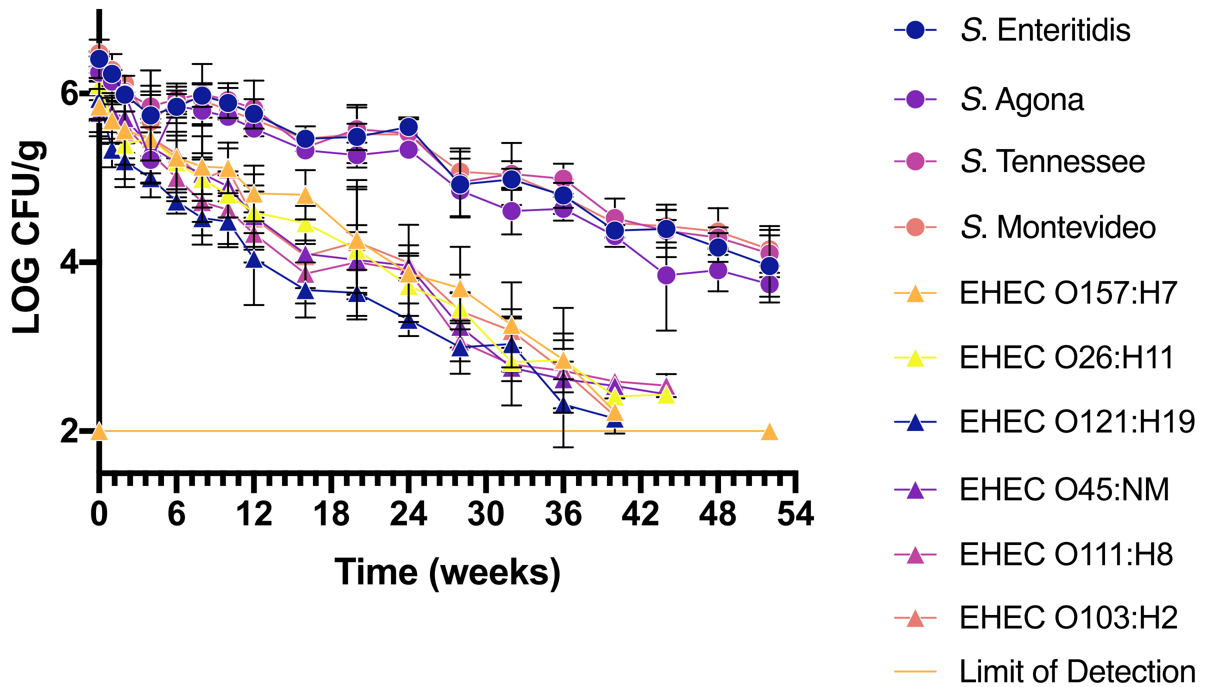

Figure 1. Long-term survival of 4 *Salmonella* and 6 EHEC strains stored on wheat grain at 22 ± 1°C for 52 weeks. Values are the log-transformed number of surviving cells per gram of sample and are shown as the mean of three biological replicates each with two technical replicates with standard deviation. The limit of detection was 2.0 log CFU/g.

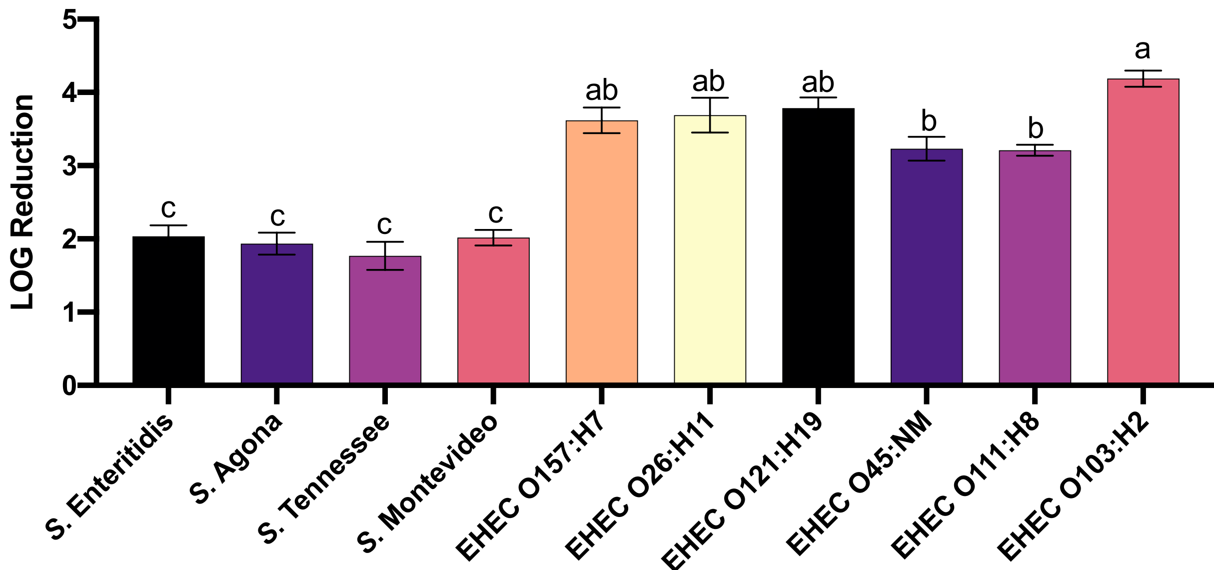

Figure 2. Total log reduction for each pathogen over 40 weeks on wheat grain. Each bar is the log-transformed value of the difference in the number in surviving cells per gram of sample from 0 to 40 weeks and is shown as the mean of three biological replicates with the standard deviation. Different letters indicate values that are significantly different (adj. $p \leq 0.05$).

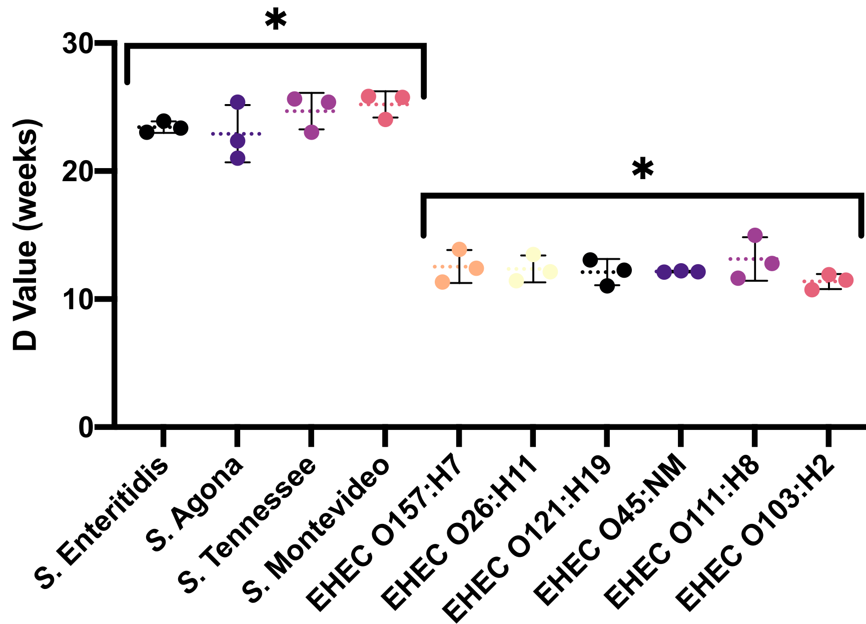

Figure 3. *D*-values, the time (in weeks) required to reduce the viable population of 4 *Salmonella* and 6 EHEC strains by one logarithmic value on wheat grain stored at 22 ± 1°C. Calculated by linear models and shown for each biological replicate. The dashed horizontal line represents the mean of the three biological replicates and the solid lines represent the standard deviation. * indicate values that are significantly different (adj. $p \leq 0.05$).

