**The Recovery and Transfer of Aerosolized *Listeria innocua***

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ABSTRACT
(academic)


Airborne pathogenic bacteria can present a significant public health risk. Pathogenic *Listeria monocytogenes* can colonize numerous surfaces as well, through direct and indirect cross contamination. The physical environment can also affect the transmission and viability of *Listeria* (distance from the source, temperature, humidity, air flow). The purpose of this work was to explore the ability of *Listeria innocua* (a surrogate for *L. monocytogenes*) to contaminate a surface after it has become aerosolized in a bioaerosol chamber and a walk-in cooler.

*L. innocua* was nebulized into a 154 L biosafety chamber (~5 log CFU in 1 mL) at two relative humidity (RH) levels (83% and 65%). Oxford Listeria agar plates, stainless steel coupons and polyethylene (HDPE) coupons in the chamber were exposed to the aerosolized bacteria for 5, 10, 20 or 40 minutes. Also, at these times, air samples (100 L) were collected on to gelatin filters which were transferred to Oxford agar plates. In the second part of the research, *L. innocua* was nebulized into an 11 m$^3$ walk-in cooler where RH ranged from ~29-37%. Aerosolized bacteria were collected on to Oxford agar plates for 10 min intervals and with 50 or 100 L air samples.

Recovery of *L. innocua* from steel, plastic and agar was significantly higher at 83% RH (2.7 cells/cm$^2$) compared to 65% RH (0.45 cells/cm$^2$). Mean cell recovery from air samples (gelatin filters) was significantly higher (p<0.05) when collected 5 or 10 minutes after nebulization at 83% humidity (mean 2.2 CFU/L) compared to collection

after 20 or 40 minutes or compared to all times under 65% humidity (mean 0.4 CFU/L).

Recovery from HDPE coupons (1.21 CFU/cm$^2$) was 2.5 X recovery from Oxford agar

(0.49 CFU/cm$^2$). In the walk-in cooler, total estimated mean recovery from Oxford

media at 10 min after nebulizing was 0.48%, but only 0.04% for samples collected after

60 minutes. The recovery of *L. innocua* from air samples after 60 min was one-fourth of

the number recovered 5 min after nebulizing. No significant difference in recovery was

found between plates at different distances (2 – 2.5 m) from the nebulizer in the walk-in

cooler. Understanding the survival of aerosolized *Listeria* and how it can colonize over

time on a food contact surface will enhance our efforts to prevent transmission on a

small and large scale. The food industry will be able to implement better safety

measures to prevent contamination by *Listeria* species.

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Airborne pathogenic bacteria, including *Listeria monocytogenes,* can present a significant public health risk.  Pathogenic bacteria can colonize numerous surfaces as well through direct and indirect cross contamination.  The physical environment can also affect the transmission and viability of *Listeria* (distance from the source, temperature, humidity).  The purpose of this work was to explore the ability of *Listeria innocua* to contaminate a surface after it has become aerosolized in a bioaerosol chamber and a walk-in cooler. Environmental factors of distance from the source, temperature, and relative humidity were explored.

*L. innocua* was nebulized into a 154 L biosafety chamber (~5 log CFU in 1 ml) at two relative humidity (RH) levels (83% and 65%).  Oxford Listeria agar plates, stainless steel coupons and polyethylene (HDPE) coupons in the chamber were exposed to the aerosolized bacteria for 5, 10, 20 or 40 minutes.  Also, at these times, air samples (100 L) were collected on to gelatin filters which were transferred to Oxford agar plates.  In the second part of the research, *L. innocua* was nebulized into an 11 $m^3$ walk-in cooler where RH ranged from ~29-37%.  Aerosolized bacteria were collected with 50 or 100 L air samples.  And, Oxford media was placed on the cooler floor in layers (attached to poster boards) at various locations for surface analysis.

The three surface samples yielded a greater mean recovery of 2.7 cells/cm$^2$ at 83% humidity compared to 0.45 cells/cm$^2$ at 65% humidity.  Mean cell recovery from air samples (gelatin filters) was significantly higher ($p<0.05$) when collected 5 or 10 minutes after nebulization at 83% humidity (mean 2.2 CFU/L) compared to collection after 20 or 40 minutes or compared to all times under 65% humidity (mean 0.4 CFU/L).  Recovery from HDPE coupons (1.21 CFU/cm$^2$) was 2.5 X recovery from Oxford agar (0.49 CFU/cm$^2$).  In the walk-in cooler, total estimated mean recovery from the Oxford media at 10 min after nebulizing the *Listeria innocua* was 0.48%, but only 0.04% for samples collected after 60 minutes.  The recovery of *L. innocua* from air samples after 60 min was one-fourth of the number recovered 5 min after nebulizing.  Understanding the survival of aerosolized *Listeria* and how it can colonize over time on a food contact surface will enhance our efforts to prevent transmission on a small and large scale.  The food industry will be able to implement better safety measures to prevent contamination by *Listeria* species.

