CHARACTERIZATION OF THE GROWTH/SURVIVAL OF *FRANCISELLA TULARENSIS* IN SELECTED FOOD MATRICES

by

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Abstract

Francisella tularensis is a Gram-negative bacterium that can cause tularemia in humans. The disease can be acquired through several routes, one of which is the ingestion of contaminated food and water. The pathogen has the potential to be used as a biological weapon, and its intentional introduction in the food supply is a possible way to cause public harm. The purpose of this study was to evaluate the growth and/or survival of *F. tularensis* in food matrices under various storage conditions. Cystine Heart Agar enriched with 2% hemoglobin and supplemented with antibiotics was used to aid the enumeration of the target organism by suppressing the levels of indigenous microflora in foods. During the first portion of the study, the growth/survival of highly virulent *F. tularensis* subsp. *tularensis* SCHU S4 strain was investigated in prepared bagged iceberg lettuce stored at two temperatures. *F. tularensis* counts were significantly different ($p \leq 0.05$) among temperature levels for days 1-3, but not day 4. After the first 24 h of incubation, the mean estimates of *F. tularensis* counts were 1.00 log cfu g^{-1} lower in lettuce stored at 23±1°C compared to lettuce stored at 6±1°C. Lower recovery rates at higher temperatures are likely due to the more rapid proliferation of naturally present bacteria which can inhibit the growth of *F. tularensis*. After 48 and 72 h, differences in *F. tularensis* counts between temperatures were 0.55 log cfu g^{-1} and 0.3 log cfu g^{-1}, respectively. For the second portion of the experiment, the ability of *F. tularensis* to grow or survive was evaluated in nine food matrices with variable compositional content (red delicious apples, green bell pepper, shredded iceberg lettuce, strawberries, whole liquid eggs, boneless ham steak, beef hot dogs, 80-20 ground beef, and 2% UHT milk) at 4, 21, and 37°C. *F. tularensis* grew well in pasteurized whole liquid eggs stored at 21 and 37°C. With the exception of ham, liquid whole eggs, and bell pepper, the

pathogen was detected infrequently throughout the duration of the study. Very low recovery rates were obtained for shredded lettuce, hot dogs, and ground beef.

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Chapter 1 - Introduction

The use of intentionally contaminated food and water to cause harm dates back to ancient cultures. Nevertheless, only in recent years, concerns about food biosecurity have increased on both national and international levels (Kennedy & Busta, 2007). Some microorganisms and their toxins have significant potential to pose a threat to national security. The Centers for Disease Control and Prevention (CDC) has classified such select agents as Category A because of their ease of dissemination, high mortality rates, and capacity to cause public panic (CDC, 2013a).

Francisella tularensis, the causative agent of tularemia, is an example of a Category A select agent. Humans can acquire the disease through arthropod bites, handling contaminated animal tissue, inhalation of contaminated aerosols, and ingestion of contaminated food and water. The symptoms vary based on the route of exposure and include cutaneous ulcers, swollen lymph nodes, pharyngitis, and fever (CDC, 2011). Biological weapon scientists from several countries (Japan, United States, Soviet Union) studied *F. tularensis* during World War II and thereafter. Many believe that aerosol release of the agent may cause the most damage to public health because of its low infectious dose. However, *F. tularensis* could be used as a biological weapon in several ways (Dennis et al., 2001). The intentional introduction of the pathogen to the military food supply as an act of bioterrorism is one possible scenario of significant concern. Perishable foods, such as fresh produce and dairy, are typically procured from sources in areas where military personnel may be targeted (Mara & McGrath, 2009), which elevates the food safety risks our troops may be facing.

As described by Kennedy and Busta (2007), select agents are more likely to be used for the intentional contamination of foods if they possess certain characteristics. For example, the lack of methods for detection of a given pathogen in foods could lead to its ingestion and

eventually cause illness before the causative agent is identified. Additionally, it is concerning if the food containing the pathogen supports its growth or preserves its viability. Therefore, understanding the growth behavior of *F. tularensis* in specific food matrices can provide critical information for the effective assessment of food safety risks and risk management in military food systems.

The purpose of this study was to evaluate the ability of *F. tularensis* to grow or survive in: 1) prepared bagged iceberg lettuce and 2) nine food matrices with various compositional profiles using multiple storage conditions. This research was a collaborative effort between the U.S. Army Natick Soldier Systems Center (Natick, Massachusetts) and Kansas State University (Manhattan, KS).

Chapter 2 - Literature Review

Francisella tularensis

The causative agent of tularemia, *Francisella tularensis*, was first isolated in 1911 by Drs. George McCoy and Charles Chapin from rodents with "plague-like" disease (McCoy & Chapin, 1912). Initially observed by McCoy in ground squirrels, the illness was found to be fatal in guinea pigs, rats, grey mice, gophers, and monkeys after inoculation of these species in a laboratory setting (McCoy, 1911). At the time of its discovery, the pathogen was named *Bacterium tularense* after Tulare county in California where it was originally found (McCoy & Chapin, 1912), and later was re-named *Francisella tularensis* in honor of Dr. Edward Francis because of his devoted efforts and significant contributions to multiple areas of tularemia research (Jellison, 1972). Unaware of the virulent nature of the organism, Francis became infected with tularemia in the beginning stages of his work during his trip to Utah where he was to investigate the disease then referred to as "deer-fly fever" (Jellison, 1972). Interestingly, throughout the years Francis developed tularemia for a total of four times while working with the pathogen (Siderovski, 2006).

As described by Wherry and Lamb (1914), the ability of *F. tularensis* to cause disease in humans became known after a 21-year old male in Ohio sought medical attention due to inflammation in his left eye in 1913. It was assumed that the patient contracted the disease while handling infected raw meat at work (Wherry & Lamb, 1914). During 1919 and 1920 Francis diagnosed seven tularemia cases in humans (Siderovski, 2006). By the mid 1920's, tularemia was no longer a disease specific to the North American region, since scientists in Japan and the Soviet Union also recognized it (Bell, 1980).

Since its discovery, the bacterium has been incorrectly classified in the genera *Bacterium*, *Brucella,* and *Pasteurella,* as its properties have not shown sufficient similarities to the properties of microorganisms belonging to these taxa (Olsufiev at al., 1959). The need for proper classification became apparent in the 1947 when Dorofeev proposed placing the etiological agent of tularemia into a separate genus called *Francisella* (Olsufiev et al., 1959). In later years, Dorofeev's recommendation was supported by findings in Ritter and Gerloff's DNA hybridization analysis, which showed differences between the genetic profiles of the pathogen and other members of the genus *Pasteurella* (Ritter & Gerloff, 1966). Additionally, scientific investigations showed that *F. tularensis* has a smooth cellular membrane, unlike other Gram-negative organisms such as *Brucella* and *Pasteurella* species, which are characterized with wavy cell membranes (Pavlova et al., 1967).

The genus *Francisella,* the only genus in the family Francisellaceae, contains only two species: *F. tularensis* and *F. philomiragia*. Furthermore, *F. tularensis* is divided into four subspecies (*tularensis, holarctica, mediasiatica,* and *novicida*) (Sjöstedt, 2005). Table 1 represents differences between *Francisella* subspecies based on geographic distribution, virulence, and biochemical properties.

Table 2.1 Characteristics of the subspecies of *Francisella tularensis*[a,b].

Characteristics	***F. tularensis* subsp. *tularensis***	***F. tularensis* subsp. *holarctica***	***F. tularensis* subsp. *mediasiatica***	***F. tularensis* subsp. *novicida***
Primary Geographic Location[a]	North America	Europe, Far East, Kazakhstan, North America	Central Asia and parts of the former Soviet Union	North America and Australia
LD_{50} in rabbits[b] (cfu)	$<10^1$	$>10^6$	$>10^6$	$>10^6$
Est. LD_{50}* in humans[a] (cfu)	$<10^1$	$<10^3$	ND	$>10^3$
Size[†b] (µm)	0.2-0.7 x 0.2	0.2-0.7 x 0.2	0.2-0.7 x 0.2	0.7 x 1.7
Capsule[b]	+	+	+	-
Cysteine required for growth[§b]	+	+	+	-
β-lactamase[b]	+	+	-	+
Acid production from[b]:				
Maltose	+	+	-	d
Lactose	-	-	-	-
Sucrose	-	-	-	+
D-Glucose	+	+	-	+
Glycerol	+	-	+	d

*LD_{50}, 50% lethality dose; the human LD_{50} is estimated for all of the subspecies except *F. tularensis* subsp. *tularensis*; cfu, colony-forming units; ND, not determined.
[†]Size in liquid medium during logarithmic growth phase
[§]A few strains of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* do not require cysteine for growth
This table was adapted from:
[a]Oyston et al., 2004; and [b]Sjöstedt, 2005.

Among the four *F. tularensis* subspecies, subspecies *tularensis* (Type A) is most virulent and is specific to North America. Subspecies *holarctica* (Type B) is less virulent but widely distributed throughout the Northern Hemisphere (Dennis et al., 2001; Sjöstedt, 2005; WHO, 2007). The majority of the illnesses that occur in Europe and Asia are generally caused by Type B strains, however, highly virulent Type A strains have also been isolated in parts of Europe

(Gurycova, 1998). This difference in virulence was investigated by Olsufiev and colleagues who determined that the American SCHU strain used in their experiment was more virulent for domestic rabbits (lethality dose was 1-10^5 organisms) compared to strains isolated from Europe and Asia (lethality dose of 10^9-10^{10} organisms). Additionally, the American strain utilized glycerol, unlike the other strains (Olsufiev et al., 1959). Type A strains also differ from Type B strains by their ability to metabolize citrulline (their possession of citrulline ureidase system), although the possession of a citrulline ureidase system was not found to be directly related to virulence (Marchette & Nicholes, 1961). Limited information is available about subspecies *mediasiatica* but research with rabbits suggests a virulence similar to that of subspecies *holarctica* (WHO, 2007). There have been reports of human cases of tularemia associated with subspecies *novidica*, usually in immunocompromised individuals; however, these strains have low virulence (Hollis et al., 1989; WHO, 2007). Strains of subspecies *mediasiatica* are common only in parts of Central Asia, whereas subspecies *novicida* have been isolated only in defined regions in North America and Australia (Petersen & Schriefer, 2004).

Francisella tularensis is a Gram-negative pleomorphic coccobacillus the size and shape of which are dependent on growth phase and growth medium (Sjöstedt, 2005). As shown in Table 1, when grown in a liquid medium and during the logarithmic phase of their growth, the bacterial cells of subspecies *novicida* tend to be larger and lack a capsule compared to members of the other three subspecies. Additionally, subspecies *novicida* does not depend on cysteine for growth; however, when present, cysteine enhances growth. Subspecies can be differentiated based on their ability to degrade carbohydrates and produce acid without producing gas. For instance, *F. tularensis* subsp. *tularensis* is distinguished from the other subspecies by its ability to form acid by degrading maltose, D-glucose, and glycerol. Strains of subspecies *holarctica*

produces acid from maltose and D-glucose, whereas subspecies *mediaciatica* only degrades glycerol. The causative agent of tularemia is non-motile, non-spore forming, and aerobic. It grows poorly at 26^{o}C, with the exception of *F. tularensis* subsp. *novicida* (Oyston, 2006) but all strains grow well at 37^{o}C.

Virulence and Pathogenicity

F. tularensis is an intracellular pathogen the virulence of which has not been associated with the formation of an exotoxin but rather with the microorganism's potential to disrupt a host cell's functions by multiplying within the cell and causing an inflammatory response (Oyston, 2008). Although the mechanisms of cell invasion are not clearly understood, it has been determined that a cluster of 19 genes called *Francisella* Pathogenicity Island (FPI) plays a major role in the virulence of *F. tularensis* (Jones et al., 2014; Nano et al., 2004; Oysten at al., 2008). Subspecies *tularensis* and *holarctica* carry two FPI copies each, whereas subspecies *novicida* carries only one FPI that is 97% similar to the FPI of the more pathogenic strains (Barker & Klose, 2007; Oyston, 2008).

Cellular components such as the lipopolysaccharide (LPS) and the bacterial capsule of *F. tularensis* have been recognized as important virulence factors. The LPS (endotoxin) is a component of the outer membrane of Gram-negative bacteria and consists of three units: lipid A, core oligosaccharide, and O-antigen. A critical function of LPS is inducing immune response through recognition of lipid A by the TLR4 receptor (Gunn & Ernst, 2007; Trent et al., 2006). Unlike most Gram-negative bacteria that display strong endotoxic activity even at low numbers, the LPS of *F. tularensis* is characterized by poor ability to generate host recognition. The inability of *F. tularensis* LPS to activate the TLR-4 receptor could be explained with the structural differences in *F. tularensis* lipid A component compared to other bacterial species. For

instance, in *Francisella* species, lipid A does not contain free phosphate groups and possesses long tetra-acylated acid chains usually 16 to 18 carbons long. In enteric bacteria, a lipid A is typically described by hexa-acylated acid chains that are only 12 to 14 carbons long and present phosphate groups (Gunn and Ernst, 2007; Jones et al., 2014).

Literature suggests that the pathogenicity of *F. tularensis* is also influenced by the O-antigen portion of the LPS (Gunn & Ernst, 2007). As described by Raynaud et al. (2007), the loss of O-antigen by inactivation of the *wbtA* gene in *F. tularensis* subsp. *holarctica* LVS resulted in diminished virulence of the strain in a murine model. Decline in resistance to human serum and lack of ability to replicate were also observed in the genetically altered organism (Raynaud et al., 2007). Earlier, Eigelsbach and Downs (1961) described the presence of two colony types of the Soviet *F. tularensis* live vaccine strain (LVS) when plated on peptone cysteine agar: a blue variant (BV) and a gray variant (GV). Inoculation of mice and guinea pigs with each variant indicated that the BV was more virulent to both species compared to the GV (Eigelsbach & Downs, 1961). The work of Hartly and others (2005) demonstrated that BV bacteria possessed O-antigen units, whereas such were absent in the GV. Additionally, the vaccination with only the BV bacteria displayed a protective effect in mice challenged with the virulent *F. tularensis* SCHU S4 strain. Such findings are also indicative of the O-antigen's influence in the development of tularemia (Hartley et al., 2006).