3.4.2. Rate of Population Decline Differs Between *Salmonella* and EHEC

D-values were found to be significantly different between *Salmonella* and EHEC (adj. $p \leq 0.05$) as shown in Figure 3. *Salmonella D*-values ranged from 22.9 ± 2.2 weeks to 25.2 ± 1.0 weeks. EHEC *D*-values ranged from 11.4 ± 0.6 weeks to 13.1 ± 1.8 weeks. There were no significant differences amongst the four *Salmonella* strains or amongst the six EHEC strains (adj. $p > 0.05$).

It is evident that the survival capabilities of enteric pathogens are dependent on many factors. Multiple pathogen desiccation studies have concluded that the food product, its structure, the inoculation protocol and density, along with interactions between low a_w and storage temperature all effect pathogen survival (*32, 44, 60*). The results from this study coincide with previous observations that enteric pathogens are capable of surviving from months to years on

low moisture foods, but there are some differences in the survival of *Salmonella* and EHEC on wheat grain. First, it is seen that the survival of both *Salmonella* and EHEC, above the 2.0 log CFU/g limit of detection, on wheat grain lasted over three times as long than on wheat flour. There were no significant differences noted between *Salmonella* and EHEC survival on wheat flour in this shorter time frame, but it was noted that between weeks 8 and 16 the *Salmonella* survival rate had a slower decline than EHEC (*30*). There were significant differences between the survival capabilities of *Salmonella* and EHEC on wheat grain, and while both of these pathogens have been linked to recalls and outbreaks of foodborne disease on flour and other low moisture foods, there is limited data available comparing their desiccation survival capabilities. There are also notable differences in the survival parameters and total log reductions seen over time under similar environmental conditions with variable food products, inoculation protocols, and inoculation densities. The study performed by Forghani et al. had an inoculum density of approximately 8.5 log CFU/g and the inoculation preparation was performed through the resuspension of a bacterial pellet from a broth growth (*29*). The study performed by Malekmohammadi et al. had a inoculum density of approximately 8 log CFU/g and the inoculation preparation was performed in the same method as this study (*64)*. These two studies, along with the study presented here, while all performed under similar temperatures and a_{w}, had varying inoculation densities, inoculum preparation protocols, and food products and all observed varying survival parameters and total log reductions seen over time. This highlights the importance of further research into the specific effects each of these variables can have on the long-term survival of enteric pathogens in dry environments as well as the comparison of the long-term survival capabilities of different enteric pathogens in low moisture environments. This

information would provide insight into high-risk low-moisture food products that would be valuable for food safety.

A recently published survey highlights that many consumers are unaware of the risk of consuming raw flour and of outbreaks or recalls of flour and flour products due to contamination with foodborne pathogens (*21*). Studies regarding the presence and survival of foodborne pathogens on these and related food products are important to supply information for the proper food safety messaging to consumers. *Salmonella* and EHEC were both found to survive at levels high enough to cause infection for extended periods of time on wheat grain, and the survival parameters of these two pathogens were found to be significantly different. The findings presented in this study provide valuable information for the risk assessment of low-moisture food safety through the knowledge of the survival capabilities of EHEC and *Salmonella* on wheat grain.

4. DESICCATION TOLERANCE OF *SALMONELLA ENTERICA* AND ENTEROHEMORRHAGIC *ESCHERICHIA COLI* ON A PLASTIC SURFACE

4.1. Abstract

The persistence of enteric pathogens on low-moisture surfaces poses a threat of cross-contamination in food manufacturing and has led to multiple outbreaks of foodborne illness. Knowledge of the desiccation tolerance capabilities of *Salmonella enterica* and Enterohemorrhagic *Escherichia coli* (EHEC) and what factors can affect it is sparse and is key information for risk assessments in the food industry. This study aimed to evaluate the tolerance of various enteric pathogens to sudden desiccations stress, and to compare this tolerance between species, serotypes, and strains of *Salmonella* and EHEC, as well as evaluate the differences in desiccation tolerance in different inoculum preparation methods. This was accomplished through exposing strains of four serovars of *Salmonella enterica* (Enteritidis, Agona, Tennessee, and Montevideo) and seven serotypes of EHEC (O157:H7, O26:H11, O45:H2, O121:H19, O45:NM, O111:H8, and O103:H2) grown from either a liquid culture or a lawn growth to desiccation stress for 24 and 48 hours and monitoring the reduction seen in the bacterial population by calculating the total log reduction. Desiccation tolerance was found to vary between *Salmonella* and EHEC, between growth methods, among the four serovars of *Salmonella* and among the seven serotypes of EHEC. Among six strains of the serovar *S.* Agona, a consistent trend of higher desiccation tolerance under the liquid culture growth method was seen and the effect growth method on total log reduction was significant. The total log reduction of the *S.* Agona strains ranged from 0.26 ± 0.10 log CFU/mL to 0.37 ± 0.18 log CFU/mL under the broth growth condition and ranged from 0.53 ± 0.34 log CFU/mL to 0.89 ± 0.22 log CFU/mL under the lawn growth condition. Among six strains of EHEC serotype O157:H7, the interaction of strain and

growth as well as RpoS functionality had a significant effect on the total log reduction. The strains that were RpoS negative had a significantly higher total log reduction than the strains that were RpoS positive. Our results show that there is not one, uniform response to desiccation stress from enteric pathogens and also emphasized the importance in using a broad range of strains when testing desiccation characteristics.

4.2. Introduction

Wide-spread outbreaks of foodborne pathogens linked to low-moisture foods are becoming common occurrences. *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) are two of the most common bacterial foodborne pathogens and cause high morbidity and large economic losses globally. Numerous outbreaks of foodborne illness linked to the consumption of low-moisture foods contaminated with *Salmonella spp.* have been reported in the United States. Recent outbreaks include foods such as almonds, flour, cereal, and pistachios (*11).* The same is true for EHEC, with recent outbreaks linked to flour, nut butter, and hazelnuts (*11*). The magnitude of outbreaks show that these pathogens are capable of surviving in dry environments, even after experiencing desiccation stress.

Desiccation tolerance has been evaluated for *Salmonella* and EHEC on various surfaces. A study conducted by Hiramatsu et al. evaluated the desiccation tolerances of multiple strains of *Salmonella enterica,* EHEC O157, O26, and O111 by drying the bacteria on paper discs (*44*). With a starting inoculum density of 7 LOG CFU, the observed bacterial population decreases over 24 hours ranged from approximately 2 to 3 LOG CFU and there was a variation in the desiccation tolerances of different species, serotypes, and strains. Another study conducted by Gruzdev et al. evaluated the desiccation tolerance of *Salmonella enterica* serotypes by drying bacteria on plastic and glass surfaces (*39*). This study also noted varying desiccation tolerance

levels among the serovars tested. This data highlights the importance of strain specificity in assessing the tolerance of common foodborne pathogens to desiccation (*44,39,70*). Due to the ability of these pathogens to survive in dry environments on various surfaces, the risk of cross-contamination onto food products has also been noted. The cross-contamination between raw and cooked foods via various food contact surfaces has been identified as a significant risk factor in the transmission of foodborne pathogens (*15*). Kusumaningrum et al. observed that various pathogens can remain viable on dry stainless-steel surfaces and as such, presents a contamination hazard for food products that come in contact with these surfaces (*59*). There are multiple examples of outbreaks confirming this hazard. A 2006 outbreak of *Salmonella* Tennessee was linked to peanut butter where the outbreak strain was isolated from environmental swabs in the manufacturing plant and environmental contamination was determined to be the likely source of the outbreak (*91*). It was also suggested that the contamination was persistent for a prolonged period of time. An outbreak of *Salmonella* Agona in 2008 was linked to dry cereal. Environmental swabs again indicated the presence of the outbreak strain. In this case, the same strain had been implicated in a previous outbreak at the same facility in 1998. It was hypothesized that a recent construction project was the point of reintroduction of *Salmonella* into the production chain (*85*). Both of these outbreak cases highlight both the persistence and resilience of *Salmonella* in a dry production environment.