# Acknowledgements

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**Introduction**

Listeria monocytogenes is one of the world's primary bacterial food safety threats and causes listeriosis in humans.  The genus *Listeria* includes at least 10 species, and while both *L. ivanovii* and *L. monocytogenes* are pathogenic for mice, only *L. monocytogenes* is consistently associated with human illness (Goh et al., 2012). *Listeria monocytogenes* is spread easily by direct food contact with a contaminated surface and through air borne transmission.  This organism is psychrotrophic (able to grow at low temperatures), grows under low-oxygen conditions, and can survive for long periods of time in the environment, on foods, in processing plants, and in household refrigerators.  Specifically, extremely slow growth of *L. monocytogenes* has been recorded at temperatures as low as −1.5⁰C and the maximum temperature for growth is generally accepted as 45⁰C (Lawley et al., 2008).  Its optimum temperature for growth is between 30 ⁰C and 37 ⁰C. The organism survives well in frozen foods, but survival times can be shortened under acid conditions.  In general, *Listeria monocytogenes* is one of the more heat resistant foodborne pathogens compared to others, such as *Salmonella* and *E. coli* O157:H7. It is readily inactivated at temperatures above 70⁰C and heat processes such as commercial milk pasteurization will destroy numbers typically found in milk.  The pH range for the growth of *L. monocytogenes* is 4.3 - 9.4 under otherwise ideal conditions, but the minimum pH is likely to be higher in real foods and at low temperatures (Lawley et al., 2008). However, *L. monocytogenes* can survive for extended periods in acid conditions, particularly in refrigerated foods.  Also, the minimum water activity for the growth of *L. monocytogenes* is 0.92. It can tolerate high sodium chloride levels and is able to grow in environments of up to 10% salt, and to

survive in concentrations of 20 – 30% (Lawley et al., 2008). *L. monocytogenes* is also able to survive for some time in low water activity environments, and may survive food drying processes.

*Listeria* is the agent of listeriosis; a serious infection caused by eating food contaminated with the bacteria. Listeriosis has been recognized as an important public health problem in the United States. The disease affects primarily pregnant women, newborns, and adults with weakened immune systems. Listeriosis is a serious disease for humans; the overt form of the disease has a mortality greater than 25 percent (Todar, 2012). The two main clinical manifestations are sepsis and meningitis. Meningitis is often complicated by encephalitis, a pathology that is unusual for bacterial

*Listeria* spp. are common in the environment. They are found in soil, where they can survive for extended periods leading to the contamination of plant material, and has been isolated from a wide variety of fresh produce. *Listeria* have been found in marine environments and are often associated with fish and seafood products. Animals such as sheep, goats and cattle are recognized carriers of *Listeria*, often acquired from the consumption of contaminated (usually poor quality) silage (Lawley, 2013). Healthy humans can also be carriers. Kitchen and food processing environments, particularly those that are cold and wet, can be reservoirs for *Listeria*. The organism can be particularly persistent and difficult to control because of its resistance to unfavorable environmental conditions and ability to grow at low temperatures, especially within biofilms.

Bioaerosol control in food production environments is important because of the potential for contamination to foods and food contact surfaces. Aerosolized bacteria are

those which are suspended in liquid, and then become airborne by physical action (Gold, 2010).  Airborne bacteria are typically described as bacteria which are attached to dust particles that can be dispersed into the air by physical action (CDC, 1996).  The physical environment of a processing facility can affect the transmission and viability of *Listeria* (growth and death of cells) or other bacteria.  The ability of *Listeria* spp. and other foodborne microorganisms to transmit to food or other surfaces, through the air, has not been well studied.  This research will explore the ability of *Listeria innocua* (a non-pathogenic surrogate for *Listeria monocytogenes*) to contaminate a surface after the organisms become aerosolized or airborne. Environmental factors that may influence the ability of the organisms to survive and attach to surfaces will also be explored. The surface material, surface characteristics and spatial orientation can influence its susceptibility to contamination.  Bacteria may attach to a horizontal wet surface differently than a vertical dry surface due to gravitational forces, for example.

If the transmission, survival and growth of *Listeria innocua* is similar to that of *Listeria monocytogenes* under similar conditions, we may be able to make predictions of the presence or concentration of pathogenic *Listeria* on foods, food contact surfaces or the environment. Even though *Listeria monocytogenes* is considered less prevalent, if it behaves in the same manner as *Listeria innocua*, then the food industry could apply proper precautions when either organism is detected.

Research Objectives:

1) To determine if environmental factors affect the survival and transmission of aerosolized *Listeria innocua* to a food contact surface.

- Determine the time that aerosolized bacteria will survive within a bioaerosol chamber.

- Determine the distance from a source that aerosolized bacteria can contaminate a surface in a bioaerosol chamber.

- Determine the effect of relative humidity on the survival (time) and transmission (distance).

2) To determine if the relationships of survival and transmission of aerosolized *Listeria innocua* to a food contact surface are valid for a larger environmental study.

- Quantitate the proportion of aerosolized *L. innocua* that can be recovered over time (as a function of relative humidity and surface composition).

- Quantitate the proportion of aerosolized *L. innocua* that can be recovered from air or surfaces at varied distances from a source (as a function of relative humidity and surface composition).

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I.  Literature Review


A.  _Listeria_ species

     _Listeria_ spp. bacteria are common in nature and found widely in such places as water, soil, infected animals, human and animal feces, raw and treated sewage, leafy vegetables, effluent from poultry and meat processing facilities, decaying corn and soybeans, improperly fermented silage, and raw (unpasteurized) milk. The genus _Listeria_ comprises at least 10 recognized species including _L. monocytogenes, L. innocua, L. grayi, L. welshimeri, L. seeligeri, L. ivanovii, L. marthii, L. rocourtiae, L. fleischmannii_, and _L. weihenstephanensis_ (den Bakker, et al., 2014). Only _L. monocytogenes_ appears to be the causative agent of listeriosis in humans.