The capsule of *F. tularensis* has a molecular mass between 100 and 250 kDa and contains sugars identical in composition to those of the LPS O-antigen (Apicella et al., 2010; Jones et al., 2014). It is present in both Type A and Type B *Francisella* species. Hood (1977) investigated the effect of capsule removal on the virulence and immunogenicity of *F. tularensis*. Capsule-free organisms demonstrated reduced virulence in guinea pigs, but not in mice. The injection of

capsule material was unable to provoke immune response in animals challenged with a virulent *F. tularensis* strain (Hood, 1977). In a later study, the presence of a capsule was found to be critical for resistance against the bactericidal effects of human serum (Sandström et al., 1988).

Sources of *Francisella tularensis* and Modes of Transmission

The number of known susceptible hosts of *F. tularensis* exceeds 300 species, which include both invertebrates and vertebrates (Keim et al., 2007). The common hosts and vectors of tularemia vary by geographic region. For instance, in North America, where disease is caused by both Type A and B strains, the most important hosts are cottontail rabbits (*Sylvilagus* spp.) and hares (*Lepus* spp.) (Keim et al., 2007). However, more human cases of tularemia have been associated with rabbits because rabbits are more susceptible to infection than hares (Sjöstedt, 2007). Tularemia is also known as "rabbit fever" because of its common occurrence in these animals. Various other animals such as voles, beavers, muskrats, squirrels, foxes, cats, sheep, deer, skunks, raccoons, and even reptiles and birds have been reported as sources of the pathogen. In the North American regions, important tularemia vectors include several ticks (the lone star tick (*Amblyoma americanu),* the American dog tick (*Dermacentor variabilis*), and the Rocky mountain wood tick *(D. andersoni*)) and biting flies (Craven & Barnes, 1991; Keim et al, 2007; Matyas, 2007).

In world regions where tularemia is primarily caused by *F. tularensis* Type B (Europe and Asia), hares and rodents are considered the major sources of tularemia to humans (Keim et al., 2007; Sjöstedt, 2007). Among the most common species are brown hares, mountain hares, and water and common voles. Black rats, mice, and Siberian chipmunks have also been infected with *F. tularensis* Type B. Arthropods such as ticks, mites, biting flies, mosquitoes, and rarely

fleas play a major role in transmitting the disease from infected to healthy mammals and maintaining its presence in nature (Keim et al., 2007).

Humans can acquire tularemia through several routes: arthropod (tick, deerfly, mosquito) or animal bites, contact with infected animals or animal tissue, inhalation of contaminated aerosols, or through consumption of contaminated food or water (CDC, 2011).

The environment itself can serve as a reservoir of *F. tularensis*, as the pathogen has been detected in water, mud, soil, hay, and animal feces samples (Petersen et al., 2009; WHO, 2007). The epidemiology of the pathogen is greatly influenced by its persistence in nature. Forsman et al. (2000) investigated the survival of *F. tularensis* in chilled (8^{o}C) water. Findings showed that viable cell counts were still detectable on a solid medium for at least 40 days after inoculation (Forsman et al., 2000). A later study determined that the pathogen can survive for at least 21 days at the same temperature (Gilbert & Rose, 2012). Multiple occurrences of water-borne tularemia have been documented (Anda, et al., 2001; Chitadze et al., 2009; Helvaci et al., 2000; Mignani, 1988; Reintjes, et al., 2002).

The disturbance of infected animal carcasses during landscaping or farming activities and the inhalation of dust or hay aerosols can lead to the development of pneumonic tularemia (Matyas, 2007). A good example of such occurrence is the pneumonic tularemia outbreak on Martha's Vineyard during 2000 when individuals became infected after exposure to aerosolized pathogen with an environmental origin (Feldman et al., 2001). In nature, grain and straw can become contaminated through urine or feces of infected animals, or from the tissue of decomposing infected animal carcasses. One study found that in oats and ray straw *F. tularensis* was able to survive for more than 4 months at 10-18^{o}C, up to 35 days at 15-25^{o}C, and up to 19

days at 20-30^{o}C, while during frozen storage the pathogen remained alive for longer than 6 months (Pomanskaia, 1957).

Clinical Forms of Tularemia

The severity of tularemia in humans depends on the route of infection, the number of infective organisms, virulence of the strain, and the immune status of the host (Craven & Barnes, 1991). The infectious dose of tularemia varies by mode of transmission. For instance, Saslaw and colleagues (1961a) determined that the introduction of only 10 to 50 organisms of the virulent strain *F. tularensis* subsp. *tularensis* SCHU via the respiratory route is sufficient to cause illness in humans. Similarly, as few as 10 organisms were able to cause ulceroglandular tularemia when the pathogen was administered intradermally (Saslaw et al., 1961b). However, a much larger dose (10^8 cells) was required to cause disease via the oral route (Hornick et al., 1966). Without antibiotic treatment, the mortality rate for virulent Type A strains is between 5 and 15%, and the fatality rates range from 30% to 60%. The overall fatality rate in the United States today is less than 2%. Illnesses caused by Type B strains are usually not fatal (CDC, 2013b; Dennis et al., 2001).

F. tularensis can enter the human body through the skin, eyes, mouth, and nose. Six forms of tularemia have been recognized based on the route of acquisition: ulceroglandular, glandular, oculoglandular, oropharyngeal, pleuropulmonary (pneumonic), and typhoid. Generally, tularemia is characterized by an abrupt onset and typically develops 3-5 days after exposure. The incubation time ranges from 1 to 25 days, depending on the virulence of the strain, route of infection, and the dose of the pathogen. Common symptoms include fever, chills, muscle and joint pain, sore throat, nausea, and diarrhea (Bossi et al., 2006). Six clinical forms have been recognized. Ulceroglandular and glandular forms occur as a result inoculation of the skin,

typically following direct contact with contaminated animals or materials, or as a result of an arthropod or animal bite. This is the most common form of tularemia responsible for approximately 80% of reported tularemia cases. Initially, a small papule appears at the site of infection, which in a few days develops into a larger pustule, which after rupture turns into a painful cutaneous ulcer. These symptoms are accompanied by enlarged lymph nodes that can persist for up to 3 years after exposure. Glandular tularemia is also described by fever and enlarged lymph nodes, but ulcers are absent (Bossi et al., 2006, Dennis et al., 2001; Gill & Cunga, 1997; Sjöstedt, 2007; WHO, 2007).

Oculoglandular tularemia affects the eye and accounts for up to 5 % of tularemia cases in the United States (Gill & Cunha, 1997). Individuals can acquire this form after touching the eye with contaminated fingers, or possibly from splashes and infective dust. This condition is characterized by ocular pain, conjunctivitis, itching, lid edema, increased tear production, and enlarged lymph nodes in the vicinity of the affected area. Although rare, complications may lead to corneal ulceration and loss of vision (Gill & Cunha, 1997; WHO, 2007).

The ingestion of contaminated food or water can lead to oropharyngeal and gastrointestinal tularemia. Food and water can become contaminated from contact with contaminated animals or their feces. Consuming undercooked infected game meat can result in infection. Inhalation of contaminated aerosols is a possible route of infection with this particular form. Symptoms of the oropharyngeal form include painful sore throat, pharyngitis or tonsillitis, and inflamed cervical lymph nodes (Ellis et al., 2002, Dennis et al. 2001). Typically oropharyngeal tularemia is not fatal, however, if untreated, it can develop into the most deadly form – pneumonic. The colonization of the intestines by *F. tularensis* leads to gastrointestinal tularemia, the severity of which dependents on the inoculum dose. Its symptoms can range from

mild persistent diarrhea to pervasive bowel ulcerations that can lead to a fatal outcome (Day & Whiting, 2009; Ellis, 2002).

Typhoidal tularemia is typically characterized with flu-like symptoms such as sore throat, nausea, headaches, fever, chills, muscle and joint pain, sometimes accompanied by diarrhea and vomiting (Bossi et al., 2006). This form does not have a specific location of the infection, nor is it clear how to pathogen enters the host's body. The lack of tularemia specific symptoms makes the diagnoses of this form very challenging, and consequently, it may be associated with higher mortality rates (Dennis et al., 2001).

Pneumonic tularemia can follow the inhalation of infected aerosols (primary) or result as a complication of typhoidal and ulceroglandular tularemia when bacteria spread to the lungs (secondary). It is most often presented along with typhoidal tularemia, and can be severe with high fatality rate if untreated. Typically symptoms appear abruptly and include high fever, headache, non-productive cough, and in some cases pleuritic chest pain (Gill & Cunha, 1999; Thomas & Schaffner, 2010). The diagnosis of pneumonic tularemia is difficult due to lack of tularemia specific symptoms and often can be misdiagnosed as Lyme disease (Matyas, et al, 2007).

Detection methods

Multiple detection methods for *F. tularensis* have been employed in clinical and research laboratories to aid the identification and diagnosis of tularemia. Commonly used procedures include bacterial culturing, molecular detection, and serological testing (WHO, 2007). The culture method is considered the gold standard for detecting the pathogen, however, it possesses several drawbacks. *F. tularensis* is a slow growing microorganism which requires cysteine supplemented media and may take up to a few days to become visible on agar plates. Isolation

from blood specimens and contaminated environmental or tissue samples may be difficult due to the specific growth requirements of the pathogens and the presence of competing flora. *F. tularensis* is highly infectious; therefore, its cultivation presents a high risk for laboratory-acquired infections to laboratory personnel (Dennis et al., 2001, Evans et al., 1985; Simsek et al., 2012; WHO, 2007).

When culture methods are not feasible, molecular detection methods such as polymerase chain reaction (PCR) and 16S rRNA analysis can be utilized (Forsman et al., 1990; WHO, 2007). PCR is a technology used for the exponential amplification of one or more DNA (deoxyribonucleic acid) sequences and typically targets the *tul 4* or *Fop A* gene, or the IS*Ftu2* element that are specific for *F. tularensis* (Higgins et al., 2000; Simsek et al., 2012, WHO, 2007). Versage et al. (2003) described the use of a multi-target real time TaqMan PCR in which IS*Ftu2*, *23kDa*, and *tul4* are targeted simultaneously.

Serological techniques such as enzyme-linked immunosorbent assay (ELISA), agglutination, and Western blotting have also been applied for the detection and identification of *F. tularensis* (Syrjala et al., 1986; WHO, 2007). Combining ELISA (a screening step) with a Western blot (confirmation step) has been shown to be a highly efficient way to detect *F. tularensis* compared to other serological tests alone (Porsch Ozcurumez et al., 2004).

Comparisons between detection methods found in literature suggest that PCR is more sensitive than culture and serological approaches. For instance, for the detection of tularemia in wound specimens from patients with ulceroglandular tularemia in Sweden, PCR identified 75% of the specimens positive for *F. tularensis*, whereas culture identified 62% (Johansson et al., 2000). PCR showed greater sensitivity than a highly sensitive capture ELISA test performed on infected hare tissue (Grunow, et al., 2000). Other advantages of molecular methods include low

risk of infection to laboratory personnel and short detection time, which is critical for the timely diagnosis and administration of proper therapy (Dolan et al., 1998; Maurin, et al., 2010). However, molecular detection methods such as PCR, unlike bacterial culturing, are often unable to differentiate between viable and nonviable cells (Day & Whiting, 2009). Additionally, PCR may not be a suitable method for detection of *F. tularensis* in selected clinical, food, or environmental samples which contain compounds that have inhibitory effects on the amplification of DNA (Day & Whiting, 2009; Wilson, 1997).

Tularemia Treatments and Vaccines

Several classes of antibiotics have shown efficacy in treating tularemia (Table 2.2). For decades aminoglycosides, such as streptomycin and gentamicin, have been used as primary therapy. Evans (1985) noted the successful recovery of 28 out of 30 tularemia patients (93%) after administering streptomycin for 10 to 14 days. Streptomycin has a bactericidal effect and is rarely associated with relapses (Evans et al., 1985). However, its availability is limited, therefore gentamicin is commonly used as a substitute (Dennis et al., 2001; Oyston et al., 2004). It is recommended for aminoglycosides to be given to patients for 10 days. Tetracyclines such as doxycycline and quinolones such as ciprofloxacin, levofloxacine, and ofloxacin, as well as chloramphenicol are suggested alternative treatments. Quinolones have been found effective as a first-line treatment in some cases, including two immunocompromised individuals (Limaye & Hooper, 1999). Ciprofloxacin should be administered 10 to 14 days and its use in children should be limited due to associations of this drug with adverse effects in animals. Chloramphenicol and doxycycline require a treatment of 14 to 21 to avoid relapses (Dennis et al, 2001; Bossi et al., 2006).

Table 2.2 Recommendations for treatment of tularemia.

	Dennis et al., 2001		Bossi et al., 2006	
	Treatment	Recommended dose	Treatment	Recommended dose
Adults and pregnant women	Streptomycin*	2 g daily	Streptomycin*	2 g daily
	Gentamicin*	5 mg/ kg daily	Gentamicin*	5mg/kg once or twice daily
	Doxycycline	200 mg daily	Ciprofloxacin	800-1000 mg daily
	Ciprofloxacin	800 mg daily	Ofloxacin	800 mg daily
	Chloramphenicol†	15 mg/kg 4 times daily	Levofloxacin	500 mg daily
			Doxycycline	200 mg daily
Children	Streptomycin*	max 2 g daily	Streptomycin*	max 2 g daily
	Gentamicin*	2.5 mg/kg 3 times daily	Gentamicin*	2.5 mg/kg 3 times daily
	Doxycycline	2.2 mg/kg twice daily	Ciprofloxacin	10-15 mg/kg twice daily
	Chloramphenicol†	15 mg/kg 4 times daily	Doxycycline	max 200 mg daily
	Ciprofloxacin	max 1 g daily		

*Preferred (first-line) treatment
†Not recommended for use in pregnant women

An attenuated live vaccine strain (LVS) of *F. tularensis* is the only available vaccine for preventing tularemia in the United States, and it has not yet received approval by the Food and Drug Administration (FDA) for general use. It was developed by US Army researchers from a Soviet vaccine strain that demonstrated efficacy in protecting millions of people during the 1950's (Ellis et al., 2002; Siderovski, 2006). The live vaccine, which was developed from a *F. tularensis* subsp. *holarctica*, was successfully used in volunteers during a respiratory challenge with the highly virulent *F. tularensis* SCHU S4 strain (Saslaw et al., 1961a). Vaccine administration in laboratory workers resulted in less laboratory-acquired infections at Fort Detrick, Maryland (Burke, 1977). The incidence of laboratory-acquired cases after the emergence of LVS has been investigated. Between 1950 and 1959, before the emergence of LVS, employees were given a killed Foshay vaccine as means of protection from tularemia.