Some of the responses of pathogens to desiccation have been described, however there is still much that is unknown. The main consequences of desiccation and the resulting loss of water includes the shrinkage of the cell and increase in intracellular salt and macromolecule concentration along with reduced fluidity of membrane lipids and damage to proteins and DNA (*9*). A pathogen's ability to tolerate desiccation stress and the resulting effects on the cell is

thought to contribute to the persistence of pathogens in dry foods and food processing environments. A study by Margas et al. assessed the survival of 15 isolates of *Salmonella* dried onto stainless-steel surfaces over 30 days and an initial reduction in viability in less than 72 h with no significant further reduction was seen (*67*). A significant difference in the different strain's desiccation survival was noted and their model indicated that time was an insignificant resistance factor. This suggests that we may be able to link initial desiccation tolerance to the ability of a pathogen to survive for prolonged time periods in low-moisture environments. The issue that arises is that there is no single, uniform response to desiccation among foodborne pathogens. Not only have differences been noted between species like *Salmonella* and EHEC, but differences have also been noted between serotypes and even strains within a serotype. The growth method used to prepare the bacteria for desiccation may also be an important factor that affects desiccation tolerance but has not been directly compared. Along with the differing environmental conditions in the studies performed by Hiramitsu et al. and Gruzdev et al., they also used different growth methods. The first used bacteria from a suspended lawn growth, while the latter used bacteria from a liquid culture.

All of these studies indicate the desiccation tolerance capabilities of common foodborne pathogens and that the survival capabilities are dependent on many factors. For pathogens like *Salmonella* and EHEC which have low infectious doses and high morbidity, it is important to be aware of their tolerance to low a_w and their potential to survive long-term as a contaminant within a food processing environment. The goal of this study was to evaluate the tolerance of various enteric pathogens to sudden desiccations stress, and to compare this tolerance between species, serotypes, and strains of *Salmonella* and EHEC, as well as evaluate the differences in desiccation tolerance in different inoculum preparation methods.

4.3. Materials and Methods

4.3.1. Strain Selection

The strains used in this study are described in Table 2. Group 1 consisted of four *Salmonella* strains and seven enterohemorrhagic *Escherichia coli* (EHEC). The *Salmonella* strains were selected to include serovars commonly studied in low moisture environments and involved in outbreaks associated with low-moisture foods. These strains were obtained from the

Table 2. *Salmonella* and *Escherichia coli* strains used in the desiccation tolerance study.

Group [a]	**Species**	**Serovar/Serotype**	**Strain**	**Isolation Source**
Group 1	*Salmonella enterica*	Enteritidis	ATCC BAA-1045	Food
Group 1	*Salmonella enterica*	Agona	FSL S9-0322	Food
Group 1	*Salmonella enterica*	Tennessee	FSL R6-0494	Human clinical
Group 1	*Salmonella enterica*	Montevideo	FSL R8-3881	Human clinical
Group 1	*Escherichia coli*	O157:H7	RIMD 0509952	Human clinical
Group 1	*Escherichia coli*	O26:H11	TW16501	Human clinical
Group 1	*Escherichia coli*	O121:H19	PNUSAE002568	Human clinical
Group 1	*Escherichia coli*	O45:NM	TW07947	Human clinical
Group 1	*Escherichia coli*	O111:H8	TW7926	Human clinical
Group 1	*Escherichia coli*	O103:H2	TW08101	Human clinical
Group 1	*Escherichia coli*	O45:H2	TW09183	Human clinical
Group 2	*Salmonella enterica*	Agona	R8 8615	Environmental
Group 2	*Salmonella enterica*	Agona	R8 8619	Environmental
Group 2	*Salmonella enterica*	Agona	S10 1750	Environmental
Group 2	*Salmonella enterica*	Agona	S10 1759	Environmental
Group 2	*Salmonella enterica*	Agona	FSL M8-0485	Food
Group 2	*Escherichia coli*	O157:H7	EDL-933	Food
Group 2	*Escherichia coli*	O157:H7	93-111	Human clinical
Group 2	*Escherichia coli*	O157:H7	TW08263	Human clinical
Group 2	*Escherichia coli*	O157:H7	TW14585	Human clinical
Group 2	*Escherichia coli*	O157:H7	TW14588	Human clinical

[a]Group 1 is the first 11 strains tested including 4 strains of *Salmonella* and 7 strains of EHEC. Group 2 contains 5 more strains of *S.* Agona, and 5 more strains of EHEC O157:H7.

food safety lab at Cornell University, with the exception of S. enteritidis which was obtained from the American Type Culture Collection. The EHEC strains were selected to include serotype O157:H7 and other common non-0157 serotypes. These strains were obtained from the Thomas S. Whittam STEC Center at Michigan State University, with the exception of the O121:H19 strain, which was obtained from the Michigan Department of Health. Group 2 consisted of 5 more strains of EHEC serotype O157:H7 and 5 more strains of S. Agona.

4.3.2. Inoculum Preparation

Each bacterial strain was tested using two growth methods in triplicate. The first growth method used was the broth growth method. Each strain was streaked onto Luria-Bertani, Miller (LB) agar plates from stock cultures stored at -80°C and incubated for 20h at 37°C. A single colony was selected from this plate and transferred to 5mL LB Broth (VWR International) and incubated for 16h at 37°C while shaking at 200 RPM. Bacterial cells were harvested from the broth through centrifugation at 3800g for 5 min at room temperature (22 ± 1°C) and washing with sterile deionized water (SDW) a total of three times as described by Gruzdev et al. (*39*). After the third centrifugation, the final pellet was suspended in 10mL of SDW to achieve an initial inoculum of 10^8 CFU, giving the final liquid culture solution.

The second growth method used was the lawn growth method. Each strain was streaked onto LB agar plates from stock cultures stored at -80°C and incubated for 20h at 37°C. A single colony was selected from this plate and transferred to 5mL LB broth and incubated for 16h at 37°C while shaking at 200 RPM. From the LB broth culture, 250µL was spread uniformly onto an LB agar plate (100mm x 15 mm) using a sterile spreader (Fisher Scientific Inc, Waltham, MA). These plates were incubated for 24 h at 37°C to achieve confluent growth over the entire plate. To achieve an initial inoculum of 10^8 CFU, the bacterial lawn from half of a plate was

collected with a sterile spreader and suspended in 40mL of SDW. This suspension was vortexed for approximately 15 seconds, giving the final lawn growth inoculum solution.

4.3.3. Preparation of Desiccated Bacteria

All biological replicates for every strain under both growth methods were desiccated in duplicate. 100μL of the final inoculum solutions of each growth method was placed in a 6-well polystyrene plate (NEST Biotechnology Co., Jiangsu, China) and air dried for either 24h or 48h at room temperature (22 ± 1°C) and 40% relative humidity (RH). To obtain the desired RH, the 6-well plates were placed in a closed chamber (Coleman cooler 60.96 cm × 40.64 cm, Coleman Company, Inc., Kingfisher, OK) with 20g of MgCl (Avantor, Radnor, PA) that had been weighed in plastic trays (Fisher Scientific Inc.). The RH inside the chamber was measured and recorded using a HOBO temp/RH logger (Onset, Bourne, MA). These desiccation conditions were chosen based on the maximum dehydration results presented by Gruzdev et al. (*39*). This study showed that the water activity (a_w) reached 0.53 after 20hrs under these conditions and no subsequent changes in a_w occurred following dehydration for additional time. Serial dilutions (1:10) were made of the initial inoculum solutions which were plated on LB agar, incubated for 24h at 37°C and enumerated using Q-count (Advanced Instruments Inc., Norwood, MA). These were recorded as the starting bacterial populations at t=0.