     Foods commonly identified as sources of _Listeria_ infection include improperly pasteurized fluid milk, cheeses (particularly soft-ripened varieties, such as traditional Mexican cheeses, Camembert and ricotta), ice cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry, and cooked, ready-to-eat (RTE) sliced meats—often referred to as "deli meats" (Tauxe, 2001).  In correlation, _Listeria_ may enter the environment of processing plants and subsequently contaminate RTE meat or poultry products, as well as other ingredients.  The microorganism has ample opportunity to occupy and thrive in various niches in a production facility, such as on floors, in drains, or in standing water.  With the lack of proper sanitation and employee hygiene practices, it can easily cross-contaminate processing equipment, gloves or aprons of employees, and product.

B. <u>Listeriosis</u>

  1. <u>Symptoms of *Listeria* Infection</u>

   When some contracts the *Listeria* infections and its symptoms, the resulting

illness is called listeriosis.  Only a small percentage of those who ingest the *Listeria*

bacteria become ill, but for those who do the resulting illness is either mild or quite

severe—sometimes referred to as a "bimodal distribution of severity" (CAST, 1994).

For a mild infection, listeriosis usually consists of fever, chills, severe headache,

vomiting, and other influenza-type symptoms (Lorber, 2000).  Along these same lines,

the CDC notes that infected individuals may develop fever, muscle aches, and

sometimes gastrointestinal symptoms such as nausea or diarrhea.  Diarrhea usually

lasts anywhere from 1 to 4 days.  Most healthy adults and children who consume

infected food experience only mild to moderate symptoms. The infection is usually self-

limited since exposure to *Listeria* stimulates the production of tumor necrosis factor and

other cytokines, which activate monocytes and macrophages to eradicate the organism

(in healthy hosts) (Stearns, 2015).  As for pregnant women, the illness appears as an

acute fever, muscle pain, backache, and headache.  The illness usually occurs in the

third trimester and infection can lead to can lead to premature labor, miscarriage,

infection of the newborn, or even stillbirth (Silver, 1998). Twenty-two percent of such

infections result in stillbirth or neonatal death (Lorber, 2000).  In the case of newborns,

the may exhibit early-onset (less than 7 days) or late-onset forms of infection (7 or more

days).  Those with the early-onset form are often diagnosed in the first 24 hours of life

with sepsis (Lorber, 2000). Early-onset listeriosis is most often acquired through trans-

placental transmission. Late-onset neonatal listeriosis is less common than the early-onset form.  Symptoms include irritability, fever, and poor feeding (Stearns, 2015).

For those who suffer a severe *Listeria* infection that does not resolve on its own, there can be numerous complications. The most common is septicemia (bacterial pathogens in the blood, also known as bacteremia), with meningitis being the second most common (Stearns, 2015). Other significant complications include inflammation of the brain or brain stem (encephalitis), brain abscess, inflammation of the heart-membrane (endocarditis), and localized infection, either internally or of the skin (Lorber, 2000).  Death is the most severe consequence of listeriosis, and it is tragically common. For example, based on 2009 FoodNet surveillance data, 89.2% of *Listeria* patients ended up in the hospital, the highest hospitalization rate for pathogenic bacterial infection (CDC, 2009).  In persons 50 years of age and older, there was a 17.5% fatality rate—also the highest relative to other pathogens (CDC, 2009).

2. <u>People at Risk to Infection</u>

Various groups of the population are at risk to *Listeria* infection.  The body's defense against the bacteria is called "cell-mediated immunity" because the success of defending against infection depends on our cells (as opposed to our antibodies) (Cossart, 2001).  As a result, those whose cell-mediated immunity is suppressed are more susceptible to the devastating effects of listeriosis, including especially HIV-infected individuals, who have been found to have a *Listeria*-related mortality of 29% (Cossart, 2001).  Pregnant women naturally have a lower cell-mediated immune system as well as fetuses and newborns.  Others include transplant recipients and lymphoma

patients.  They are given necessary care with the intent of depressing T-cells, and these individuals become especially susceptible to *Listeria* as well*.*  According to the CDC and other public health organizations, persons at risk for being infected and becoming seriously ill with *Listeria* include the following groups: Pregnant women (who are about 20 times more likely than other healthy adults to get listeriosis), newborns (who can suffer serious effects of infection in pregnancy), persons with weakened immune systems (including persons with AIDS, who are almost 300 times more likely to get listeriosis than, people with normal immune systems), persons with cancer, diabetes, or kidney disease, persons who take glucocorticosteroid medications, elderly persons (CDC, 2016)

3. Incidence of *Listeria* infections

In the food industry and medical field, *Listeria* is known as an "opportunistic pathogen" and is noted to cause an estimated 2,600 cases per year of severe invasive illness (Tauxe, 2001). When ingested by mouth, *Listeria* is among the most virulent foodborne pathogens, with up to 20% of clinical infections resulting in death. These bacteria primarily cause severe illness and death in persons with immature or compromised immune systems. Consequently, most healthy adults can be exposed to *Listeria* with little to no risk of infection and illness. With that being said, it is estimated that this foodborne pathogen causes approximately 1,460 hospitalizations each year in the United States, resulting in 260 deaths. In developed countries worldwide, the incidence of listeriosis is 0.36 to 5 cases annually per million people (Ferreira et al, 2013).

4. Diagnosis and Treatment of *Listeria* Infections

There are few symptoms that are unique to listeriosis, doctors must consider a wide variety of potential factors and causes for infection, including viral infections and other bacterial infections. Early diagnosis and treatment of listeriosis in at-risk patients is crucial due to the infection being potentially fatal. This is especially true for pregnant women because of the increased risk of spontaneous abortion and preterm delivery. Depending on the risk group, rates of death from listeriosis range from 10% to 50%, with the highest rate among newborns in the first week of life (Stearns, 2015).