During that period the incidence of typhoidal and ulceroglandular tularemia was 5.70 cases per 1,000 at-risk employee-year and 0.76 cases per 1,000 at-risk employee-year, respectively. Incidence of typhoidal tularemia dropped significantly (0.27 cases per 1,000 at-risk employee-year) between 1960 and 1969 when attenuated LVS was the vaccine of choice. LVS was not effective in protecting from ulceroglandular tularemia, however, vaccinated individuals generally experienced milder course of illness (Burke, 1977).

The LVS is available as an investigational new drug to individuals with high risk of exposure to *F. tularensis,* such as laboratory personnel. The need to better understand the mechanisms of protective response and attenuation have made the licensing of the vaccine challenging (Ellis et al., 2002). A protective effect is generated 2 weeks after vaccination, therefore LVS is not an alternative for post-exposure preventive measures (Bossi et al., 2006; Dennis et al., 2001; Siderovski, 2006).

Tularemia Outbreaks

According to data of the Centers for Disease and Control (2013b), there are approximately 120 tularemia cases on average reported each year in the United States. In the last 2 decades (2001-2010 and 1990-2000), these numbers have remained relatively steady, and the majority of reported cases have occurred in states located in the central or western part of the country (except Massachusetts). The highest incidence of reported tularemia cases took place in the 1930's and 1940's but these numbers significantly dropped in the 1950's. The number of cases varied throughout the year with most cases documented in the spring and summer months associated with arthropod bites and landscaping/farming activities, and outbreaks in the fall and winter months are related to rabbit hunting. Regardless of season or age, more cases have been reported in males versus females (CDC, 2002; CDC, 2013b).

Individuals of any age or sex are susceptible to tularemia, however, certain occupations (Ex.: laboratory workers, farmers) or activities (Ex.: hunting, landscaping) increase the risk of infection (Dennis et al., 2001). Individuals who mow lawns or cut brush in areas where *F. tularensis* is typically present in the environment are more likely to develop tularemia via the inhalation route (Feldman et al., 2001).

Tularemia cases have been reported in numerous parts of the Northern Hemisphere. Table 2.3 describes several outbreaks that have occurred in the last two decades and demonstrates the diversity in geographic locations, bacterial strains, and possible sources of tularemia infection.

Table 2.3 Tularemia outbreaks throughout the Northern Hemisphere (1997-2008).

Location	Subspecies	Year	Source of infection	Number of cases	Reference
Spain	*holarctica*	1997-1998	Hares	559 humans	Ariza Miguel et al., 2014
Kosovo	*holarctica*	1999-2000	Food and Water	327 humans	Reintjes et al., 2002
United States	*tularensis*	2000	Environmental	15 humans	Feldman et al., 2001
Bulgaria	*holarctica*	1997-2005	Possibly food and water	285 humans	Kantardjiev et al., 2006
Germany	*holarctica*	2005	Hares	10 humans	Hauri et al., 2010
Spain	*holarctica*	2007-2008	Common vole	507 humans	Ariza Miguel et al., 2014

Airborne tularemia is not a new occurrence. In endemic regions such as Sweden and Finland, pneumonic tularemia is one of the most reported clinical forms (Tärnvik, 2004). In the United States, a primary pneumonic tularemia outbreak was described in 2000 on Martha's Vineyard, Massachusetts. Patients developed the disease after performing landscaping activities such as lawn mowing and brush cutting and inhaling aerosolized contaminated particles. The

source of *F. tularensis* was determined to be the environment and the pathogen most likely was introduced through animal feces (Feldman et al., 2001). Infection through inhalation was also described during a tularemia outbreak in Germany in 2005 when hare hunters acquired the disease through inhalation of aerosolized particles that were generated during rinsing infected hare carcasses (Hauri et al., 2010). An outbreak in Spain in 2007-2008 was characterized by numerous typhoidal and pneumonic tularemia cases suggestive of transmission through the aerosol route. The source of infection is believed to be the common vole because the outbreak occurred during the time when the vole population numbers were the greatest (Ariza Miguel et al., 2014).

The ingestion of *F. tularensis* has resulted in multiple tularemia outbreaks. Between 1999 and 2000, Kosovo experienced its first tularemia outbreak affecting 327 individuals. The disease was determined to be contracted by ingesting food and water contaminated by rodents. Disordered environmental conditions in post-war Kosovo allowed for a vast number of food storage areas and water wells to become vulnerable to contamination spread by the increased rodent populations (Reintjes et al., 2002). The high number (96.5%) of oropharyngeal tularemia cases during the tularemia outbreak in Bulgaria between 1997 and 2005 is suggestive of transmission via the oral route. Additionally, bacterial strains isolated from well water showed similarities with strains isolated from infected patients. Given the increased rodent populations in the area, rodents and their feces were considered a possible source of contamination (Kantardjiev et al., 2006). Water-borne outbreaks of tularemia have also been described in Turkey and Georgia (Chitadze et al., 2009; Helvaci et al., 2000).

Tularemia is no longer a disease limited to the Northern Hemisphere. The first case of tularemia south of the Equator was reported in Australia after a 53-year old male contracted the

disease through a cut on his foot that was exposed to brackish water. Laboratory tests suggested that the strain responsible for the disease was very closely related to one strain of *F. tularensis* subsp. *novicida*, however, its proper classification in the *F. tularensis* species is still to be determined (Whipp et al., 2003).

F. tularensis as a Biological Weapon

The potential of *F. tularensis* to be used as a biological weapon has been acknowledged for many decades. During the 1930's and 1940's, both Japanese and Western military warfare researchers studied the pathogen (Dennis et al., 2001). Japanese experiments with biological agents on humans resulted in the deaths of thousands. Along with *F. tularensis*, many other highly infectious pathogens were studied for use as biological weapons, some of which include, *Bacillus anthracis*, *Vibrio cholerae*, *Yersinia pestis*, *Shigella* and *Salmonella* species. The release of infected insects and the contamination of food and water are some of the methods used to intentionally introduce biological agents to human populations (Siderovski, 2006). It is believed that during World War II tularemia was intentionally introduced to Soviet and German soldiers. The U. S. military conducted extensive research on tularemia in the 1950's and 1960's, which resulted in the development of weapons for the dissemination of the agent, along with attenuated live vaccine, as well as several antibiotic treatment options for the disease. In the late 20th century, the Soviet Union developed a strain of *F. tularensis* resistant to both antibiotics and vaccines for use as a biological weapon (Dennis et al., 2001; Siderovski, 2006).

The intentional release of aerosolized *F. tularensis* could have a catastrophic effect on public health. To demonstrate such a phenomenon, in a report in 1970, the World Health Organization (WHO) Group of Consultants included population models estimating the casualties in the case of biological warfare attack. The models indicated that aerosolizing 50 kg of dried

powder that contains 6 x 10^{15} organisms over an urban area with five million inhabitants would result in a quarter million incapacitated cases, including 19,000 deaths (WHO, 1970). A model on the economic impact of intentional release of tularemia developed by the CDC in 1997 indicated that the minimum cost of such biological attack would be 5.4 billion dollars per 100,000 exposed individuals (Kaufmann et al., 1997). Some of the expenditures considered to generate these estimates included cost of hospitalization, doctor visits, loss of life, medical treatments, and prophylaxis.

By 1973, the program for developing bioweapons in the United States was ceased, and biological weapon reserves were destroyed in response to President Nixon's order (Dennis et al., 2001). Today, *F. tularensis* is classified as a Category A agent by the CDC. Category A agents are of highest priority and have a significant potential to be used in the act of bioterrorism or bio-warfare because they are easily dispersed or transmissible, can lead to high mortality rates, may cause public panic, and call for public health preparedness measures. Other high-risk organisms and toxins that fall in this category are the causative agents of anthrax, botulism, pneumonic plague, smallpox, and Viral Hemorrhagic Fevers (CDC, 2013a). It is also recognized as a Tier 1 agent, meaning it belongs to a subgroup of select agents and toxins that "present the greatest risk of deliberate misuse with the most significant potential for mass casualties or devastating effects to the economy, critical infrastructure; or public confidence" (HHS, 2012).

Francisella tularensis, Biosafety, and Biosecurity

F. tularensis, has been recognized as the third most common cause for laboratory acquired infections following brucellosis and typhoid (Pike, 1976). Although the number of laboratory acquired tularemia infections has dramatically decreased since the 1970's (Titball et al., 2007), laboratory workers continue to be at high risk for acquiring infections. In 2004, three

laboratory employees at Boston University contracted tularemia after handling what they believed was a live vaccine strain (LVS) not harmful to humans. Investigations revealed that the LVS culture was contaminated with highly virulent *F. tularensis* Type A from an unknown source (Barry, 2005). Another accidental exposure of laboratory workers and autopsy personnel to *F. tularensis* was described by Shapiro and Schwartz (2002). Medical staff caring for a 43-year old patient with pulmonary tularemia symptoms failed to inform the microbiology lab and autopsy personnel about the possible case of tularemia. Twelve lab technicians became exposed to the pathogen while handling the patient's specimens and subculturing of what they believed was *Haemophilus* species. No infections were reported after laboratory and autopsy workers received prophylactic treatment (Shapiro & Schwartz, 2002).

In order to protect laboratory personnel and the environment from exposure to infectious materials, safe laboratory practices, safety equipment, and proper facility design must be implemented. Microbial agents can be manipulated safely in one of four biosafety levels based on the severity of the disease those agents have on humans and the availability of treatment for infections. Agents that are not known to cause disease are manipulated in BSL-1 laboratories. When the manipulated organisms have the ability to cause illness typically through oral, percutaneous, or mucous membrane transmission, they should be handled in BSL-2 facilities. BSL-3 facilities should be used when the handled pathogens can cause serious illness or death through inhalation and other routes of exposure. The handling of any exotic agents that can cause life-threatening infections via aerosolation and with no available treatments or vaccines must occur in BSL-4 facilities (HHS, 2009).

F. tularensis research can be conducted in laboratories with either containment level 2 or 3 based on the virulence of the strain and the nature of the experimental procedure applied. For

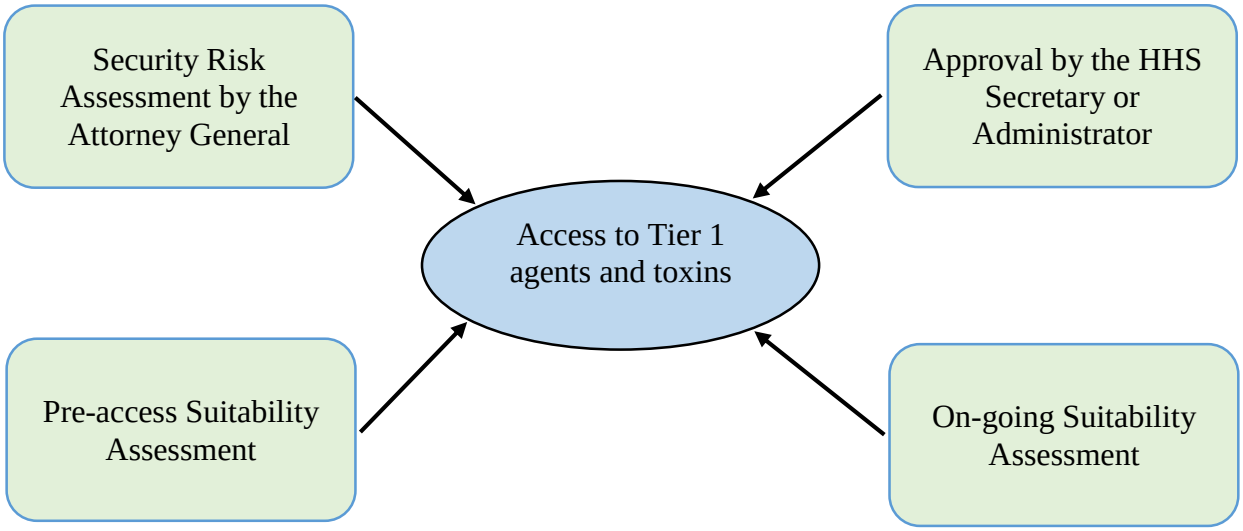
instance, research that involves attenuated LVS of *F. tularensis* and low virulence strains of *F. tularensis* subsp. *novicida* can be performed at biosafety level 2 (Titball et al., 2007). Practices such as specimen handling and the collection and processing of clinical materials are also carried out at containment level 2 (Nulens & Voss, 2002). Because of their high virulence, *F. tularensis* subsp. *tularensis* stains must be manipulated only at containment level 3. The same conditions apply to research involving aerosolizing of the pathogen even when the strains are of lower virulence, such as *F. tularensis* Type B (Titball et al., 2002). Research with Type A tularemia requires the use of facilities with negative airflow and secure access, where all microbial work is performed inside a biosafety cabinet. Required personal protective equipment includes protective laboratory clothing that must be decontaminated prior to laundering, as well as gloves and respiratory protection (HHS, 2009). Staff need to be properly trained and made aware of potential risks associated with handling highly virulent strains of *F. tularensis* prior to starting work with the pathogen (Titball et al., 2007).

According to Miller (2006), under the Select Agent Rule, all laboratories with select agent research are closely overseen by either the CDC or the Animal and Plant Health Inspection Services (APHIS) based on whether agents pose a severe threat to public health or animal and plant health and products. Since *F. tularensis* can be life-threatening to humans, any entities involved with the possession, use, or transfer of the pathogen must be registered with the CDC Select Agent Program. Any release of the agent outside of primary containment during manipulation has to be reported to the CDC immediately. The same rule applies in case of theft or loss. Access to select agents is limited and is only allowed for individuals approved by the Select Agent Program. Laboratory personnel with access to select agents have to undergo a

security risk assessment by the FBI and be rendered suitable to perform work with select agents (Miller, 2006).

Tier 1 select agents such as *F. tularensis* are subject to more stringent rules because they pose a greater risk to public or animal health. As noted in the Federal Register (HHS, 2012), an individual must not be granted access to Tier 1 select agents unless that person meets certain requirements (Figure 2.1). In addition to successfully completing a security risk assessment by the Attorney General and obtaining approval by the HHS Secretary, which are necessary for work with any select agent or toxin, individuals involved with Tier 1 research must complete a pre-access suitability assessment and participate in an on-going suitability assessment program.

Figure 2.1 Requirements for access to Tier 1 select agents and toxins.



Adapted from HHS, 2012.