4.3.4. Enumeration of Viable Cells Following Desiccation

Following desiccation, 1mL of SDW was added to each well and incubated at room temperature for 5 min. The bacteria were then resuspended by pipetting 10 times and transferred to a microcentrifuge tube, and the bacterial suspension was vortexed for approximately 5 seconds using a Vortex-Genie 2 (Stellar Scientific, Baltimore, MD) and serially diluted (1:10) and plated

onto LB agar. The plates were incubated for 24h at 37°C and the colony counts were performed using Q-count.

4.3.5. RpoS Functionality Test

The functionality of RpoS was assessed for the six strains of EHEC O157:H7 and the six strains of *S.* Agona through a catalase screen using 30% concentrated hydrogen peroxide. Each strain was streaked onto LB agar plates from stock cultures stored at -80°C and incubated for 20h at 37°C. A single colony was selected from this plate and transferred to 5mL LB Broth and incubated for 16h at 37°C while shaking at 200 RPM. Serial dilutions of this liquid culture were made and plated onto LB agar. Each strain was tested in triplicate as follows. A single drop of concentrated (30%) hydrogen peroxide was placed on an isolated colony on the agar plate. Positive catalase activity, and thus positive RpoS functionality, was confirmed by the presence of vigorous and rapid bubbling upon the addition of hydrogen peroxide. Negative catalase activity, and thus negative RpoS functionality was confirmed by either nonexistent bubbling or delayed and minimal bubbling.

4.3.6. Data Analysis

Desiccation tolerance was quantified as the log reduction observed at each time-point, calculated using the following equation.

$$L = -\log_{10}(\frac{n_t}{n_0})$$

Where L is the log reduction, n_t is the bacterial population (CFU/mL) at time-point (t) and n_0 is the bacterial population at t=0. The total log reduction was determined between time points t=0h and t=48h. The desiccation tolerance was observed for each strain in three biological replicates and two technical replicates. The results are reported as mean values with standard deviations. A Bartlett test of homogeneity of variances was run on all biological replicates. To compare the

desiccation tolerance between growth methods of one strain, a two-sample t-test was used. The total log reductions of each group of strains were compared using an analysis of variance (ANOVA) model and Tukey honest significant differences. The relative importance metrics were calculated using the package relaimpo (Grömping 2006). All statistical analyses were completed using R version 4.0.3. Differences were considered significant at adjusted (adj.) $p < 0.05$.

4.4. Results

4.4.1. Desiccation Tolerance Varies Between Salmonella and EHEC

The desiccation tolerance of strains of *Salmonella enterica* and EHEC are visualized through the total log reduction observed after 48h of desiccation in Figure 4. The Bartlett test of homogeneity of variances found no significant differences in variances among biological replicates. A consistent trend seen between species, is the majority of the bacterial cell population reduction is seen within the first 24 hours (Fig. 4). Other than this, the desiccation tolerance between species has a wide variation. Between *Salmonella* and EHEC, the relative variance from strain was 21% and the relative variance from growth method was <1%. The interaction between species and growth method had a significant effect on the total log reduction ($p \leq 0.05$). The average total log reduction of *Salmonella* desiccated from the liquid culture was significantly different than the average total log reduction of *Salmonella* desiccated from the lawn growth and the average total log reduction of EHEC desiccated from the liquid culture ($p \leq 0.05$). Desiccation tolerance varied considerably among strains and by growth method (Fig. 5). The lowest desiccation tolerance was seen from EHEC O111:H8 desiccated from the liquid culture at a total log reduction of 1.23 ± 0.26 log CFU/mL and the highest desiccation tolerance was seen from *S.* Agona desiccated from the liquid culture at a total log reduction of 0.43 ± 0.06 log CFU/mL.

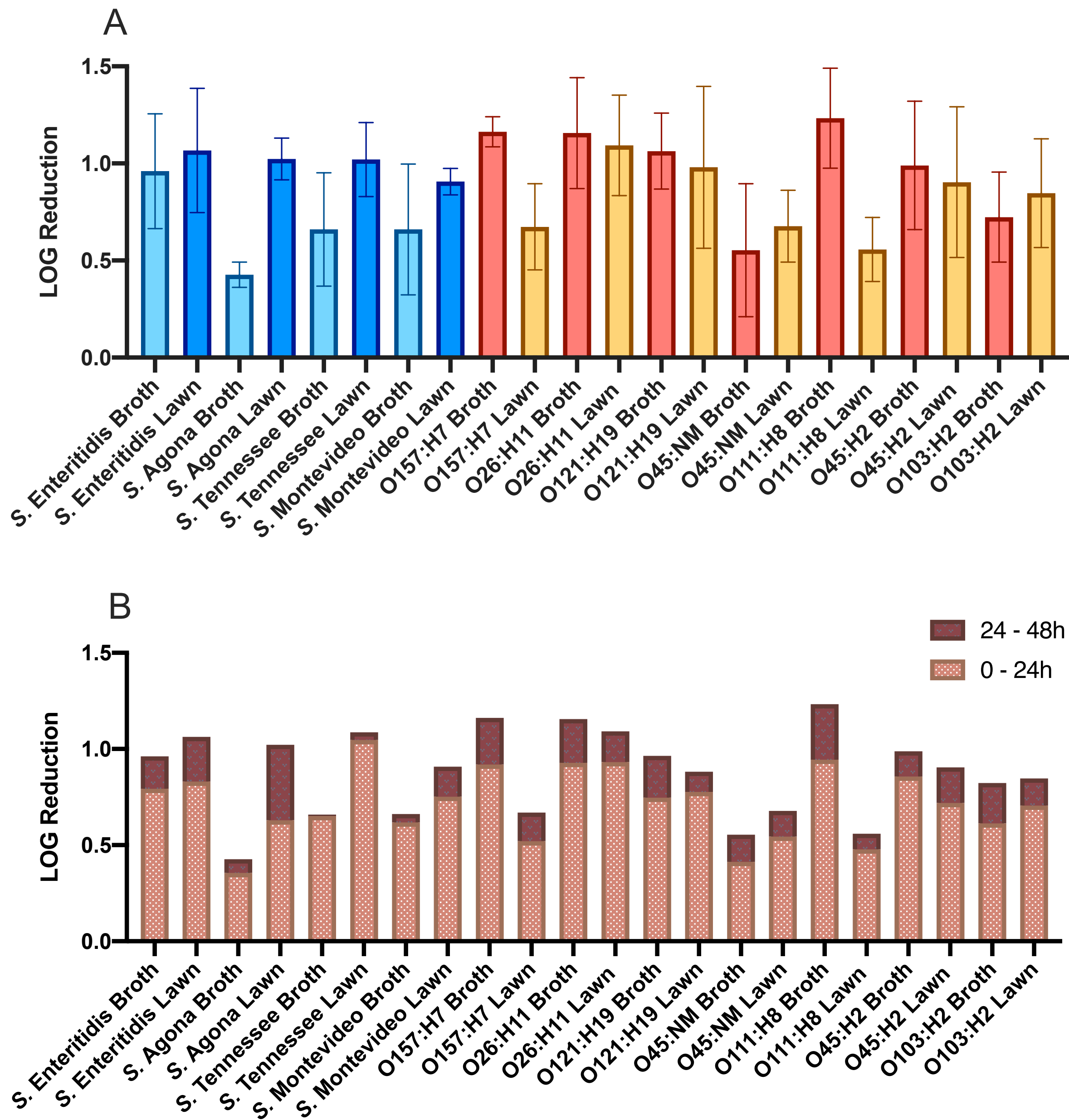

Figure 4. Total log reduction for each strain after 48 hours of desiccation on a plastic surface. Panel A shows the log-transformed value of the difference in the number in surviving cells from time 0 to 48h and is shown as the mean of three biological replicates with standard deviation. Panel B shows the log-transformed value of the difference in the number in survival cells from time 0 to 24h and from time 24 to 48h and is shown as the mean of three biological replicates. * indicate growth methods that were significantly different within a strain ($p \leq 0.05$).