Generally, routine methods are effective for isolating *Listeria* from spinal fluid, blood, and joint fluid. Magnetic-resonance imaging (MRI) is used to validate or dismiss brain or brain stem involvement. The common methods used to identify diarrhea-causing bacteria in stool cultures interfere with the growth of *Listeria*, making it less likely to be identified and isolated for further testing. As a result, these methods are not used in testing.

Listeriosis is usually a self-limited illness, which means most of the infected persons with improve in health without the need for medical care. However, with patients having symptoms such as high fever, a stool culture and antibiotic-treatment may be enough for otherwise healthy individuals. The recommended drug treatment for this infection is ampicillin (Stearns, 2015). Invasive infections with *Listeria* can be treated with antibiotics. For pregnant mothers, antibiotics are administered promptly and often to prevent infection of the fetus or newborn. Babies with listeriosis receive the same antibiotics as adults under the physician's discretion.

C.  <u>Reducing the Impact of Foodborne Listeriosis</u>

    1.  <u>Recent Outbreaks of *Listeria*</u>

Even with the numerous prevention methods, protocols, and precautions set in place, there have still been significant *Listeria monocytogenes* outbreaks in the United States within the past couple decades.  Three major outbreaks that had a major impact on food production and food processing industries in America occurred in 1998, 2002, and 2011.  In 1998, molecular subtyping helped to identify *L. monocytogenes* cases linked to this nationwide outbreak.  The cases were eventually found in 24 states and a control study implicated meat frankfurters, or hotdogs, as the likely source. It affected at least 100 people, causing 14 adult deaths, and four miscarriages. The contamination affected over nine brands, including Sara Lee Deli Meat. This outbreak spread from Bil Mar Foods' manufacturing plant in Zeeland, Michigan (Healthline, 2017).  To help rid the tarnished image of the Sara Lee Corporation, the company actually recalled 34,500 pounds of Ball Park hot dogs in 2000 in addition to the 1998 national recall of 15 million pounds of hot dogs and luncheon meat (Healthline, 2017).

2002 marked a multistate outbreak of *Listeria monocytogenes* infections with 46 culture-confirmed cases, seven deaths, and three stillbirths or miscarriages in eight states (CDC, 2002).  It was linked to sliceable turkey deli meat from Pilgrim's Pride and the company recalled 27.4 million pounds of poultry products.  The company also voluntarily suspended all operations.

The second deadliest bacterial foodborne outbreak in the United States occurred in 2011.  The outbreak of *Listeria monocytogenes* food poisoning went across 28 US

states that resulted from contaminated cantaloupes linked to Jensen Farms of Holly, Colorado.  It was reported that FDA officials found *Listeria* on dirty equipment used by Jensen Farms, which had been bought used and was previously utilized for potato farming. Officials said that the "equipment's past use may have played a role in the contamination".  Water contaminated with *Listeria* was also found on the floor of the packing plant and it was determined that the workers moving around the plant had spread it, as the contaminated water was also found on the cantaloupe conveyor belt (CDC, 2011).  A final report stated that there were 30 deaths and 147 total confirmed cases of infection.  Jensen Farms issued a voluntary recall on September 15th of the entire harvest crop of 300,000 cantaloupe that it had distributed to its chain stores and temporarily shut down its processing plant.

   2. Surveillance for *Listeria*

   *Listeria monocytogenes* a potentially lethal pathogen that cause extreme determent nationwide and worldwide if not controlled and monitored properly. Government surveillance does this by providing valuable insights into the foods, germs, and settings linked to foodborne diseases.  The CDC has conducted surveillance for *Listeria* infections to learn more about the germ, how it causes/spreads disease, and other information to help protect the public from illness for over 30 years.  There are five main systems of the CDC in place that serve a different purpose in monitoring *Listeria* overall.  All together they provide a comprehensive view of listeriosis in the United States as well as potential threats to other nations.

The *Listeria* Initiative is one of the enhanced surveillance systems that collects reports of laboratory-confirmed cases of listeriosis in the United States.  Its main goal is to find and stop outbreaks by interviewing all the patients with *Listeria* infection using the *Listeria* Case Report, a questionnaire developed to collect detailed information about the foods people ate before they got sick (CDC, 2017).  In addition, when investigators identify a group of illnesses with a possible common source, they rapidly compare information on the foods eaten by patients in the possible outbreak with foods eaten by listeriosis patients who are not involved in the possible outbreak.  This comparison gives investigators important clues about possible sources, which helps to solve outbreaks. The system also uses molecular subtyping data ("fingerprinting") from clinical, food, and environmental isolates, or samples, of *Listeria* to identify clusters of possibly related cases. Laboratories subtype isolates using pulsed-field gel electropho-resis (PFGE, a type of DNA fingerprinting) and submit the results to PulseNet, the National Molecular Subtyping Network for Food-borne Disease Surveillance (CDC, 2016).

Another surveillance system in place is called FoodNet.  This network collects information to track rates and report trends for nine germs transmitted commonly by food, including *Listeria*. It is a collaborative program among the Centers for Disease Control and Prevention, 10 state health departments, the U.S. Department of Agriculture's Food Safety and Inspection Service, and the Food and Drug Administration.  This particular system has four main objectives:

1.  Determine the burden of foodborne illness in the United States
2.  Monitor trends in the burden of specific foodborne illness over time

3. Attribute the burden of foodborne illness to specific foods and settings

4. Disseminate information that can lead to improvements in public health practice and the development of interventions to reduce the burden of foodborne illness.