Francisella tularensis and Foods

Although *F. tularensis* presents a significant concern due to its potential to be used a biological weapon and its ability to cause disease through ingestion, only a limited amount of research has been conducted on the behavior of this pathogen in foods. Below is an overview of the current knowledge on *F. tularensis* survival characteristics in foods during high temperature and high pressure processing, as well as during long-term storage under various atmospheric conditions. A study focused on the use of ultraviolet light for the inactivation of the pathogen on foods and food contact surfaces is also described.

Day and colleagues (2008) investigated the thermal stability of *F. tularensis* LVS in liquid infant formula and fruit juices (apple, mango, and orange). Inoculated samples were exposed to five different temperatures ranging from 47.5ºC to 57.5ºC. The pathogen was only able to survive in infant formula and apple juice at temperatures greater than 55ºC, and it did not survive in any of the tested matrices above 58ºC. The range of D-values for infant formula (12 s to 580 s) was much broader than the range of D-values for apple juice (8 s to 59 s) determined at temperatures between 50ºC and 57.5ºC. The ability of *F. tularensis* to survive for a longer period of time in infant formula was explained by the higher fat content of the beverage, which may have had a protective effect on the pathogen. Additionally, the shorter survival times in apple juice may have been due to no fat content and higher acidity. It has been previously suggested that foods with low pH inhibit the growth of certain Gram-negative foodborne pathogens (Day et al., 2008).

As a part of a study on high pressure processing inactivation of *F. tularensis* LVS in beverages, researchers assessed the survival of the pathogen during storage (Schlesser & Parisi,

2009). Inoculated ultra-high temperature pasteurized (UHT) skim milk, UHT 2% milk, pasteurized orange juice, and reduced-acid orange juice samples were sealed in pouches and stored for 24 h at 25 to 28°C. Results showed no reduction in bacterial levels in UHT skim milk, UHT 2% milk, and reduced acid orange juice. In regular orange juice, *F. tularensis* LVS counts were seen to decrease by 1 log after 4 h of incubation (Schlesser & Parisi, 2009).

The survival of *F. tularensis* has also been investigated in dehydrated infant formula stored for up to 12 weeks at three different atmospheric conditions (ambient air, dry, and nitrogen) (Day et al., 2009). In this study, two strains of freeze-dried *F. tularensis* (subspecies *holarctica* LVS and subspecies *novicida* (Utah 112)) were introduced into an infant formula suspension. This mixture was freeze-dried and used for the inoculation of powdered infant formula. The greatest decrease in viable cells was observed at ambient air storage, where bacterial counts were reduced by 4.15 log cycles in 12 weeks. In nitrogen and dry atmosphere, counts decreased by 3.72 and 3.37 log cycles, respectively. Based on these findings, *F. tularensis* demonstrated the longest survival time when stored in a desiccation chamber in dry atmosphere, whereas ambient air storage was associated with the most rapid bacterial die off. Although Day et al. (2009) concluded that during long-term storage in infant formula *F. tularensis* does not survive in large enough numbers to cause oropharyngeal or gastrointestinal tularemia, the possibility of infection through accidental inhalation of contaminated product was not excluded.

More recently, a study conducted by Sommers et al. (2013) focused on the inactivation of *F. tularensis* on foods and on food contact surfaces by utilizing ultraviolet light (UV-C). The research indicated that the application of UV-C at various doses (0.25 to 2.0 J/cm^2) to foods (chicken, beef steak, fish, frankfurters, and bratwurst) inoculated with the *F. tularensis* Utah-112 strain was as effective (or more effective) in inactivating the target organism compared to other

food borne pathogens. UV-C doses of 0.5 J/cm^2 and 1 J/cm^2 applied to food contact surfaces (stainless steel coupons and high density polypropylene) inoculated with *F. tularensis* containing food exudates achieved pathogen reductions of > 4 log CFU and > 7 log CFU, respectively (Sommers et al., 2013).

Viable But Nonculturable State of Bacteria

The so called viable but nonculturable (VBNC) state is a condition that non-sporeforming bacteria enter as a response to a stress factor such as depletion of nutrients, change in temperature, change in salinity or oxygen concentration, application of sanitizers or preservatives, or incubation at temperatures unfavorable for growth (Montville & Matthews, 2005; Oliver, 2009). During such state bacteria are not able to be detected via culture methods, however, they maintain some percentage of metabolic activity. Although some pathogens are unable to cause disease while in VBNC state, virulence is often preserved and disease can result after pathogens are resuscitated (Oliver, 2009). It has been proposed that some *Vibrio* species may be able to cause disease after resuscitation in the intestine after being ingested while in the VBNC state (Baffone et al., 2003).

Cappelier et al. (2005) investigated the virulence of four strains of *Listeria monocytogenes* after entering a VBNC state when maintained in microcosm water incubated at 20 and 4^{o}C. No viability was determined by culturing, but 10^4 active cells per mL were detected by direct microscopy and viable staining methods. VBNC cells were injected into a human adenocarcinoma cell line and a mouse model, with the conclusion that *L. monocytogenes* became avirulent upon losing its culturability. These authors suggested that *L. monocytogenes* can remain in the environment for extended periods in the VBNC state, but under the conditions of their study, these cells are non-pathogenic. However, it is still unknown if VBNC *L. monocytogenes*

regains pathogenicity after recovery (becoming culturable) or through some resuscitation protocol not investigated in their study.

Pawlowski et al. (2011) demonstrated that *Yersinia pestis*, the plague agent, could be induced to enter a VBNC state, whereby it could not be recovered by normal laboratory culturing procedures, upon exposure in low-temperature tap water for 21 days. However, they contended that the cells were still viable based upon cellular membrane integrity determinations, uptake and incorporation of radiolabeled amino acids, and protection of DNA from DNase I digestion. They were able to resuscitate a low number of VBNC cells in one (turbidity of 1/8 strength BHI broth at room temperature in an MPN assay format) of numerous culturing methods, but pathogenicity of resuscitated cells were not evaluated.

F. tularensis is a pathogen reported to maintain its metabolic activity but was not culturable in water samples at 5^{o}C or 25^{o}C (Gilbert and Rose, 2012). Additional investigations have shown that after entering a VBNC state as a result of extended nutrient starvation in cold water, *F. tularensis* LVS was no longer virulent to mice, nor were cells able to resuscitate once injected in mice (Forsman et al., 2000). Passage through animals has been shown to be a requirement of some VBNC pathogens such as *Vibrio cholerae* (Colwell et al, 1985) and *Campylobacteri jejuni* (Jones et al., 1991) to regain their ability to multiply in culture media. Various other strain-specific resuscitation requirements, some complex, have been reported for various pathogens (Forsman et al., 2000). Forsman et al. (2000) failed to resuscitate VNBC *F. tularensis* cells by using room temperature pre-incubation without nutrients, pre-incubation in spent nutrient media, extended incubation in various culture media, and passage through mice.

Not all scientists are convinced that bacterial cells actually enter into a VBNC state, based on the complexities of determining the differences between cultures containing culturable,

injured, and VNBC cells (Bogosian & Bourneuf, 2001). Others state that the detection of bacteria in VBNC state can be achieved by utilizing microscopic methods or molecular procedures. Fluorescent microscopy uses stains to differentiate between living and non-living cells, which typically appear in different colors (Fakrruddin et al., 2013). The commonly used LIVE/DEAD *Bac*Light bacterial viability kit relies on two stains to discriminate between dead (red fluorescence) and alive (green fluorescence) based on membrane integrity (Gunasekera at al., 2002; Makino et al., 2000; Nicolo et al., 2011). The metabolic activity of cells can also be measured with flow cytometry after staining with rhodamine 123 (Forsman et al., 2000). Molecular methods include both DNA and RNA-based techniques, however, only RNA-based methods, such as reverse transcriptase PCR, are able to discriminate between viable and nonviable cells (Fakruddin et al., 2013).

Chapter 3 - Recovery media for *Francisella tularensis*

Introduction

Francisella tularensis is a fastidious slow-growing organism, the culturing of which is difficult. Therefore, adding sulfhydryl compounds such as cysteine (or cystine), thiosulfate, or IsoVitaleX to media is essential for the growth of the organism. Thioglycollate, tryptic soy, and Mueller-Hinton broth supplemented with up to 2% IsoVitaleX provide desirable nutrients for the growth of the pathogen. Recovery and isolation are typically done on chocolate agar (CA), cysteine heart agar with 9% chocolatized blood (CHAB), buffered charcoaled yeast extract (BCYE), or Thayer-Martin (TY) agar (Chu & Weyant, 2003).

The presence of indigenous flora in field specimens and tissue samples could negatively influence the growth of fastidious *F. tularensis* and impede the process of detection. In such cases, supplementing the media with antibiotics may be useful for controlling the growth of the competing microorganisms. Petersen et al. (2004) studied the use of CHAB medium supplemented with five antibiotics (colistin, amphotericin, lincomycin, trimethoprim, and ampicillin) (CHAB-A) to recover *F. tularensis* from contaminated prairie dog tissue. Their results indicated that the recovery rate of the pathogen was improved by 81.1% by plating on CHAB-A compared to CHAB without any supplements.

In a later study (Petersen et al., 2009), CHAB was supplemented with another set of antibiotics (polymyxin B, amphotericin B, cefepime, cyclohexamide, and vancomycin) to successfully isolate *F. tularensis* from seaweed and seawater. Mueller-Hinton agar modified with colicin, amphotericin, lincomycin, trimethoprim, and ampicillin was used for confirmatory identification of *F. tularensis* LVS in lettuce (Day & Whiting, 2009).

Minimally processed fresh produce, such as pre-cut bagged lettuce, contains various levels of native microorganisms which tend to increase over the shelf life of the products (Jay et al., 2005). Therefore, the recovery of *F. tularensis* from such foods may be problematic, unless naturally present microbial loads are inhibited. The goal of this portion of the study was to develop a useful medium for the recovery of *F. tularensis* from packaged pre-cut iceberg and lettuce and raw ground beef.

Materials and Methods

Media Types

Three media types were used to evaluate the natural microbial load of iceberg lettuce and ground beef samples. Prepared plates with Chocolate II Agar (GC II Agar with Hemoglobin and IsoVitaleX™) (Becton Dickinson, Sparks, MD) were purchased from Fisher Scientific. Cystine Heart Agar (CHA, Becton Dickinson, Sparks, MD) enriched with 2% hemoglobin (Remel, Lenexa, KS) was prepared according to manufacturer's instructions. CHA enriched with 2% hemoglobin was supplemented with 7.5 mg colistin (Sigma-Aldrich, St. Louis, MO), 4 mg trimethoprim (Research Products International Corp., Mt. Prospect, IL), and 10 mg ampicillin (Fisher Scientific, Fair Lawn, NJ) per liter to prepare modified CHA (mCHA).

Sample Preparation for Background Flora Evaluation

Iceberg lettuce salad mix and ground beef (80-20) were purchased from Walmart in Manhattan, KS. Twenty-five grams of lettuce only (no carrots or cabbage from the mix were included) and ground beef were measured in Whirl-Pak™ sterile filter bags (Nasco, Fort Atkinson, WI). Filter bags were sealed and stored at 21±1°C for lettuce and ground beef, or 4°C for ground beef only, for 3 days.

Inoculum Preparation

Inoculum was prepared by transferring a single colony of *F. tularensis* SCHU S4 to 10 mL of BBL™ Fluid Thioglycollate Medium (THIO; Becton Dickinson, Sparks, MD) supplemented with 2% (vol/vol) BBL™ IsoVitaleX Enrichment Medium (THIO/S; Becton Dickinson) and incubated at 37ºC for 24 h. The overnight culture was centrifuged (Eppendorf 5804 R, Hamburg, Germany) at 3,500 × g for 10 min at 4ºC to generate a cell pellet. Supernatant was discarded, and the cell pellet was resuspended in sterile 0.1% peptone water (Becton Dickinson) to a final volume of 5 mL. Serial dilutions (1:10) were prepared in sterile peptone water, and 100 µl of the fifth and sixth dilutions were plated with Whitley Automated Spiral Plater 2 (WASP 2; Microbiology International, Frederick, MD) in duplicate on Chocolate II agar and mCHA. Plates were incubated at 37ºC for up to 4 days.

Sample Preparation for Inoculated Lettuce

Bags with iceberg lettuce salad mix from two different lots were purchased from Walmart in Manhattan, KS. Twenty-five grams of pre-cut iceberg lettuce (carrots and cabbage were not included) were measured into Whirl-Pak™ sterile filter bags (Nasco, Fort Atkinson, WI) that were double bagged in Stomacher 400 classic standard bags (Seward, West Sussex, UK) to prevent leakage during sample processing, and the bags were stored at 4ºC until inoculation. A volume of 500 µL from the second 1:10 dilution of the prepared inoculum (cell suspension) was transferred to each bag with pre-weighed lettuce to yield a concentration of approximately 1×10^4 cfu g^{-1} of lettuce.

Sample Processing (Background Flora Evaluation)

Fifty mL of sterile 0.1% peptone water were added to each sample bag, and samples were homogenized for 60 seconds (Stomacher R 400 Circulator; Seward, West Sussex, UK). During

the first run of the experiment, 50 µl of the homogenate were plated in duplicate on Chocolate II agar, CHA, and mCHA. During the second run, 100 µl of homogenate were plated in duplicate on all three media types. Plates were incubated for 3 days at 37ºC.

Sample Processing of Inoculated Lettuce

Twenty-five mL of sterile 0.1% peptone water were added to each bag, and the sample was homogenized for 60 seconds (Stomacher R 400 Circulator; Seward, West Sussex, UK). Five mL of the homogenate were filtered using disposable filter columns (Fisher Scientific, Pittsburg, PA) (to prevent clogging of spiral plater) and serially (1:10) diluted in 0.1% sterile peptone water. A 100 µl of the filtrate and dilutions were plated in duplicate onto mCHA using a spiral plater. Plates were incubated at 37ºC for up to seven days.

Results and Discussion

Three different media types were used to evaluate the volumes and appearance of indigenous microflora in iceberg lettuce (low protein/low fat food) and ground beef (high protein/high fat food) samples containing no artificially inoculated *F. tularensis*. To generate heavy loads of natural microbial populations (worst case scenario for being able to detect *F. tularensis*), lettuce and ground beef samples were stored at 21±1ºC for three days. To attempt suppression of the natural microflora in these two products, three antibiotics were added to CHA enriched with 2% hemoglobin. All Chocolate II agar plates contained heavy microbial loads beyond countable ranges (data not shown). CHA plates contained microbial volumes comparable to those on Chocolate II agar. Although some variability among samples was seen, the addition of antibiotics resulted in substantial reduction in the background microbial presence for both lettuce (Figure 3.1) and ground beef (Figure 3.2) samples.