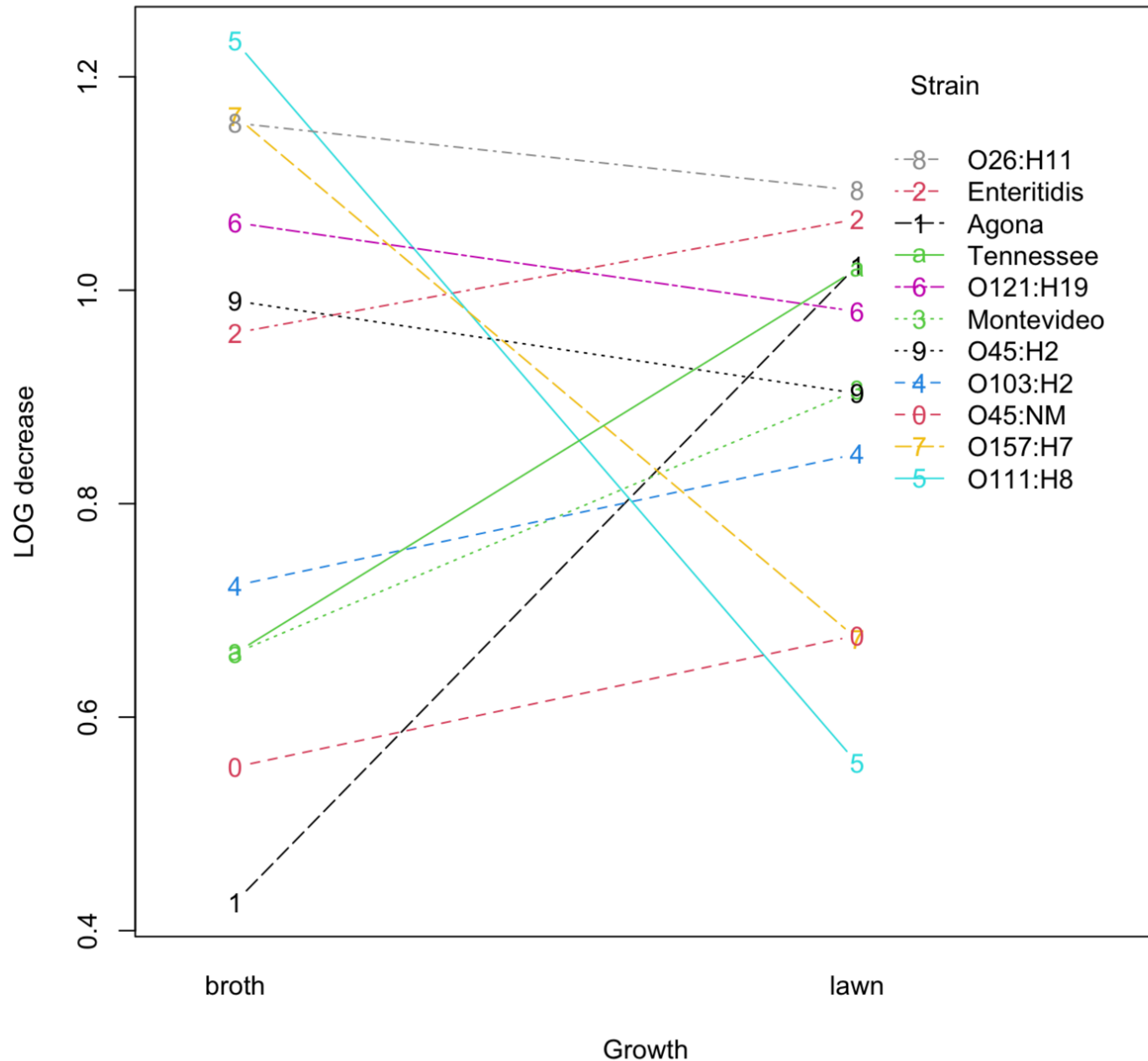

Figure 5. Interaction plot between total log reduction and growth method for the 11 strains in group 1. The total log reduction is the log-transformed value of the difference in the number in surviving cells from time 0 to 48h and is shown as the mean of three biological replicates for each growth method for each strain.

4.4.2. Desiccation Tolerance Varies Among Four Strains of Different Serovars of *Salmonella*

For all four *Salmonella* strains tested and shown in Figure 4, the bacterial cells desiccated from a liquid culture had a higher desiccation tolerance than the cells from a lawn growth and the effect of the growth method on the total log reduction was significant ($p \leq 0.05$). Among these

Salmonella strains, the relative variance from strain was 14% and the relative variance from growth was 32%. *S.* Agona's total log reduction after 48h of desiccation was significantly different ($p \leq 0.05$) between the broth and lawn growth method, with a total log reduction of 0.43 ± 0.06 log CFU/mL under the broth growth condition and a total log reduction of 1.02 ± 0.11 log CFU/mL under the lawn growth condition. The total log reduction among the *Salmonella* strains under the broth growth condition ranged from 0.43 ± 0.06 log CFU/mL to 0.96 ± 0.30 log CFU/mL and ranged from 0.91 ± 0.07 log CFU/mL to 1.06 ± 0.32 log CFU/mL under the lawn growth condition, and the variation in total log reduction between strains and growth methods is seen in Figure 5.

4.4.3. Desiccation Tolerance Varies Among Seven Strains of Different Serotypes of EHEC

A wide variation in desiccation tolerance was noted among the seven strains of EHEC. There were no consistent trends observed of the effect of growth method or strain on the total log reduction and the observed total log reduction varied widely. The relative variance from strain was 23% and the relative variance from the growth method was 7%. The total log reduction among the seven EHEC strains under the broth growth condition ranged from 0.55 ± 0.34 log CFU/mL to 1.23 ± 0.26 log CFU/mL and under the lawn growth condition ranged from 0.56 ± 0.17 log CFU/mL to 1.09 ± 0.25 log CFU/mL. Some strains had little to no difference in desiccation tolerance under each of the growth conditions while others had a significant difference. The total log reduction seen for O111:H8 and O157:H7 were significantly different under the broth and lawn growth condition. For O111:H8, the total log reduction was 1.23 ± 0.26 log CFU/mL under the broth growth condition and was 0.56 ± 0.17 log CFU/mL under the lawn growth condition. For O157:H7, the total log reduction was 1.16 ± 0.08 under the broth growth

condition and was 0.67 ± 0.22 under the lawn growth condition. The largest differences in total log reduction between growth methods is further visualized in Figure 5.

4.4.4. Consistent Desiccation Tolerance Trends Seen Among Six Strains of *Salmonella* Agona

S. Agona was the one serovar of the four strains of *Salmonella* tested in group 1 that had a significant difference in desiccation tolerance between growth methods, and five more strains of this serovar were tested in group 2 and analyzed along with the first strain (Fig. 6). The effect of growth method on total log reduction was significant ($p \leq 0.05$) with a consistent trend of a higher desiccation tolerance under the broth growth condition and a lower desiccation tolerance under the lawn growth condition (Fig. 7). The relative variance from strain was 10% and the relative variance from growth method was 47%. No significant differences were observed between the strains. The total log reduction of the *S.* Agona strains ranged from 0.26 ± 0.10 log CFU/mL to 0.37 ± 0.18 log CFU/mL under the broth growth condition and ranged from 0.53 ± 0.34 log CFU/mL to 0.89 ± 0.22 log CFU/mL under the lawn growth condition.