A third system of surveillance is a network called PulseNet. It connects foodborne illness cases by using DNA fingerprints of the bacteria making people sick. Basically when PulseNet identifies a possible cluster of *Listeria* by finding the same molecular pattern in patients' samples, epidemiologists can rapidly investigate the source (CDC, 2016). PulseNet has also added whole genome sequencing to find more outbreaks and help stop them while they are in beginning stages of development. This tool allows investigators to find the source, alert the public sooner, and identify gaps in our food safety systems that would not otherwise be recognized.

Another CDC system is the National Notifiable Diseases Surveillance System (NNDSS). It is a reporting system that enables all local, state, territorial, and federal health agencies to share health information to monitor, control, and prevent the occurrence and spread of nationally notifiable infectious diseases (CDC, 2015). This includes listeriosis and some noninfectious diseases and conditions. The NNDSS includes policies, laws, electronic messaging standards, people, partners, information systems, processes, and resources at all jurisdictive levels. Facets of the NNDSS are used by numerous health departments and partner organizations, such as the Council of State and Territorial Epidemiologists (CSTE), to:

- facilitate collecting, managing, analyzing, interpreting, and disseminating health related data for diseases designated as nationally notifiable,

- develop and maintain national standards (for example, consistent case definitions for nationally notifiable diseases) applicable across states,

- maintain the official national notifiable diseases statistics,

- provide detailed data to CDC programs to aid in identifying specific disease trends,

- work with states and partners to implement and assess prevention and control programs, and

- publish summarized data findings from 57 state, territorial, and local reporting jurisdictions weekly and annually in the Morbidity and Mortality Weekly Report (MMWR). (CDC, 2015)

The last CDC system used is known as the Foodborne Disease Outbreak Surveillance System. It collects reports of foodborne disease outbreaks from local, state, tribal, and territorial public health agencies. FDOSS contains information on foods, settings, and germs linked to specific outbreaks (CDC, 2015). In conjunction, the Foodborne Outbreak Online Database (FOOD Tool) was developed by the Centers for Disease Control and Prevention to make Foodborne Disease Outbreak Surveillance System data more available to the public and stakeholders. This tool lets users search foodborne disease data by year, state, location of food preparation, food and ingredient, and cause. It also provides information on numbers of illnesses, hospitalizations, deaths, the germ, and the confirmed or suspected cause.

3.  The Economic Impact of *Listeria*

The USDA Economic Research Service (ERS) published its first comprehensive cost estimates for sixteen foodborne bacterial pathogens in 1989 (Roberts, 1989). Five years later, it was estimated that, in 1993, there were 1,795 to 1,860 *Listeria* infections that required hospitalization, with 295-360 of these cases involving pregnant women (Buzby, 1996). Based on these estimates, the medical costs that *Listeria* infections had caused each year were said to run from $61.7 to $64.8 million, including those individuals who ultimately died as a result of their infections (Buzby, 1996). For these same cases, productivity costs were estimated to run from $125.8 to $154.4 million a year (Buzby, 1996). The productivity costs associated with *Listeria*-related chronic illness was estimated to be an additional $38 million a year (Buzby, 1996). In total, estimates of costs for the 1,795 to 1,860 cases of listeriosis range from $232.7 million to $264.4 million annually. In 2000, the USDA updated the cost-estimates for four pathogens. They included Campylobacter, Salmonella, E. coli O157:H7, *and Listeria monocytogenes* (Crutchfield, 2000). The 2000 estimates were based on the CDC's then newly-released estimates of annual foodborne illnesses, and put the total cost in the United States for these four pathogens at $6.5 billion a year (Crutchfield, 2000). For *Listeria* specifically, it was estimated that costs amounted to $2.3 billion per year, based on 2,493 cases, which involved 2,298 hospitalizations and 499 deaths (Crutchfield, 2000). More recently, in 2007, it was estimated that the worldwide cost of all foodborne disease was $1.4 trillion per year (Buzby, 2009). These numbers truly display how devastating *Listeria* and other foodborne illness are to society.

4. <u>Prevention of *Listeria* Infections</u>

In general, the majority of *Listeria* infections are the result of consuming contaminated food or water.  These infections are very prevalent in the environment and it is an absolute necessity to control these infections to prevent illness and death.  The pathogenic strain of *Listeria monocytogenes* presents a concern to food handling because it can grow at refrigerated temperatures (4°C to 10°C).  Freezing also has little effect on the bacteria.  The processing method of pasteurization is effective, however failure to reach the desired temperature in large packages can allow the organism to survive (Stearns, 2015).  Products can also be cross contaminated after processing by the introduction of unpasteurized material, as happens sometimes in cheese manufacturing.  Other ways of spreading include contact with contaminated hands, equipment, and counter tops.

The use of irradiation to reduce *Listeria* to safe levels is one very effective methods many food companies employ.  Gamma irradiation, for example, has been shown to effectively control *L. monocytogenes* in uncooked meats but has not been extensively studied in ready-to-eat meats.  One study showed that bacteria counts for ready-to-eat ham and cheese sandwiches treated with 3.9 kGy decreased by 5 log units initially and then decreased further during storage at 4°C (Clardly, 2002).  Also, ready-to-eat meats, such as hot dogs, have already been introduced to a pathogen-killing step when the meat is cooked at the factory, so contamination is typically the result of contamination after that step (Stearns, 2015).  Improvement in sanitation in many plants has reduced the incidence of infection by half since 1986, but the risk is still

a threat.  The CDC provides a comprehensive list of recommendations and precautions to avoid becoming infected with *Listeria*, as follows (Stearns, 2015):

- Thoroughly cook raw food from animal sources, such as beef, pork, or poultry to a safe internal temperature. For a list of recommended temperatures for meat and poultry, visit http://www.fsis.usda.gov/PDF/IsItDoneYet_Magnet.pdf.