Figure 3.1 Recovery of naturally present microbial flora from iceberg lettuce plated on Cystine Heart Agar enriched with 2% hemoglobin (left) and Cystine Heart Agar enriched with 2% hemoglobin supplemented with colistin, trimethoprim, and ampicillin (right).

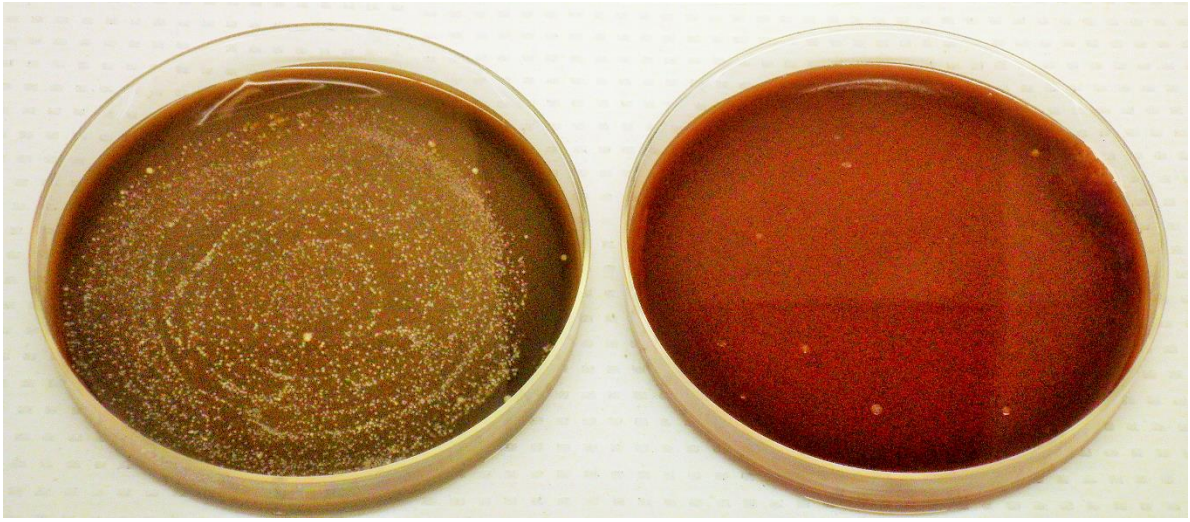
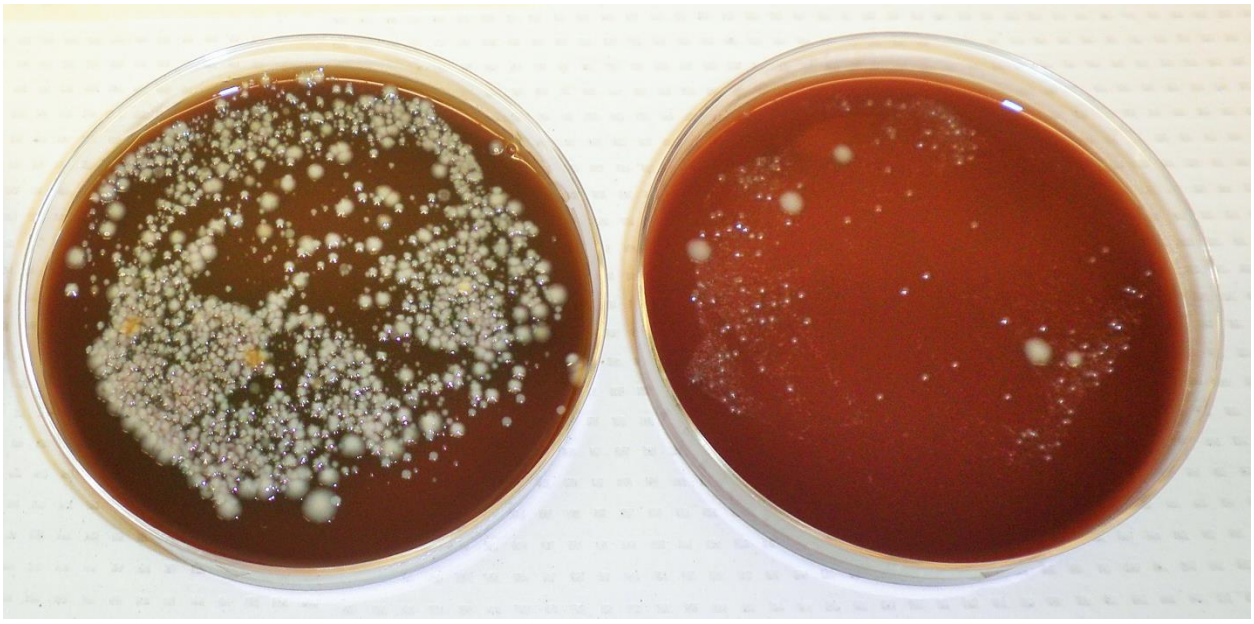


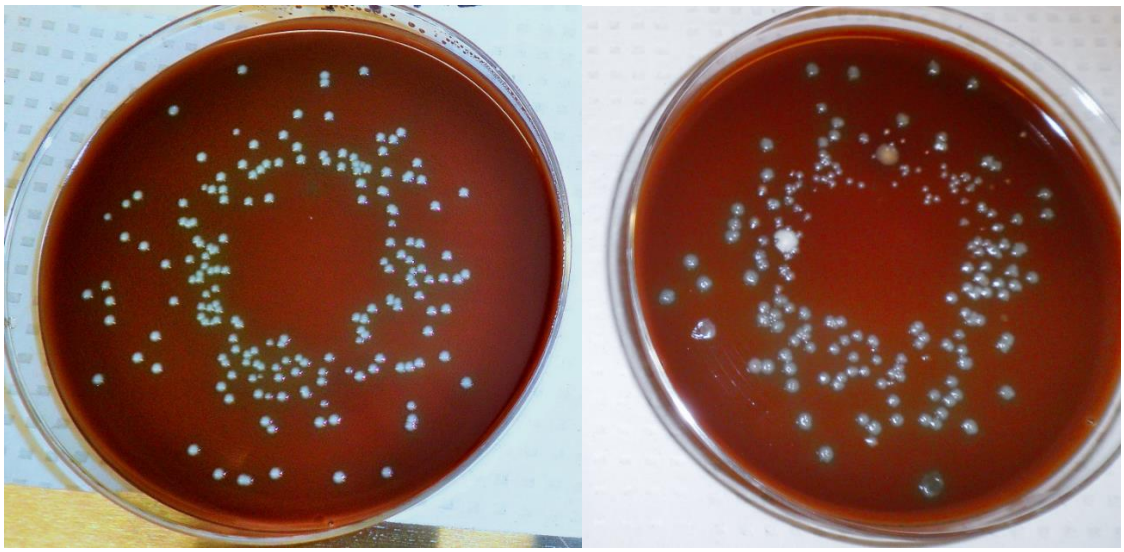
Figure 3.2 Recovery of naturally present microbial flora from ground beef plated on Cystine Heart Agar with 2% hemoglobin (left) and Cystine Heart Agar with 2% hemoglobin supplemented with colistin, trimethoprim, and ampicillin (right).



As described by Chu and Weyant (2003), *F. tularensis* has unique morphology when plated on CHAB; colonies are pearl-white to ivory with opalescent sheen and green tint. Green discoloration of the agar is also typical. These characteristics of *F. tularensis* allow for the

differentiation of the pathogen from other microorganisms that may be present on the growth medium. Figure 3.3 shows the morphology of the *F. tularensis* SCHU S4 strain on mCHA from a pure culture and from an inoculated lettuce sample. The colonies with green tint can easily be recognized among other microbial species from the lettuce sample.

Figure 3.3 Recovery of *Francisella tularensis* from pure culture (left) and inoculated iceberg lettuce (right) on Cystine Heart Agar with 2% hemoglobin supplemented with colistin, trimethoprim, and ampicillin.



Our observations indicated that *F. tularensis* colonies from pure culture typically developed color within three days of incubation. On some occasions, the color formation in *F. tularensis* colonies recovered from lettuce samples was delayed until day 5 or 6 of incubation. Therefore, it was determined that the incubation period for lettuce samples should be a minimum of 6 days. A delay in color formation for certain *F. tularensis* strains has been reported previously (Peterson et al., 2009) during an experiment focused on the recovery of the pathogen from environmental samples.

Conclusions

Observations suggest that the addition of colistin, trimethoprim, and ampicillin to CHA was found to be effective in reducing natural background flora counts from lettuce and ground beef samples (Figure 3.1 & Figure 3.2) while allowing the target *F. tularensis* to grow. *F. tularensis* colonies could be differentiated among other microbial colonies due to their opalescent sheen and green tint. Therefore, mCHA was deemed adequate for use in our studies on characterization of the growth or survival of *F. tularensis* in iceberg lettuce and raw ground beef.

Chapter 4 - Determination of the Ability of *Francisella tularensis* to Grow/Survive in Bagged Iceberg Lettuce

Introduction

Francisella tularensis, the causative agent of tularemia, has been classified as a Category A select agent because it is easily distributed, extremely infective, and capable of causing severe illness or death in humans (Dennis et al., 2001). Tularemia can be acquired through the ingestion of food and water contaminated with *F. tularensis* (CDC, 2013b). Introducing the agent intentionally into the food supply could be done as an act of bioterrorism or bio-warfare. Fresh produce is considered a high-risk commodity to military food systems because it is often produced and distributed locally in regions where military personnel may be targeted (Mara & McGrath, 2009). Fresh produce is very frequently consumed raw, and it does not undergo effective treatment for inactivation of any potential biological threats. Therefore, understanding and predicting the behavior of select agents in food matrices is of great importance for the safety of the military food supply in particular, but also of the national food supply. There are no published reports assessing the growth and/or survival of the *F. tularensis* in fresh produce. The goal of the current study was to investigate whether the highly virulent *F. tularensis* subsp. *tularensis* SCHU S4 strain would be able to grow or survive in pre-cut iceberg lettuce at two temperatures over a defined storage period.

Materials and Methods

Francisella tularensis subsp. *tularensis* strain SCHU S4 (FSC237), NR-643, was obtained from the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH (Manassas, VA). Stock cultures were stored at -80^{o}C, and working plates of BBL^{TM} Chocolate II agar were maintained at 4^{o}C for up to 2 weeks. The identity of the culture was

confirmed by performing real-time PCR using a Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.) instrument (BioFire Defense, Salt Lake City, UT). Due to high virulence of the strain, all handling procedures were performed under biosafety level 3 containment.

Inoculum Preparation

For the preparation of each inoculum, a single colony of *F. tularensis* SCHU S4 was transferred into 10 mL of BBL™ Fluid Thioglycollate Medium (THIO; Becton Dickinson, Sparks, MD) supplemented with 2% (vol/vol) BBL™ IsoVitaleX Enrichment Medium (THIO/S; Becton Dickinson) and incubated at 37ºC for 24 h. The overnight culture was centrifuged (Eppendorf 5804 R, Hamburg, Germany) at 3,500 × g for 10 min at 4ºC to generate a cell pellet. Supernatant was discarded, and the cell pellet was resuspended in sterile 0.1% peptone water (Becton Dickinson) to a final volume of 5 mL. Serial dilutions (1:10) were prepared in sterile peptone water. One hundred µl of the fifth and sixth dilutions were plated with a Whitley Automated Spiral Plater 2 (WASP 2; Microbiology International, Frederick, MD) in duplicate on Cystine Heart Agar enriched with 2% hemoglobin and supplemented with colistin, trimethoprim, and ampicillin (modified CHA (mCHA)). Plates were incubated at 37ºC for up to 4 days.

Sample Preparation

Packages (340 g each) of iceberg lettuce salad mix were purchased from Walmart, Manhattan, KS. To minimize the variability in lettuce, the contents of the packages were mixed well in a disinfected plastic tub. From the mixed lettuce, 25 g of pre-cut iceberg lettuce (carrots and cabbage were not included) were measured into Whirl-Pak™ sterile filter bags (Nasco, Fort Atkinson, WI) that were double bagged in Stomacher 400 classic standard bags (Seward, West Sussex, UK) to prevent leakage during sample processing, and the bags were stored at 4ºC until inoculation (within 24 hours). The surface pH of duplicate lettuce samples was obtained using an

Accumet^{TM} Excel XL60 instrument (Fisher Scientific, Pittsburg, PA). The water activity (AquaLab 4TEV, Decagon Devices, Inc., Pullman, WA) of duplicate samples was measured twice per sample at the time of sample preparation. A 150 g representative lettuce sample was collected and shipped overnight to IEH Warren Analytical Laboratory (Greeley, CO) for proximate analysis. A volume of 500 µL from the second 1:10 dilution of the prepared inoculum (cell suspension) was transferred to bags with pre-weighed lettuce to yield a *F. tularensis* concentration of approximately 1×10^4 cfu $g^{-1}$of lettuce. Bags were sealed, placed into sealed secondary containers and stored at either 6±1^{o}C or 23±1^{o}C for 10 and 5 days, respectively. The temperature of the storage environment was monitored using a temperature data logger (SM300/325, Dickson, Addison, IL). Microbial analyses were performed after each 24-h storage period. Sample bags were opened inside the biosafety cabinet (BSC) once a day to introduce fresh air to samples, re-sealed, carefully wiped with Spor-Klenz disinfectant (Steris, Mentor, OH), and returned to their assigned storage locations.

Sample Processing and Enumeration Procedures

Each bag of lettuce to be sampled was removed from its assigned storage location and transferred to the BSC. Twenty-five mL of sterile 0.1% peptone water were added to each bag, and the sample was homogenized for 60 seconds (Stomacher R 400 Circulator; Seward, West Sussex, UK). Five mL of the homogenate were filtered using disposable filter columns (Fisher Scientific, Pittsburg, PA) (to remove particulates and prevent clogging of spiral plater) and serially (1:10) diluted in 0.1% sterile peptone water. A 100 µl aliquot of the filtrate and each serial dilution were plated in duplicate onto mCHA using a spiral plater. Plates were incubated at 37^{o}C for up to seven days.