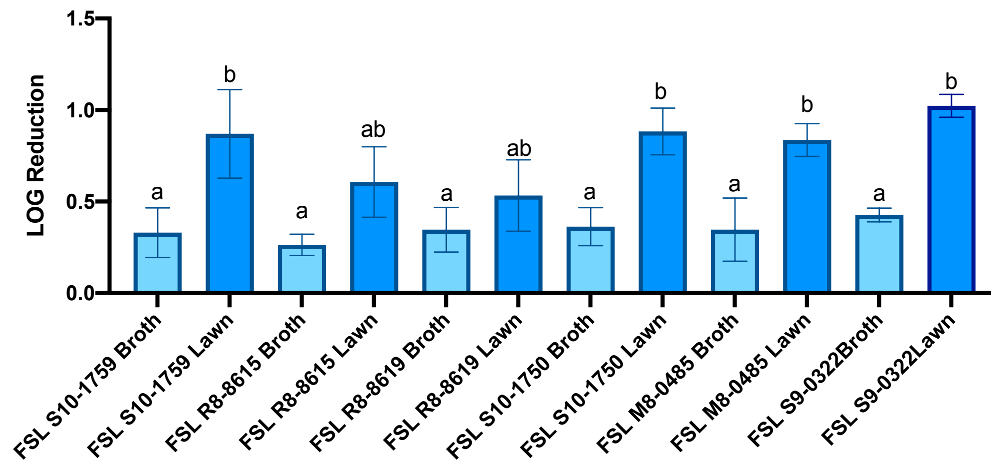

Figure 6. Total log reduction for each strain of *Salmonella* Agona after 48 hours of desiccation on a plastic surface. Each bar represents the log-transformed value of the difference in the number in surviving cells from time 0 to 48h and is shown as the mean of three biological replicates with standard deviation.
Different letters indicate values that are significantly different (adj. $p \leq 0.05$).

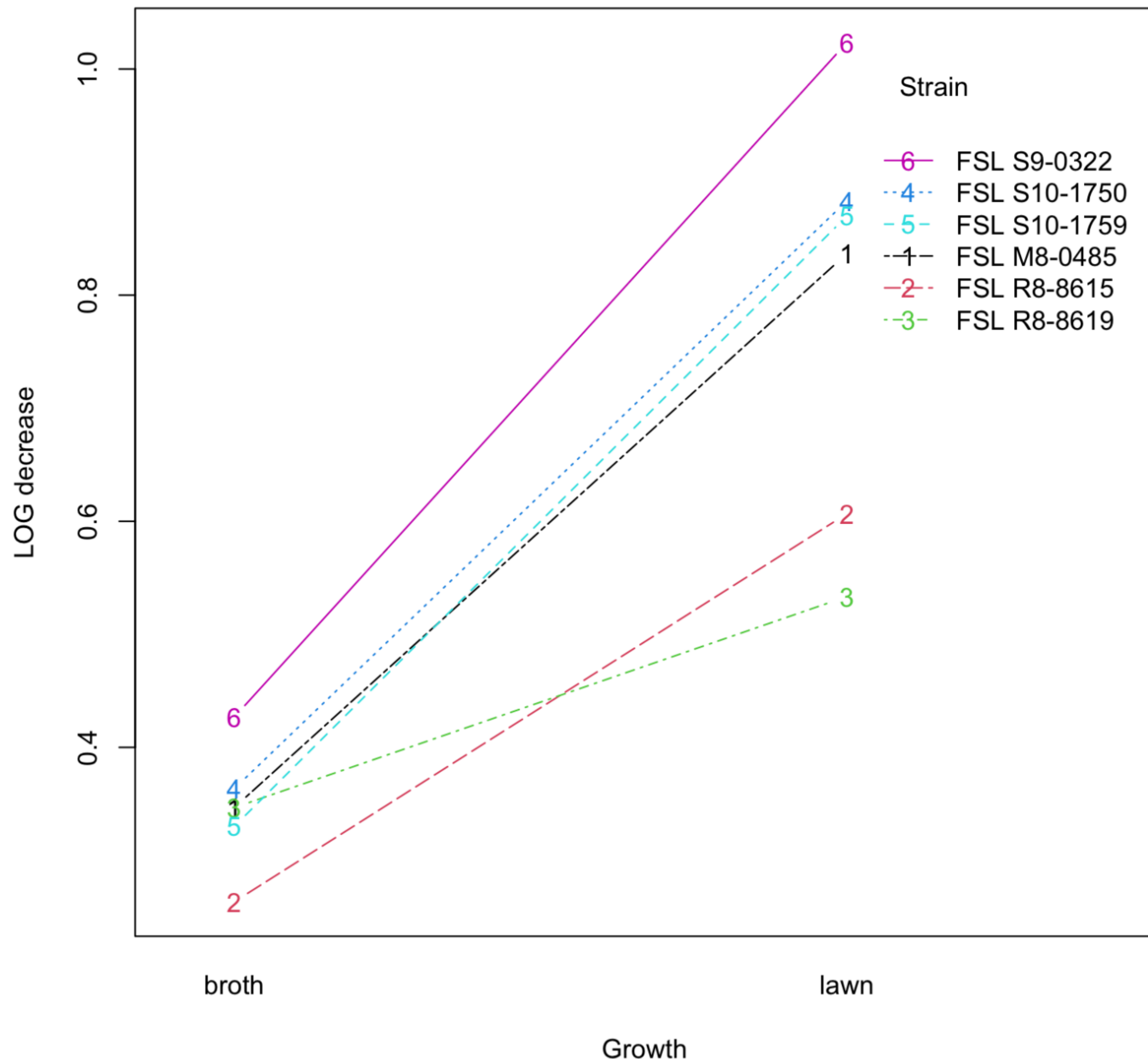

Figure 7. Interaction plot between total log reduction and growth method for the 6 strains of *Salmonella* Agona. The total log reduction is the log-transformed value of the difference in the number in surviving cells from time 0 to 48h and is shown as the mean of three biological replicates for each growth method for each strain.

4.4.5. Strain and Growth Method Led to Differing Desiccation Tolerance Among Six EHEC O157:H7 Strains

O157:H7 was one of the two strains of EHEC tested in group 1 that had a significant difference in growth methods, and five more strains of this serotype were tested in group 2 and analyzed along with the first strain (Fig. 8). The interaction of strain and growth had a significant

effect on the total log reduction of EHEC O157:H7, with the relative variance from strain being 93% and the relative variance from growth method being <1%. RpoS functionality also had a significant effect on the total log reduction. The results of the RpoS functionality test are shown in Table 3. The strains that were RpoS negative had a significantly higher total log reduction than the strains that were RpoS positive. Among the RpoS negative strains, the interaction between strain and growth method had a significant effect on total log reduction, and the same was true among the RpoS positive strains. The high variability between strains, RpoS functionality, and growth method can be seen in Figure 8 and Figure 9 The total log reduction of the RpoS negative strains ranged from 2.34 ± 0.18 log CFU/mL to 2.58 ± 0.31 log CFU/mL under the broth growth condition and ranged from 2.55 ± 0.35 log CFU/mL to 3.49 ± 0.21 log CFU/mL under the lawn growth condition. The total log reduction of the RpoS positive strains ranged from 0.33 ± 0.21 log CFU/mL to 1.16 ± 0.08 log CFU/mL under the broth growth

Table 3. Strain and result of hydrogen peroxide catalase screen for RpoS functionality.