- Rinse raw vegetables thoroughly under running tap water before eating.

- Keep uncooked meats and poultry separate from vegetables and from cooked foods and ready-to-eat foods.

- Do not drink raw (unpasteurized) milk, and do not eat foods that have unpasteurized milk in them.

- Wash hands, knives, countertops, and cutting boards after handling and preparing uncooked foods.

- Consume perishable and ready-to-eat foods as soon as possible.

In addition, recommendations for persons at high risk, such as pregnant women and persons with weakened immune systems include (Stearns, 2015):

Meats:

- Do not eat hot dogs, luncheon meats, cold cuts, other deli meats (e.g., bologna), or fermented or dry sausages unless they are heated to an internal temperature of 165°F or until steaming hot just before serving.

- Avoid getting fluid from hot dog and lunch meat packages on other foods, utensils, and food preparation surfaces, and wash hands after handling hot dogs, luncheon meats, and deli meats.

- Do not eat refrigerated pâté or meat spreads from a deli or meat counter or from the refrigerated section of a store. Foods that do not need refrigeration, like canned or shelf-stable pâté and meat spreads, are safe to eat. Refrigerate after opening.

Cheeses:

- Do not eat soft cheese such as feta, queso blanco, queso fresco, brie, Camembert, blue-veined, or panela (queso panela) unless it is labeled as made with pasteurized milk.

Seafood:

- Do not eat refrigerated smoked seafood, unless it is contained in a cooked dish, such as a casserole, or unless it is a canned or shelf-stable product. Refrigerated smoked seafood, such as salmon, trout, whitefish, cod, tuna, and mackerel, is most often labeled as "nova-style," "lox," "kippered," "smoked," or "jerky." These fish are typically found in the refrigerator section or sold at seafood and deli counters of grocery stores and delicatessens. Canned and shelf stable tuna, salmon, and other fish products are safe to eat.


D. Transmission of *Listeria* in Food Environments

    1. Aerosolized Transfer and Infection by *Listeria*

In food microbiology, it is widely assumed that *Listeria monocytogenes* can be transmitted to processed foods by means of airborne or aerosol transmission. "Airborne" bacteria may best be defined as bacteria which are attached to dust particles, while "aerosolized" bacteria may be defined as bacteria which are suspended

in liquid, which then become airborne by physical actions which develop droplets. For

example, a recent study has shown that airborne soil particulates could serve as a

vehicle for *Salmonella* contamination of fruit (Kumar et al., 2017). *Listeria*, on the other

hand, will most likely form into bioaerosols and these may be created from foot and

wheeled (e.g., forklifts, handcarts) vehicle traffic through standing water in which

microorganisms have grown, the application of high-pressure washers to contaminated

surfaces, the use of compressed air lines that do not have reliable filters in which

condensate has accumulated, and various unhygienic designs of Air Handling Units

(Kornacki, 2014). Depending upon different environmental factors, bioaerosols will be

suspended in the air for various lengths of time. Their settling rate is related to particle

diameters. Vegetative (nonspore) cells liberated into an open space will become diluted

and injured as they mix with the air in the environment. These bioaerosols will move

from high to low pressure on air currents in the plant. This highlights the importance of

appropriately filtered, positive-pressure air in rooms requiring elevated hygienic controls,

such as filling and packaging rooms, compared with basic Good Manufacturing Practice

(GMP) areas of the plant (Kornacki, 2014). In general, the infective dose is estimated to

be between 10–100 million viable bacteria (or colony forming units "CFU") in healthy

individuals, and only 0.1–10 million CFU in people at high risk of infection (Tauxe,

2001).

2. Other Transmissions of Bacteria

There are many other unorthodox ways bacteria can be transferred. One such

way is the transaction of money in society. Globally, money is one of the items most

frequently passed from hand to hand. During its passing, money can get contaminated and may play a role in the transmission of microorganisms to other people. For instance, money may get contaminated with microorganisms from the respiratory- and gastro-intestinal tract during counting (Gedik, 2013).  Money is usually not prime for the survival of microorganisms, except for some that are resistant to external conditions and non-resistant forms of spores.  The hygiene levels of a particular community may also contribute to the amount of microbes found on currency.  This mode of transmission has shown that money may be a potential pathway, especially in countries that use polymer-based banknotes.

   3. _Listeria_ spp. Attachment to Food and Food Contact Surfaces

_Listeria_ can attach to various surfaces such as plastic and stainless steel, in addition to various ready-to-eat products.  Cross contamination can occur with any of the previously mentioned sources.  In general, bacterial cells may need only one to a few minutes to attach to a surface and spread infection (Arritt et al., 2002).  An example could be cross contamination between the deli meat on the conveyor belt, which is dropped into a plastic packaging system.  Deli meat on a conveyor belt can contain _Listeria_, and contaminate all materials and surfaces throughout processing.

   Polyethylene is an inexpensive plastic material that is chemically resistant and can be very durable for use in food industries.  Among polyethylene, low density polyethylene has the most excessive branching which causes a less compact molecular structure and lowered density. High density polyethylene has minimal branching of its

polymer chains. Due to it being denser, it is more rigid and less permeable then the LDPE.  It has a density of 0.941-0.965 g/cm$^3$ (U.S. Plastic Corp., 2008).