DNA Extraction, Risk Assessment, and PCR

All *F. tularensis* cultures used in this study were confirmed by polymerase chain reaction (PCR) assay prior to inoculations. Since PCR was to be conducted on the open benchtop (outside of the BSC), complete inactivation of *F. tularensis* was required. A single colony was transferred to 10 mL of THIO/S and incubated for 24 h at 37ºC. An IT 1-2-3 Platinum Path DNA Sample Purification Kit (BioFire Defense, Salt Lake City, UT) was used to extract DNA from the overnight liquid culture. A portion of the purified DNA sample (100 µl) was transferred to 10 mL of sterile THIO/S and incubated for 24 h at 37ºC. The remaining portion of the eluted DNA was stored at -20ºC for PCR analysis. To perform a laboratory biological risk assessment, 100 µl from the incubated THIO/S tube were plated in triplicate onto Chocolate II agar. Plates were incubated at 37ºC and checked for any bacterial growth on days 2, 3, and 4. Absence of growth after 4 days indicated that the *F. tularensis* culture was completely inactivated by the DNA extraction protocol and could be analyzed by PCR on the open laboratory benchtop.

As part of the preparation process for the PCR analysis, the extracted DNA was mixed with a Freeze-Dried Reagent Kit for the detection of *F. tularensis* (Tularemia, Target 1) (BioFire Defense, Salt Lake City, UT). Vials with negative and positive control reagents were rehydrated with 40 µl reagent grade water. The extracted DNA (40 µl) was added to a vial for unknown samples. Nineteen µl of each mixture were transferred to the kit's capillary tubes in duplicate. After capping and centrifuging, the capillary tubes were loaded into the carousel of a R.A.P.I.D. 9200 instrument. PCR tests were conducted utilizing parameters specified by the instrument's manufacturer.

After sample processing and incubation, PCR tests were also conducted on colonies that did not have the typical *F. tularensis* appearance to confirm their identities as negative for *F. tularensis*.

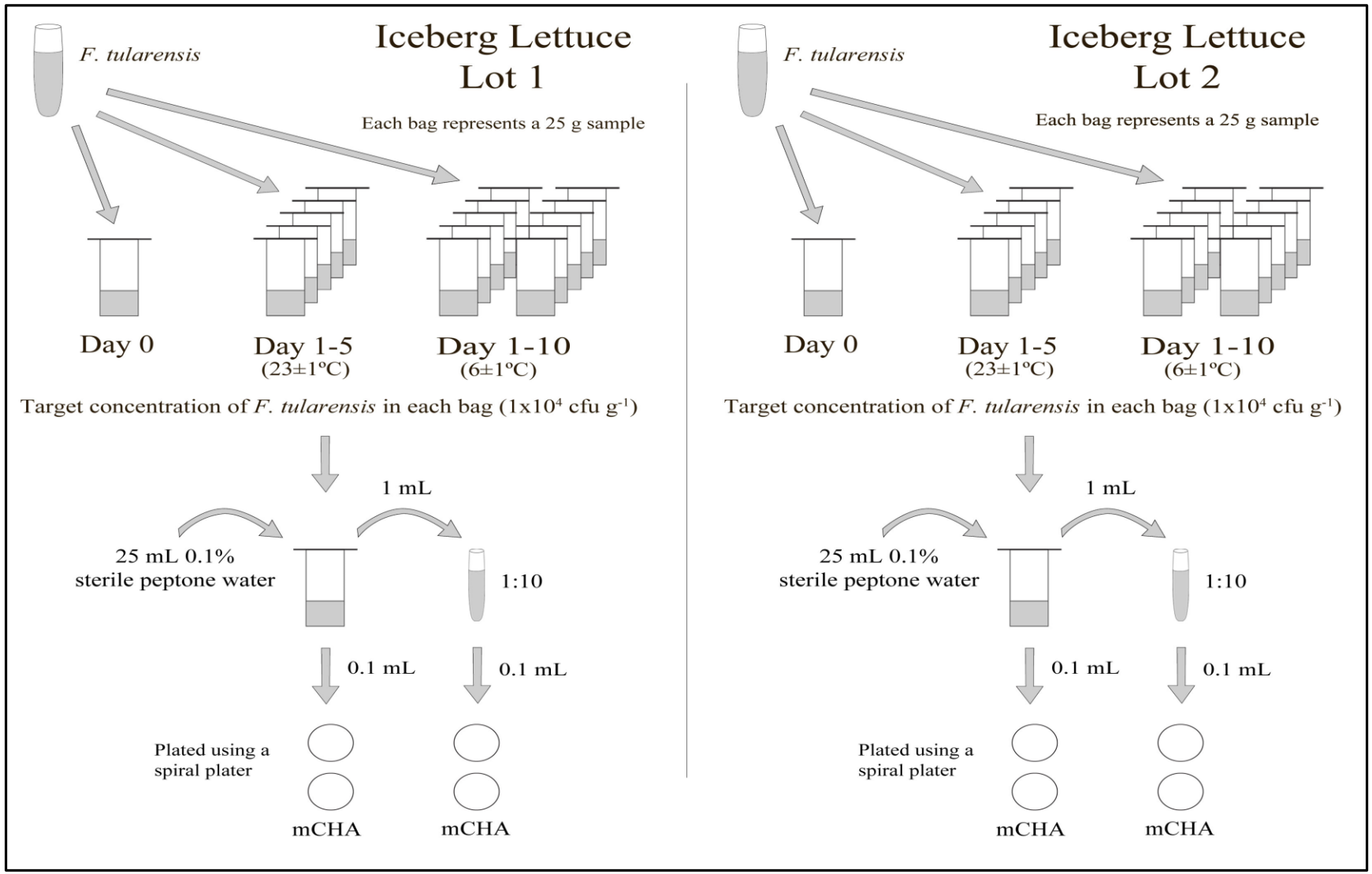
Statistical Analysis

The current study followed a randomized complete block design (RCBD) replicated over 4 experimental runs (Figure 4.1), with runs being treated as a random factor. Within each RCBD, manufacture lots of the 340 g packaged iceberg lettuce were recognized as a random blocking factor with two different expiration dates. The fixed treatment factors included a 2-level storage temperature in 23±1^{o}C and 6±1^{o}C, and a 4-level time factor in 1-4 days. The factors were arranged in a 2 × 4 factorial structure. The levels of *F. tularensis* bacterial counts (presented in $\log_{10}$ cfu g^{-1}) for each treatment combination was measured on a subsample of 25 g lettuce extracted from each lot within each run. Since the response variable (*F. tularensis* counts) was not normally distributed, a generalized linear model with Gamma distribution and log link function was applied to analyze the data, as such distribution can tolerate skewness. The model was implemented using the GLIMMIX procedure of SAS version 9.3 (SAS Institute, Cary, NC). Tukey adjustment was used to protect the overall Type I error rate in pairwise comparisons.

Results and Discussion

In this study, the survival of *F. tularensis* in lettuce was monitored for 5 days after inoculation at 23±1^{o}C and for 10 days after inoculation at 6±1^{o}C. However, viable *F. tularensis* counts were not detected on mCHA plates by the fourth day of storage at either storage temperature. Therefore, only data obtained from days 1 through 4 were used in the statistical analysis. The detection limit of *F. tularensis* by plating was 1 $\log_{10}$ cfu g^{-1}.

Figure 4.1 Inoculation of iceberg lettuce with *Francisella tularensis* and sample analysis (represents one experimental run).

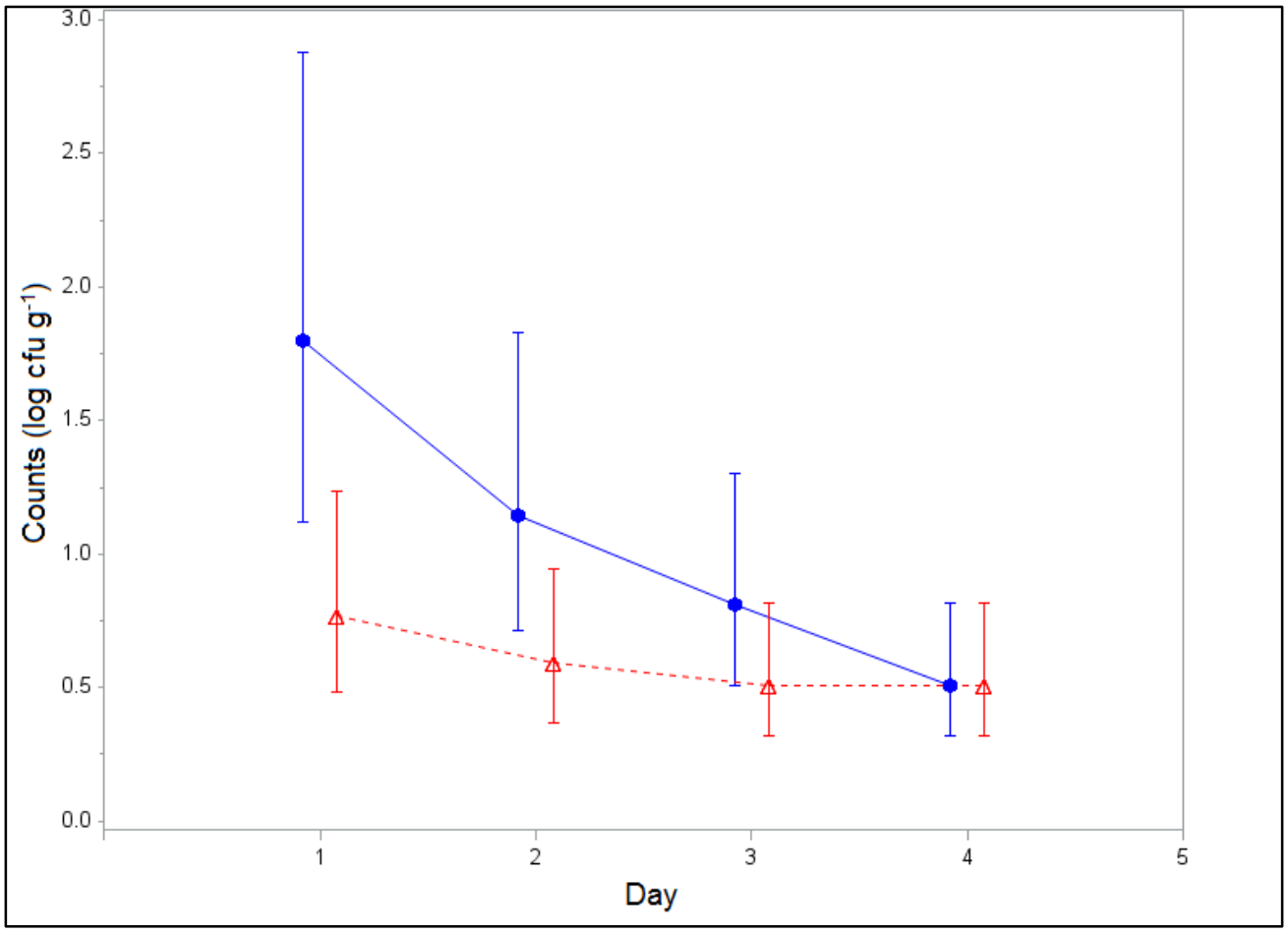


For the purposes of this statistical analysis, samples with no *F. tularensis* growth on mCHA were assigned a value of 0.5 $\log_{10}$ cfu g^{-1} (half of the analytical detection limit).

Using a significance level of 0.05, there was no evidence of an interaction effect between temperature and time (P=0.0744), suggesting that the effect of temperature on the measurement (bacterial levels) does not depend on the time. However, the main effects were found to be significantly different from each other with P=0.0002 for both temperature and time.

Although the temperature × day interaction was not significant, the presence of any trend in the survival behavior of this highly virulent organism was worth investigating. Figure 4.2 shows the survival course of *F. tularensis* at 6±1°C and 23±1°C over a 4-day storage period. *F. tularensis* levels were significantly different among temperature levels at all days except day 4. More specifically, enumeration on mCHA after 24 h of incubation indicated that *F. tularensis* counts were significantly different between storage temperatures (p=0.0011), with lower counts observed in lettuce stored at 23±1°C (0.77 log cfu g^{-1}) compared to lettuce stored at 6±1°C (1.80 log cfu g^{-1}). Similarly, after 48 h, lettuce stored at 23±1°C harbored a smaller population of viable *F. tularensis* (0.59 log cfu g^{-1}) than lettuce maintained at 6±1°C (1.14 log cfu g^{-1}) (P= 0.0074). Finally, a significant difference (P=0.049) in *F. tularensis* counts between temperatures was observed 72 h after inoculation. On the fourth day of storage, *F. tularensis* was no longer recovered by plating on mCHA from any of the lettuce samples.

Figure 4.2 Plot of mean estimates of *Francisella tularensis* counts in log scale for temperature*time. Shown are lettuce samples stored at 6±1°C (blue circles) and lettuce samples stored at 23±1°C (red triangles). Error bars indicate 95% confidence limits (n=8).



Results from the proximate analysis, pH and water activities measurements are presented in Table 4.1.

Table 4.1 Characteristics of pre-cut packaged iceberg lettuce prior to inoculation with *Francisella tularensis* (data presented in ranges).

Parameter	Measured Value
pH[†]	5.11 – 6.95
Water Activity[†]	0.998 – 0.999
Ash (%)*[§]	0.25 – 0.43
Crude Fat (%)*[§]	<0.8
Moisture (%)*[§]	95.49 – 96.53
Protein (%)*[§]	0.72 – 1.16

* Test Method: Ash = AOAC 920.153, Fat = AOAC 945.44, Moisture = AOAC 950.46, Protein = AOAC 990.03/992.23
[†] n=16; [§] n=8.

Deterioration of lettuce stored at 23±1ºC occurred more rapidly compared to lettuce stored at 6±1ºC. At the higher temperature, browning of the lettuce was observed on day 1 of incubation and by day 2, slime was starting to form on edges rendering lettuce no longer fit for consumption. Background microbial flora (colonies that did not appear as typical *F. tularensis* and were confirmed by PCR as *F. tularensis* negative) was observed in greater numbers on plates from samples stored at 23±1ºC as early as one day of storage. As described by Jay et al. (2005), minimally processed produce tends to demonstrate a substantial concentration of spoilage microorganisms even after application of antimicrobials. Data collected in Canada suggested that ready-to-eat chopped lettuce contained initial native microbial loads of 4.85 log cfu g^{-1}, and these numbers were seen to increase to 5.63 log cfu $g^{-1}$by day 4 of storage at 4ºC. Salad mix had an aerobic plate count of 5.35 log cfu g^{-1}on day 0 and 6.05 log cfu g^{-1}four days later (Jay et al., 2005).