Serotype	**Strain**	**Result (+ OR -)** [a]
O157:H7	EDL-933	-
O157:H7	TW14588	-
O157:H7	93-111	-
O157:H7	TW08263	+
O157:H7	TW14585	+
O157:H7	RIMD 0509952	+
S. Agona	R8 8615	+
S. Agona	R8 8619	+
S. Agona	S10 1750	+
S. Agona	S10 1759	+
S. Agona	FSL M8-0485	+
S. Agona	FSL S9-0322	+

[a] Strains were classified as positive (+) or negative (-) for RpoS functionality based on catalase activity.

condition and ranged from 0.32 ± 0.25 log CFU/mL to 0.67 ± 0.22 log CFU/mL under the lawn growth condition.

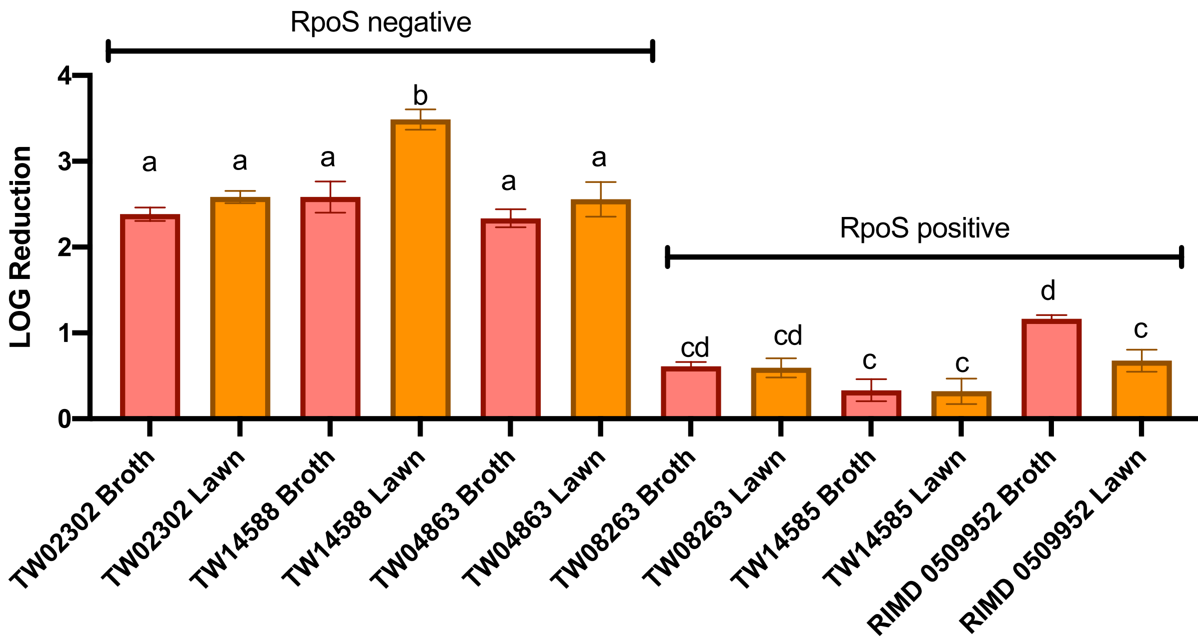

Figure 8. Total log reduction for each strain of EHEC O157:H7 after 48 hours of desiccation on a plastic surface. Each bar represents the log-transformed value of the difference in the number in surviving cells from time 0 to 48h and is shown as the mean of three biological replicates with standard deviation.
Different letters indicate values that are significantly different (adj. $p \leq 0.05$).

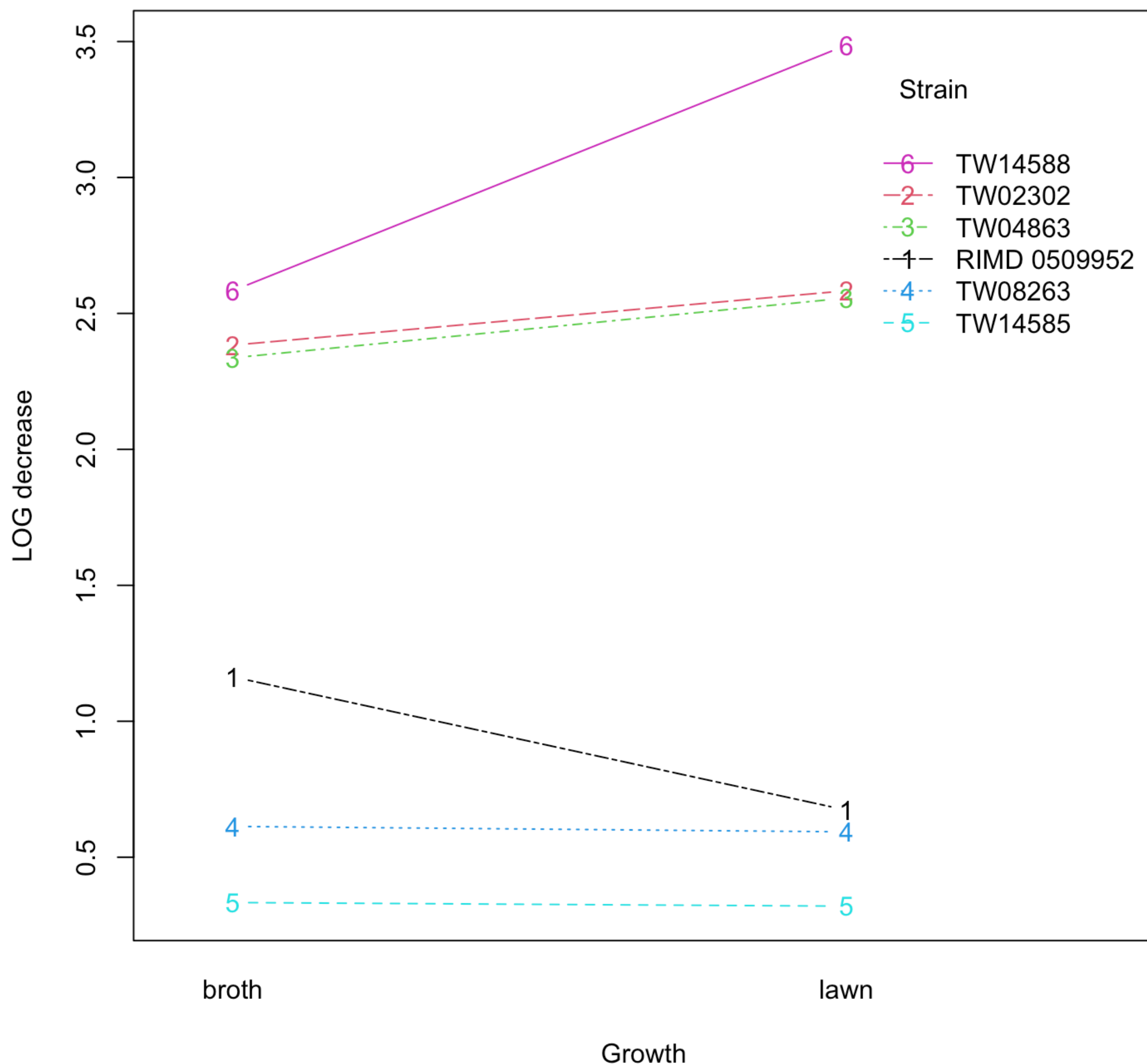

Figure 9. Interaction plot between total log reduction and growth method for the 6 strains of EHEC O157:H7. The total log reduction is the log-transformed value of the difference in the number in surviving cells from time 0 to 48h and is shown as the mean of three biological replicates for each growth method for each strain.