Many bacteria can attach to plastic food contact surfaces such as conveyor belts, storage containers, and cutting boards, especially if the surfaces have scratches or pits. Scored lines in the surface should not have bacteria as long as the surface is thoroughly washed.  If there is no food residue on a plastic surface, there is no food source for bacteria to utilize for growth.   Unlike wood, plastic boards do allow rinsing with harsher cleaning chemicals such as bleach and other disinfectants without damage to the board or retention of the chemicals to later contaminate food. Many studies have shown that the material of the surface has little or no effect on biofilm development.  In general, different cutting boards must be used for cutting raw materials and cooked food as pathogens on the surface of cutting boards can easily be transmitted to cooked food. Re-use of the same cutting board for raw and RTE food without washing is a potential source of *L. monocytogenes* transmission (Jevsnik, et al., 2008).  It is recommended to wash cutting boards with detergents and warm water between using the cutting board for raw material and RTE or cooked food.

Stainless steel may be just as susceptible to bacterial contamination as plastic. Most stainless steel containers, pipework and food contact equipment are in stainless steel is manufactured from either 304 or 316 type austenitic stainless steels (BSSA, 2013). The 17% chromium ferritic stainless steel (430 type) is also used widely for such applications as splash backs, housings and equipment enclosures, where corrosion resistance requirements are not so demanding (BSSA, 2013). To reiterate the point of surface susceptibility, Mayette (1992) noted that "Piping material that microorganisms

cannot adhere to has yet to be discovered.  Studies have shown that microbes will

adhere to stainless steel, Teflon, PVC and PVDF (Kynar) with nearly equal enthusiasm"

(Edstrom, 2013).  The finish of a steel material can also effect the bacteria formation on

its surface and should be accounted for when applying a certain antimicrobial.  In one

particular study, three common finishing treatments of stainless steel that are used for

equipment during poultry processing were tested for resistance to bacterial

contamination.  The treatments included sand-blasted, sanded, and electropolished;

and each of these treatments were exposed to natural bacterial populations from

chicken carcass rinses to allow growth of bacteria and development of biofilms on the

surfaces.  The sandblasted surface was a darker gray and uniformly pitted.  Parallel

striations could be seen on the mechanically sanded or ground surface, while the

electropolished surface was mirror-like, very smooth, and shiny. The visible differences

in the surface finishes were confirmed and extended by SEM (scanning electron

micrographs) (Arnold et al., 2000).  Sandblasting pitted the surface, and the pit-marks

observed visually with SEM appeared as "craters," but fewer bacterial cells were

present compared to the untreated control.  Sanding removed the mill finish and

showed scratches and microscopic metal debris embedded on the surface, and even

fewer bacterial cells were present.  The electropolished surface was difficult to image

with SEM because the surface was so smooth and featureless, and few bacterial cells

were present (Arnold et al., 2000). To summarize, biofilm formation significantly

decreased with each finishing treatment.  Sandblasting had the greatest amount of

dispersed cells (1,534), then sanded (1,217), and lastly electropolished (118).  This

demonstrates that the finish of a particular stainless steel surface can affect biofilm formation by bacteria.

4. <u>The Study of *Listeria monocytogenes* by Using Surrogate *Listeria* Strains</u>

*Listeria monocytogenes* has unique growth characteristics that can make it a formidable pathogen to control in the processing environment.  Specifically, it has the ability to grow in cool damp environments where other pathogens may not and is capable of surviving freezing temperatures.  It can also exhibit heat and salt tolerance and is known to form biofilms on food contact surfaces non-food contact environmental surfaces. As a result, *Listeria* persists on these surfaces despite aggressive cleaning and sanitizing.  Once it has established itself, it may persist in the environment for long periods of time until the niche is identified and eliminated.  Because of its pathogenic nature and persistence in the environment, it is not feasible to allow *L. monocytogenes* within commercial processing plants for thermal experiments. This is where surrogates such as *L. innocua* come in to play for researchers. *Listeria innocua* is frequently used as a surrogate for *L. monocytogenes*. *L. innocua* is very similar to *L. monocytogenes* in growth properties, biological characteristics, guanine-plus-cytosine DNA content, 16S rRNA sequence, electroporation transformation rate, and morphology but is not hemolytic and not pathogenic (Zhang et al., 2007).

A surrogate is a bacterium that has physiological characteristics nearly identical to a pathogenic bacterium of interest. A useful surrogate must have thermal heat resistance equivalent to or slightly greater than the target organism, and should be measured in the intended final food product formulation (Fairchild, 1993).  Surrogates

also give a margin of safety to the researchers and prevent unnecessary exposure to pathogens. *L. innocua* M1 is a strain that possesses many of the qualities of an excellent surrogate. The species has been used in several studies. For instance, Francis and O'Beirne (1997) who compared the growth and survival of *L. innocua* and *L. monocytogenes* on minimally processed lettuce under different package atmospheres and storage temperatures, concluded that their behaviors were similar and used *L. innocua* for further studies on the effects of antimicrobial dip on the fate of *L. monocytogenes* on lettuce. This proposed project will further support the claim that surrogate *Listeria* strains can be used in lieu of *Listeria monocytogenes*.

E. Survival of Airborne and Aerosolized Bacteria

    1. Effects of Relative Humidity on Microorganisms

Relative humidity is an important extrinsic factor that affects everyone. It can have a profound effect on people, food sources, as well as the development and persistence of pathogenic microorganisms that could be a threat to society. The definition for relative humidity (RH) is the ratio of the partial pressure of water vapor to the equilibrium vapor pressure of water at a given temperature. It depends on temperature and the pressure of the system of interest. It also requires less water vapor to attain high relative humidity at low temperatures and more water vapor to attain high relative humidity in warm or hot air. In the environment and in home, relative plays an important part in overall health. A healthy balance of humidity must be maintained to prevent the rise of health issues among people. For example, too much house humidity can cause an increase in biological pollutants, like mold, bacteria, viruses, fungi, and

dust mites that can trigger respiratory health ailments. Not enough house humidity can also lead to nosebleeds, painful skin irritation, difficulty breathing and damaging static electricity (Acurite, 2012).  Relative humidity also affects the intensity of chemical pollution in the air by changing the distribution rate of gas from the materials used inside the buildings and the reaction between water and chemicals in the air (Arundel et al., 1986).