Several studies have suggested that the indigenous microbial flora in fresh and minimally processed produce may compete or have inhibitory effect on the growth of pathogenic bacteria (Francis & O'Beirne, 1998; Johnston et al., 2009; Schuenzel & Harrison, 2002). In model lettuce media, *L. innocua* (used as a substitute for *L. monocytogenes*) counts have been reduced when the bacterium was co-cultivated with total lettuce microflora, lactic acid bacteria, and several *Enterobacter* spp. (Francis & O'Beirne, 1998). Additionally, background flora isolates from each step of the processing of bagged pre-cut iceberg lettuce have had an inhibitory effect on *Escherichia coli* O157:H7 (Johnston et al., 2009). The antimicrobial properties of background flora isolates from iceberg lettuce have also been described by Schuenzel and Harrison (2002). These isolates have demonstrated interference with the growth profiles of pathogens such as *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* Montevideo and *Staphylococcus aureus*. The inhibition of microbial growth is typically accomplished by producing acids, antimicrobial compounds such as bacteriocins, hydrogen peroxide, or by competing for nutrients (Francis & O'Beirne, 1998; Johnston et al., 2009).

Previous research has shown that the growth of *F. tularensis* can be inhibited by the naturally present flora in carcass tissue (Petersen et al., 2004) and water samples (Humrighouse et al., 2011). It has also been suggested that *F. tularensis* does not compete well with indigenous flora in foods (Day & Whiting, 2009).

The goal of the current study was to investigate the growth/survival profiles of *F. tularensis* in pre-cut bagged lettuce at two different temperatures over a defined storage period. Results suggest that target pathogen microbial loads continuously decreased over time and no viable *F. tularensis* cells were observed on day 4 of storage at either temperate. Decrease in quality of lettuce was seen to occur more rapidly for samples stored at 23±1^{o}C compared to those

maintained at 6±1°C. Consequently, higher concentrations of natural microbial flora were observed on mCHA. The objectives of this study did not include determination of impacting factors on *F. tularensis* growth or survival in lettuce, but only focused on determining how long the pathogen could survive if it were to be inoculated into fresh lettuce. However, the rapid decrease in *F. tularensis* counts observed in raw lettuce suggests that *F. tularensis* does not compete well with the background spoilage flora of raw lettuce. The more rapid decrease in *F. tularensis* counts at higher temperatures (23±1°C) is likely due to the more rapid proliferation of indigenous flora in lettuce which suppresses the growth of the target pathogen and inhibits its detection.

Conclusions

Highly virulent *F. tularensis* subsp. *tularensis* SCHU S4 strain was unable to grow but was able to survive for up to 3 days on pre-cut bagged iceberg lettuce stored at 6±1°C when initially inoculated at a level of 10^4 cfu g^{-1}, as detected by direct plating on mCHA. This level of inoculation was chosen to be able to determine population decay, but also growth (if occurring) of *F. tularensis* during storage. Storage at 23±1°C was associated with a more rapid decline in pathogen populations, as well as more rapid deterioration of the food product. The infectious dose of *F. tularensis* via the oral ingestion route is considered to be relatively high (consumption of 10^6-10^8 viable cfu (PHAC, 2011)). If a person consumed a typical amount of product (i.e. dinner salad or topping of a taco) that had been intentionally inoculated with a concentrated *F. tularensis* inoculum, it is possible that that person would be at significant risk for serious health consequences. Given that raw lettuce is very perishable and would oftentimes be provided to the customer within minutes to hours (if on a salad bar) or a few days (if commercially packaged as shredded lettuce or salad mix), the risk of adequate levels of *F. tularensis* being present to cause

illness must be seriously considered, especially, if that contamination was added to a small to moderate volume of raw lettuce using a concentrated inoculum (i.e. 10 mL of culture medium that had grown to 10^{7-9} cfu mL^{-1}). The other consideration would be at what point in storage time did the contamination event occur (i.e. was the pathogen introduced to lettuce on a salad bar or to lettuce in a kitchen where a meal is being prepared, or was the contamination added at a commercial lettuce processing facility where it would have to survive longer on the product while it was distributed to the consumer).

Since *F. tularensis* did not multiply in raw lettuce at either storage temperature, further research should investigate the length of survival of *F. tularensis* (considering accepted infectious dose calculations) when a high inoculum level is applied prior to chilled (long-term) storage.

Chapter 5 - Evaluation of the Ability of *Francisella tularensis* to Grow/Survive in Food Matrices with Variable Compositional Profiles

Introduction

The ingestion of food and water contaminated with *F. tularensis* can lead to the development of oropharyngeal or gastrointestinal tularemia. The oropharyngeal form may develop into the more deadly pneumonic form of the disease (CDC, 2011), and gastrointestinal tularemia may result in serious bowel ulcerations (Day & Whiting, 2009; Ellis, 2002). Multiple instances of naturally occurring outbreaks of oropharyngeal tularemia have been reported (Chitadze et al., 2009; Helvaci et al., 2000; Kantardjiev et al., 2006; Reintjes et al., 2002). For instance, a large outbreak in Kosovo (1999-2000) resulted in 327 confirmed tularemia cases after consumption of rodent-contaminated food and water (Reintjes et al., 2002). Similarly, rodents transmitted the infection to food and water during an outbreak in Bulgaria between 1998 and 2003 (Christova et al., 2004). *F. tularensis* could be intentionally introduced into to food as an act of bioterrorism. The ability of various foods to support the growth or the survival of bioweapon agents could make such foods likely vehicles for the dissemination of those agents (Kennedy & Busta, 2007). Understanding and predicting the behavior of select agents in various food matrices is of great importance for ensuring the safety of military foods, and similarly, the national food supply. The purpose of this study was to evaluate the ability of highly virulent *F. tularensis* subsp. *tularensis* SCHU S4 strain to grow or survive in nine food matrices with different composition profiles at three different temperatures over a defined storage period.

Materials and Methods

Francisella tularensis subsp. *tularensis* strain SCHU S4 (FSC237), NR-643, was obtained from the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH (Manassas, VA). Stock cultures were stored at -80°C, and cultures on working plates of BBL™ Chocolate II agar (GC II Agar with Hemoglobin and IsoVitaleX™) (Becton Dickinson, Sparks, MD) were maintained at 4°C for up to 2 weeks. The identity of the culture was confirmed by performing real-time PCR using a Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.) instrument (BioFire Defense, Salt Lake City, UT). Due to the high virulence of the strain, all experimental procedures were performed under biosafety level 3 containment, and all open manipulations were performed in a Class II biosafety cabinet.

Inoculum Preparation

For the inoculum preparation, a single colony of *F. tularensis* SCHU S4 was transferred into 10 mL of BBL™ Fluid Thioglycollate Medium (THIO; Becton Dickinson, Sparks, MD) supplemented with 2% BBL™ IsoVitaleX Enrichment Medium (THIO/S; Becton Dickinson) and incubated at 37°C for 24 h. The overnight culture was centrifuged (Eppendorf 5804 R, Hamburg, Germany) at 3,500 × g for 10 min at 4°C to generate a cell pellet. Supernatant was discarded, and the cell pellet was resuspended in sterile 0.1% peptone water (Becton Dickinson) to a final volume of 5 mL. Serial dilutions (1:10) were prepared in sterile 0.1% peptone water and 100 µl of the fifth and sixth dilutions were plated using a Whitley Automated Spiral Plater 2 (WASP 2; Microbiology International, Frederick, MD) modified Cystine Heart Agar (mCHA, Cystine Heart Agar enriched with 2% hemoglobin and supplemented with colistin, trimethoprim, and ampicillin) to determine inoculum concentration. Plates were incubated at 37°C for 4 days.

A *F. tularensis* inoculation level of 10^3 cfu g^{-1} or mL^{-1} of food product was targeted. Please see chapter 2 for a more in-depth discussion of *F. tularensis* protocols utilized in these studies.

Food Matrices

As agreed upon through discussions with the sponsor (U.S. Army Natick Soldier Center; Natick, Massachusetts), nine food matrices with various compositional profiles were investigated. Four raw produce products (red delicious apples, green bell pepper, shredded iceberg lettuce, and strawberries) and five animal-based foods (whole liquid eggs, boneless ham steak, beef hot dogs, 80-20 ground beef, and 2% UHT milk) were selected for use in this study. For ground beef only, 5-g portions were placed in trimmed plastic pouches (6.5 cm × 25.5cm) (BUNZL, Kansas City, MO) and treated with ionizing radiation (Food Technology Service, Inc., Mulberry, FL) to reduce natural microbial flora. All irradiated ground beef samples were stored at -20^{o}C until use.

Sample Preparation

Whole liquid eggs were purchased from the K-State Food Stores. The remaining food items were obtained from Walmart in Manhattan, KS. Five grams or milliliters of each product were measured into trimmed plastic pouches (6.5 cm ×25.5 cm; BUNZL, Kansas City, MO). Samples collected from apples, bell peppers, strawberries, and hot dogs were prepared in a way to primarily test the original external surface area of these products. Frozen pouches with irradiated ground beef were allowed to thaw at 4^{o}C. The pH of duplicate food samples was obtained using an Accumet^{TM} Excel XL60 instrument (Fisher Scientific, Pittsburg, PA). The water activity (AquaLab 4TEV, Decagon Devices, Inc., Pullman, WA) of duplicate samples was measured twice per sample. A representative sample from each product was collected and shipped overnight to IEH Warren Analytical Laboratory (Greeley, CO) for proximate analysis.

All samples were inoculated to achieve a target concentration of approximately 3 $\log_{10}$ cfu mL^{-1} or g^{-1}. Filled pouches were flattened to expunge the maximum amount of air possible from the packet, then each pouch was triple heat sealed using an Impulse sealer (TechnoPACK, Miami, FL) and was placed into a circulating water bath (Haake A 25, Thermo Scientific, Waltham, MA) set at either 37°C, 21°C, or 4°C. Water baths containing the sealed sample pouches were contained within a benchtop negative pressure containment enclosure (bioBUBBLE, Inc., Fort Collins, CO) to protect against any sample pouch potentially leaking and releasing *F. tularensis* into the unprotected laboratory environment.

Sample Processing and Enumeration Procedures

Inoculated sample pouches were removed from the circulating water baths at designated sampling times, thoroughly wiped with a Spor-Klenz disinfectant (Steris, Mentor, OH), and transferred in a sealed secondary container to the BCS for analysis. Each pouch was opened and the food contents were transferred into a sterile filtered stomacher 80 bag that was placed inside of a secondary bag to prevent potential sample leakage during processing. Ten mL of sterile 0.1% peptone water were added to each bag, and the samples were homogenized for 60 seconds (Stomacher R 80 Biomaster; Seward, West Sussex, UK). Five mL of the homogenate were filtered by using disposable filter columns (Fisher Scientific, Pittsburg, PA) (to prevent particulate clogging of the spiral plater) and serially (1:10) diluted in 0.1% sterile peptone water. Aliquots (100 µl) of the filtrate and selected dilutions (based on anticipated microbial loads) were plated in duplicate onto mCHA using a Whitley Automated Spiral Plater 2. Plates were incubated at 37°C for 6 days.

The study was repeated four times for samples incubated at 37 and 21°C, and samples were processed once every 24 h over a 3-day storage period. Samples stored at 4°C were analyzed on a weekly basis over a 2-week period; this portion of the study was repeated twice.

Results and Discussion

The ability of *F. tularensis* to grow or survive in nine food matrices with various compositional profiles was tested at three different temperatures. Results obtained after enumeration on mCHA are presented in Table 5.1. The recovery rates for inoculated *F. tularensis* varied widely by temperature of incubation and by product. Proximate analyses, pH, and water activity measurements (Table 5.2) of all food matrices tested were determined prior to inoculation to evaluate whether such characteristics affect the growth or survival of *F. tularensis*.

The enumeration of *F. tularensis* on mCHA resulted in highly variable and inconsistent recoveries from the nine food matrices. Inconsistencies in the recovery of specific strains of *F. tularensis* (LVS and NIB B 3-8 strains) from food matrices has been reported previously (Sommers et al., 2013). Given the variability of the results shown in Table 5.1, trends regarding the growth or survival of *F. tularensis* related to time of storage could not be established. With this being the case, the data in this chapter are presented to help generalize our observations across the food matrices with respect to the likelihood that they support growth and/or survival of *F. tularensis*, or that a *F. tularensis* population would likely decline over storage under the defined storage conditions. To conclude anything further, this generalization will require additional inoculated storage studies focused towards confirming the current recovery observations and understanding why recovery of *F. tularensis* from diverse food matrices is highly unpredictable.

Table 5.1 Recovery of *Francisella tularensis* on mCHA from nine food matrices after inoculation and storage at 37°C, 21°C, and 4°C. Data shows detection frequencies across all sampling times within storage temperature.

Food Matrix	Culture recovery (%) at:		
	37°C	21°C	4°C
Apple	1/16 (6.25)	4/16 (25)	3/6 (50)
Irradiated ground beef	1/16 (6.25)	2/15 (13.3)	1/6 (16.7)
Bell Pepper	6/16 (37.5)	5/16 (31.25)	2/6 (33.3)
Whole Liquid Eggs	11/16 (68.75)	6/16 (37.5)	1/6 (16.7)
Ham Steak	7/16 (43.75)	6/16 (37.5)	3/6 (50)
Hot Dog	2/16 (12.5)	1/16 (6.25)	0/6 (0)
Shredded lettuce	1/16 (6.25)	2/16 (12.5)	0/6 (0)
Milk (2% UHT)	3/16 (18.75)	4/16 (25)	2/6 (33.3)
Strawberry	3/16 (18.75)	3/16 (18.75)	2/6 (33.3)

Enumeration of the inoculum solution confirmed that a *F. tularensis* concentration of 7.5 to 8.1 log cfu g^{-1} was achieved; and by inoculating a calculated dilution of this inoculum into a defined weight or volume of product, our target of 10^3 cfu g^{-1} or mL^{-1} would have been attained. However, across all replications and across all nine food matrices evaluated, recoveries showed no consistency and no trends. Even on D 0 samplings (which occurred approximately 30 minutes after inoculation), in several instances no *F. tularensis* was recovered on the lowest dilution plates, but would be detected on a later sampling. Thus, general observations will be presented.