4.5. Discussion

The goal of this study was to evaluate the tolerance of various enteric pathogens to sudden desiccation stress, and to compare this tolerance between species, serotypes, and strains of *Salmonella* and EHEC, as well as evaluate the differences in desiccation tolerance in different

growth methods. The eleven strains tested in group one were chosen to have a representation of common outbreak serotypes of both *Salmonella enterica* and EHEC. Differences in desiccation tolerance were noted between species, strains, and growth methods. It was clear upon this first round of observation, that there was no uniform desiccation response. The first round of testing covered four strains of four different serovars of *Salmonella enterica.* Gruzdev et al. had previously observed the desiccation tolerance of various strains of *Salmonella enterica* grown and desiccated from a liquid culture. This study observed a total log reduction ranging from 0.31 log CFU to 2.12 log CFU after 22 h of desiccation at 25°C and 40% relative humidity on a plastic surface (*39*). While different serovars and strains were tested in that study, the environmental conditions of our study were modeled after theirs and our observed desiccation tolerance for *Salmonella enterica* falls closely within the range observed by Gruzdev et al. For the strains of *Salmonella enterica* tested in this study, the effect of growth method appeared to be more influential over the total log reduction than the strain. The EHEC strains tested, however, showed the opposite trend. While growth was still a significant factor, the effect of the strain had more of an influence over the total log reduction. A study on the desiccation tolerance of similar serotypes of EHEC was conducted by drying the inoculum on paper disks for 24 h at 35°C (*44*). A total log reduction ranging from 2.22 log CFU/disk to 2.89 log CFU/disk was observed for strains of O157, a range of 2.15 log CFU/disk to 3.36 log CFU/disk was observed for strains of O26, and a range of 2.19 log CFU/disk to 3.57 log CFU/disk was observed for strains of O111. While the magnitude of these population reductions was higher than those observed in this study, a variation in desiccation tolerance between strains was observed in both studies.

S. Agona was one of the original strains of *Salmonella enterica* on which the growth method had a large and significant effect on the total log reduction (Fig. 5). Five more strains of

S. Agona were chosen to study further to see if this trend would remain consistent for different strains among this serovar. For the strains we tested, this trend remained consistent, with the growth method significantly effecting the total log reduction (Fig.6). Higher desiccation tolerance was observed under the broth growth condition for *S.* Agona (Fig. 7). It is important for the effect of growth method on desiccation tolerance to be taken into consideration as many studies primarily use a suspended lawn growth inoculum preparation method in monitoring the desiccation tolerance and long-term survival of enteric pathogens like *S.* Agona in low-moisture environments (*61,64*).

EHEC O157:H7 was one of the original strains of EHEC on which the growth method had a large and significant effect on the total log reduction (Fig. 5). Five more strains of EHEC were chosen to study further to see if this trend would remain consistent for different strains among this serovar. Among the strains tested here, this trend did not remain consistent, but a new grouping of strains emerged. Three strains were found to have very poor desiccation tolerance, while the other two had a desiccation tolerance that was more comparable to the first strain of O157:H7 tested in group 1. When bacteria undergo hyperosmotic stress, trehalose synthesis is induced. This is dependent on the alternative sigma factor RpoS. RpoS expression is known to be induced during hyperosmotic stress like desiccation (*86*, *94*). This knowledge led to the screening of all of the tested strains of EHEC O157:H7 and *S.* Agona for RpoS functionality and the results are shown in Table 3. The results of this screening showed that all three strains that had a significantly lower desiccation tolerance were negative for RpoS functionality, and all of the other tested strains were positive. These results coincide with the previous studies and shows that RpoS functionality plays an important role in desiccation tolerance. EHEC O157:H7 has been found to be genetically diverse and multiple clades with genomic and epidemiological

variation have been detected (*65*). This knowledge, along with the differing desiccation tolerance seen among strains tested here shows the importance of strain selection when assessing the risk of EHEC O157:H7 in a low-moisture environment.

Our results show that there is not one, uniform response to desiccation stress from enteric pathogens and also emphasized the importance in using a broad range of strains when testing desiccation characteristics to ensure that the results obtained are an accurate representation of the serotype or species as a whole. It is also apparent that many environmental factors play a role in desiccation tolerance. The differing desiccation tolerances from the different growth methods must also be taken into account when performing stress response studies of these pathogens. The knowledge of the desiccation tolerance of these pathogens may also lend insight to their long-term survival in a dry environment. Low-moisture surfaces and environments are highly prevalent in the food industry, and desiccation is used on many food products as it increases their stability and shelf-life. It is critical that there is a clear understanding of how individual pathogens respond and adapt to environmental stressors like desiccation in order to develop appropriate risk reduction measures.

5. CONCLUSIONS

Salmonella enterica and Enterohemorrhagic *Escherichia coli* are tolerant to desiccation stress and can survive for extended periods of time in a low-moisture environment. On wheat grain, strains of four different serovars of *Salmonella enterica* were able to survive for 52 weeks with an average decimal reduction time of 24 weeks. Viable counts of strains from EHEC serotypes O45:NM, O111:H8, and O26:H11 were detected for 44 weeks and strains from serotypes O157:H7, O121:H19, and O103:H2 were detected for 40 weeks until they passed below the limit of detection (2.0 log CFU/g). The average decimal reduction time for the EHEC serotypes was 12 weeks. A significant difference in the survival of *Salmonella* and EHEC was noted, but both were able to persist on wheat grain for an extended period of time at a high enough level to cause infection.

We also determined that there is no uniform response to desiccation stress between *Salmonella enterica* and EHEC and that the growth method used to prepare the bacteria can also affect desiccation tolerance. A similar trend among *Salmonella* strains of higher desiccation tolerance from the liquid culture was noted. When six strains of the serovar *S.* Agona were observed, a consistent response within the serovar was seen with total log reductions ranging from 0.26 ± 0.10 log CFU/mL to 0.37 ± 0.18 log CFU/mL under the broth growth condition and ranged from 0.53 ± 0.34 log CFU/mL to 0.89 ± 0.22 log CFU/mL under the lawn growth condition. When six strains of EHEC O157:H7 were observed, the desiccation tolerance varied both among strains and between growth methods. RpoS functionality was determined to be a crucial factor in desiccation tolerance with strains that were determined to be RpoS negative having a significantly lower desiccation tolerance than the strains that were determined to be RpoS positive.

Knowledge of the desiccation tolerance and long-term survival capabilities of these pathogens is key to performing accurate risk assessments in the food industry. It was determined that there are many influential factors that can affect these responses. These factors include, growth method, the composition of the low-moisture surface, environmental conditions, and more. It was also shown here that strain selection is vitally important when evaluating desiccation tolerance and long-term survival in a low-moisture environment as it cannot always be assumed that a single strain will be representative of an entire serotype. The differing results from different bacterial growth methods must also be taken into consideration when performing studies like this in order to ensure the results obtained are an accurate representation of what would occur outside of a lab environment. In conclusion, the information provided by this research enhances the knowledge on the desiccation tolerance and long-term survival of *Salmonella enterica* and EHEC and is useful for risk assessments and determining appropriate preventative measures in the food industry.

6. FUTURE STUDIES

This research showed that *Salmonella enterica* and EHEC are capable of withstanding desiccation stress and can survive for extended periods of time in a low-moisture environment. Further investigation is still required to obtain the full picture of the response of these pathogens to a low-moisture environment. With the information obtained on the long-term survival capabilities of *Salmonella* and EHEC on wheat grain, further research into potential storage condition modifications to impede their survival would be useful. Research into inactivation treatments for the wheat grain that would not affect the quality of the flour produced would also be useful. The information obtained on the desiccation tolerance of *Salmonella* and EHEC leaves us with a few more unanswered questions that require further investigation. Research into what differs between the bacteria grown under the different conditions, how changing environmental conditions affects desiccation tolerance, and if there are bacterial cells that are entering a viable but nonculturable (VBNC) state would all help further enhance the knowledge on the response of these pathogens to desiccation stress. Overall, these studies would enhance the knowledge on the desiccation tolerance and long-term survival phenotypes of enteric pathogens, which would be useful for risk assessments and preventative measures in the food industry.

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