For 4°C stored samples, *F. tularensis* was sporadically recovered from all matrices tested except hot dogs and shredded lettuce during the 2-week storage period. In the first replication at 4°C, viable *F. tularensis* was only observed at or after week 1 in apple, UHT milk, and strawberry matrices. However, in replication 2 *F. tularensis* was recovered as long as week 2 in apple, bell pepper, egg, ham and UHT milk; once again demonstrating the unpredictable nature of recoveries. *F. tularensis* is not considered psychrotrophic, thus, growth in any matrix was not

expected. However, when recovered at one or two weeks of 4°C at levels of 1.7-3.0 log cfu g^{-1} or mL^{-1}, it must be considered possible that the organism can tolerate chilled storage for two weeks or longer in a variety of food products. Additionally, at this temperature it is likely that the growth/survival of *F. tularensis* was not inhibited by indigenous flora, the proliferation of which was possibly delayed. Further, several tested products have been either pasteurized (liquid whole eggs and UHT milk) or irradiated (ground beef) to reduce the presence of naturally present bacteria that may negatively affect the detection of *F. tularensis*.

Liquid whole eggs and ham steak presented the highest frequencies of recovery of viable *F. tularensis* at 21 and 37°C storage, with enumerated counts often equaling or exceeding the targeted 3 log cfu g^{-1} level. The levels of *F. tularensis* in liquid eggs obtained throughout the study were repeatedly greater than the initial concentration of the pathogen immediately after inoculation, reaching 7.7 log cfu g^{-1} by the Day 1 of sampling at 37°C and 5.4 by Day 2 at 21°C. *F. tularensis* counts recovered on days 1 and 2 of storage in ham steak were typically 2-3 log cfu g^{-1}, approximating the initial inoculation level, at 21 and 37°C. Liquid whole eggs are characterized by pH of 7.33 to 7.76, high protein content (12.19 - 13.01%), and due to pasteurization lack heavy loads of naturally present bacteria that may compete for nutrients with *F. tularensis*. Ham has a slightly lower pH (6.11-6.36) and is also rich in protein (16.39 - 17.92%) (Table 5.2).

Strawberry was the food matrix with the lowest pH (Table 5.2) among the nine food products used in this experiment. Recovery of the target pathogen was obtained in only one sample per tested temperature at or after the Day 1 storage sampling. The role of pH on the survival of *F. tularensis* has been suggested previously. For instance, Schlesser and Parisi (2009) observed 1 log reduction of *F. tularensis* LVS (live vaccine strain) counts in pasteurized orange

juice with pH of 4.0 in the first 4 h of incubation at 25 to 28ºC. The investigators did not observe such change in microbial loads within 24 h in reduced acid orange juice (pH = 4.2) or UHT milk (pH = 6.7). Day et al. (2008) have also proposed that low pH may negatively impact the survival of *F. tularensis*.

It is not clear what caused the inconsistencies in recovery rates among food matrices, however, the possibility that the target organism may have entered a viable but non-culturable (VBNC) state should not be ruled out. Non-sporeforming bacteria use VBNC state as a survival strategy in response to a stress factor. Nutrient limitation, incubation at temperatures not favorable for growth, change in salt or oxygen concentration, change in temperature, or exposure to preservatives or sanitizers are several examples of stresses that may cause a microorganism to enter VBNC state (Montville & Matthews, 2005; Oliver, 2009). Gilbert and Rose (2012) were unable to culture *F. tularensis* from water samples at 5ºC or 25ºC, while organisms were confirmed as viable, therefore speculated that *F. tularensis* was in VBNC state. Culture was recovered at 8ºC. The VBNC state of several microorganisms has been reported to occur in foods. One study suggested that *E. coli* O157:H7 and *Salmonella* lost their culturability 24 hours after storing pasteurized grapefruit juice at 4ºC (Nicolo et al., 2011). Another study determined that *E. coli* and *Pseudomonas putida* were not culturable but remained metabolically active after pasteurization (Gunasekera et al., 2002). Some bacterial species have been shown to maintain their virulence after recovering from VBNC state. For instance, enterohemorrhagic *E. coli* O157 in a VBNC state found in salted salmon roe has been associated with a human outbreak in Japan; cultures resuscitated from VBNC state were fatal to mice (Makino et al., 2000). Forsman et al. (2000) demonstrated that after *F. tularensis* LSV entered VBNC state, the organism was no

Table 5.2 Characteristics of nine food matrices prior to inoculation with *Francisella tularensis* (data presented in ranges).

Matrix[a]	Aw*	pH* (flesh/liquid)	pH*(skin/ outer surface)	Ash[b§] (%)	Fat[b§] (%)	Moisture[b§] (%)	Protein[b§] (%)	Nitrate[b§] (ppm)	Nitirite[b§] (ppm)	Salt[b§] (%)
A	0.976 - 0.989	3.92 - 4.74	4.67 - 5.92	0.22 - 0.35	<0.8	87.32 - 89.48	<0.5 - 0.75	n/a	n/a	n/a
BP	0.984 - 1.003	5.33 - 6.16	5.35 - 6.29	0.42 - 0.58	<0.8	74.45 - 94.83	0.81 - 0.97	n/a	n/a	n/a
E	0.998 - 1.001	7.33 - 7.76	n/a[c]	1.13 - 1.36	9.09 - 9.84	75.59 - 76.33	12.19 - 13.01	n/a	n/a	n/a
IRR GB	0.992 - 0.994	5.50 - 5.70	n/a	0.90 - 0.95	16.38 - 17.72	63.10 - 64.97	18.61 - 19.71	n/a	n/a	n/a
H	0.970 - 0.986	6.11 - 6.36	n/a	3.09 - 3.62	1.26 - 3.04	76.43 - 77.78	16.39 - 17.92	<10	<11 - 41	1.92 - 2.19
HD	0.936 - 0.973	5.90 - 6.36	5.85 - 6.39	3.33 - 3.47	26.51 - 30.31	47.95 - 51.86	8.75 - 10.95	<10	<11 - 12	2.01 - 2.18
L	0.996 - 0.999	6.57 - 7.25	n/a	0.37 - 0.43	<0.8	96.27 - 96.43	1.06 - 1.40	n/a	n/a	n/a
M	0.997 - 0.999	6.70 - 7.01	n/a	0.56 - 0.76	1.70 - 2.07	89.22 - 89.33	3.08 - 3.28	n/a	n/a	n/a
SB	0.989 - 0.998	3.54 - 4.15	3.36 - 4.47	0.29 - 0.48	<0.8	91.01 - 92.91	0.67 - 0.95	n/a	n/a	n/a

[a] A = red delicious apple, BP = green bell pepper, E = whole liquid egg, IRR GB – irradiated ground beef (80/20), H = boneless ham, HD = beef hot dog, L = iceberg shredded lettuce, M = 2% UHT milk, SB = strawberries

[b] Test Method: Ash = AOAC 920.153, Fat = Crude Fat (AOAC 960.39) and Acid Hydrolysis (AOAC 945.44), Moisture = AOAC 950.46, Protein = AOAC 990.03/992.23/992.15, Nitrate = AOAC 935.48, Nitrite = AOAC 973.31, Salt = AOAC 971.27

[c] n/a = not applicable

*n=8, except irradiated ground beef (n=4) and 2% UHT milk (n=6)

[§] n=4, except irradiated ground beef (n=2) and 2% UHT milk (n=3).

longer virulent to mice. Additionally, pathogen cells were unable to resuscitate after injection in mice. However, it is not clear whether resuscitation can occur once favorable conditions are provided (Forsman et al., 2000). Also, it is not known if such loss of virulence is typical for more virulent *F. tularensis* strains.

Conclusions

Among the nine tested food products, *F. tularensis* was able to grow in pasteurized liquid whole eggs only. Therefore, liquid eggs may be considered a high-risk food in which the pathogen can readily proliferate, particularly at 21 and 37ºC. With the exception of ham, liquid whole eggs, and bell pepper, the pathogen was detected infrequently throughout the duration of the study. Very low recovery rates were obtained for shredded lettuce, hot dogs, and ground beef. Future research efforts could focus on monitoring the growth/survival of *F. tularensis* at shorter intervals (i.e. 4 or 6 h) to better understand the dynamics of the pathogen in defined food matrices. Additionally, appropriate testing methods could be implemented to test whether bacterial cells in fact enter VBNC state and determine if such foods are potential vehicles for the distribution of *F. tularensis* throughout the food supply.

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Appendix A - Raw data from the study "Evaluation of the Ability of *Francisella tularensis* to Grow/Survive in Food Matrices with Variable Compositional Profiles

Table A.1 Recovery of *F. tularensis* (in log cfu g^{-1} or mL^{-1}) from nine* food matrices (37°C)

Food/Temp	Day	Rep1	Rep 2	Rep 3	Rep 4
App 37	0	2.38	0[a]	0	0
App 37	1	0	0	0	0
App 37	2	0	0	0	0
App 37	3	0	0	0	0
Irr B 37	0	1.88	0	0	0
Irr B37	1	0	0	0	0
Irr B 37	2	0	0	0	0
Irr B 37	3	0	0	0	0
BP 37	0	1.78	0	0	3.54
BP 37	1	0	3.07	0	2.97
BP 37	2	0	0	1.48	1.78
BP 37	3	0	0	0	0
Egg 37	0	0	0	0	1.88
Egg 37	1	TNTC &0	lawn	TNTC & 5.19	7.67
Egg 37	2	lawn	lawn & 0	lawn	7.40
Egg 37	3	0	lawn	lawn	0
Ham 37	0	2.72	2.95	0	3.17
Ham 37	1	0	2.26	0	2.60
Ham 37	2	0	lawn	2.41	0
Ham 37	3	0	0	0	0
HD 37	0	0	0	0	1.65
HD 37	1	0	0	0	0
HD 37	2	0	0	1.48	0
HD 37	3	0	0	0	0
Let 37	0	0	0	0	1.78
Let 37	1	0	0	0	0
Let 37	2	0	0	0	0
Let 37	3	0	0	0	0
UHT 37	0	0	0	0	0
UHT 37	1	0	1.65	0	1.18
UHT 37	2	0	0	1.18	0
UHT 37	3	0	0	0	0
Strwb 37	0	0	2.08	0	2.43
Strwb 37	1	0	2.18	0	0

Strwb 37	2	0	0	0	0
Strwb 37	3	0	0	0	0

* App = red delicious apple; Irr B = irradiated ground beef; BP = bell pepper; Egg = liquid whole eggs; Ham = ham steak; HD = hot dog; Let = shredded iceberg lettuce; UHT = 2% UHT milk; Strwb = strawberry.

[a]0 represents no growth of *F. tularensis* detected by plating on mCHA

Table A.2 Recovery of *F. tularensis* (in log cfu g^{-1} or mL^{-1}) from nine* food matrices (21°C)

Food/Temp	Day	Rep1	Rep 2	Rep 3	Rep 4
App 21	0	2.38	0[a]	0	0
App 21	1	0	0	0	1.78
App 21	2	0	0	1.18	1.18
App 21	3	0	0	0	0
IrrB 21	0	1.88	0	0	0
IrrB 21	1	0	0	0	2.26
IrrB 21	2	0	0	0	0
IrrB 21	3	0	0	no sample	0
BP 21	0	1.78	0	0	3.54
BP 21	1	0	0	0	0
BP 21	2	1.88	0	3.00	0
BP 21	3	0	1.65	0	0
Egg 21	0	0	0	0	1.88
Egg 21	1	1.88	0	0	0
Egg 21	2	3.49	0	5.36	3.73
Egg 21	3	0	lawn	0	0
Ham 21	0	2.72	2.95	0	3.17
Ham 21	1	0	0	0	2.32
Ham 21	2	0	0	3.13	2.18
Ham 21	3	0	0	0	0
HD 21	0	0	0	0	1.65
HD 21	1	0	0	0	0
HD 21	2	0	0	0	0
HD 21	3	0	0	0	0
Let 21	0	0	0	0	1.78
Let 21	1	0	0	0	0
Let 21	2	0	0	1.78	0
Let 21	3	0	0	0	0
UHT 21	0	0	0	0	0
UHT 21	1	1.18	0	0	2.18
UHT 21	2	0	0	1.18	0
UHT 21	3	0	1.18	0	0
Strwb 21	0	0	2.08	0	2.43
Strwb 21	1	0	0	0	0
Strwb 21	2	0	0	1.48	0
Strwb 21	3	0	0	0	0

* App = red delicious apple; Irr B = irradiated ground beef; BP = bell pepper; Egg = liquid whole eggs; Ham = ham steak; HD = hot dog; Let = shredded iceberg lettuce; UHT = 2% UHT milk; Strwb = strawberry.

[a]0 represents no growth of *F. tularensis* detected by plating on mCHA

Table A.3 Recovery of *F. tularensis* (in log cfu g^{-1} or mL^{-1}) from nine* food matrices (4ºC)

Food/Temp	Week	Rep1	Rep 2
App 4	0	2.38	0[a]
App 4	1	1.78	0
App 4	2	0	1.88
IrrB 4	0	1.88	0
IrrB 4	1	0	0
IrrB 4	2	0	0
BP 4	0	1.78	0
BP 4	1	0	0
BP 4	2	0	2.57
Egg 4	0	0	0
Egg 4	1	0	0
Egg 4	2	0	3.04
Ham 4	0	2.72	2.95
Ham 4	1	0	0
Ham 4	2	0	1.65
HD 4	0	0	0
HD 4	1	0	0
HD 4	2	0	0
Let 4	0	0	0
Let 4	1	0	0
Let 4	2	0	0
UHT 4	0	0	0
UHT 4	1	2.95	0
UHT 4	2	0	2.98
Strwb 4	0	0	2.08
Strwb 4	1	1.18	0
Strwb 4	2	0	0

* App = red delicious apple; Irr B = irradiated ground beef; BP = bell pepper; Egg = liquid whole eggs; Ham = ham steak; HD = hot dog; Let = shredded iceberg lettuce; UHT = 2% UHT milk; Strwb = strawberry.

[a]0 represents no growth of *F. tularensis* detected by plating on mCHA

Appendix B - SAS code used for the statistical analysis of the study "Determination of the Ability of *Francisella tularensis* to Grow/Survive in Bagged Iceberg Lettuce"

```
*** import data set from Excel ***;
proc import out=one
datafile=path dbms=xls replace;
sheet='sheet1';
run;

*** re-name variables ***;
data one;
set one;
rename week=run lettuce=lot;
run;

*** convert 0 to 0.5 ***;
data zerohalf;
set one;
if log = 0 then log = 0.5;
if day = 0 then delete;
run;


proc glimmix data=zerohalf ic=q;
where day le 4;
class run lot temp day;
model log = temp|day / ddfm=satterth dist=gamma link=log;
random run lot(run) run*temp*day;
lsmeans temp day /cl ilink pdiff adj=tukey;
lsmeans temp*day / cl ilink slice=day pdiff adj=tukey
plots=meanplot(sliceby=temp join ilink);
run;
```