The food we produce and market is also affected by relative humidity as well. Besides temperature, the water content of a sample as well as the relative humidity of the ambient air is an important key factor for the flow and structural properties of a broad variety of food. These parameters can be used to quantify sensory attributes like mouth feel, crispness or firmness. These properties are also strongly affected by the water content of the food. Since their water content depends on the ambient temperature and humidity, the control of these parameters is also of great interest regarding the production and storage stability (Perera, 2017).  For example, powder processing must be conducted under controlled relative humidity and temperature to facilitate storage, handling and processing operations.  As the relative humidity of the surrounding air is increased, powders tend to absorb water which may form liquid bridges between powder particles and result in greater powder cohesion and reduced flow ability. On the other hand, as the relative humidity decreases, the powders tend to desorb water, and liquid bridges will disappear for moist insoluble materials, such as glass beads.  For most food powders however, which are soluble materials (such as salt and sugar) solid bridges may remain causing the powder to cake (Teunou, 1999).  This

alone demonstrates how impactful relative humidity is on food. A balance must be obtained to produce and market an acceptable food product.

Lastly, relative humidity can have a strong influence on the development and persistence of pathogenic microorganisms in the ecosystem. Certain bacteria, including Gram-positive bacteria, are more tolerant of high humidity in the air, while others are more tolerant of desiccation and dry conditions (Pepper and Dowd, 2009). For instance, high humidity encourages the spreading of airborne sickness through increased bacteria. Bacteria cells are weakened the most when humidity is in the 40-60% range and numerous studies have shown that outside of this range, it's more plausible that bacteria will survive and multiply. For instance, it's been shown that *E. coli* is less likely to survive in the mid-humidity range. Another example would be *Mycoplasma pneumonia*, which studies have shown will survive longer in high or low humidity levels. Also, the proliferation of *Listeria* is promoted by high humidity and nutrient waste in certain food production plants. One study explored this threshold by exposing four strains of *Listeria monocytogenes* (EGDe, CCL500, CCL128, and LO28) to different RH conditions (75%, 68%, 43% and 11%) with different drying kinetics and then rehydrating either progressively or instantaneously (Zoz, et al., 2016). The main factors that affected the survival of *L. monocytogenes* were RH level and rehydration kinetics. Lowest survival rates (between 1% and 0.001%) were obtained after three hours of treatment under optimal conditions (68% RH and instantaneous rehydration) (Zoz, 2016). Studies such as this show that controlled ambient RH fluctuations could offer new possibilities to control foodborne pathogens in food processing. Similar to bacteria, extreme relative humidity levels are more conducive to the survival of viruses.

Depending on molecular structure, viruses usually prefer either very high or very low humidity levels versus a moderate humidity level. Viruses that prefer humidity under 50% include rubella and measles. However, many viruses, such has adenovirus and coxsackievirus, have a molecular structure that thrives in humidity levels above 70% (Arundel, 1986).  Certain viruses can survive well in both high and low humidity environments. For example, one experiment showed that influenza survival was highest when the humidity was very low (20% RH) or very high (80% RH) with a decrease during the moderate stages of humidity (Arundel, 1986).

2. <u>Nebulizers Used in Medicine and Research</u>

In medicine, nebulizers are drug-delivery devices used to administer medication in the form of a mist inhaled into the lungs.  They are commonly used for the treatment of cystic fibrosis, asthma, COPD and other respiratory diseases or disorders.  These devices use oxygen, compressed air or ultrasonic power to break up solutions and suspensions into small aerosol droplets that can be directly inhaled from the mouthpiece of the device. The definition of an aerosol is a mixture of gas and liquid particles.  There are two main types of medical nebulizers. They are mechanical and electrical, which can be further broken down for specific uses.

Mechanical nebulizers are powered by either oxygen or compressed air to convert liquid to a fine continuous mist.  The main purpose of this nebulizer is to improve breathing by the administration of bronchodilators, mucolytics, or other medications directly into the lungs by means of aerosol mist, and to provide high humidity to assist the breakup of pulmonary and bronchial secretions and aid the person

in coughing (OCDE, 2005).  There are two subtypes of mechanical nebulizers, which

are the soft mist inhaler and the human powered nebulizer.  The soft mist inhaler was

invented by medical company Boehringer Ingelheim in 1997 and is known as the

Respimat Soft Mist Inhaler.  This device provides a metered dose to the user, as the

liquid bottom of the inhaler is rotated clockwise 180 degrees by hand, adding a buildup

tension into a spring around the flexible liquid container. When the user activates the

bottom of the inhaler, the energy from the spring is released and imposes pressure on

the flexible liquid container, causing liquid to spray out of two nozzles, producing two

fine jets of liquid at the outlet that converge at a predetermined angle to form the

aerosol cloud (Anderson 2006).  This cloud contains an aerosol with a fine-particle

fraction (particles smaller than 5.8 μm) at least twice as high as most pMDIs and DPIs,

which would allow a higher proportion of the emitted dose to be delivered to the lungs

and less to the oropharynx (Anderson, 2006).  The human powered nebulizer is the

second subtype and designed to provide relief to patients suffering from respiratory

diseases such as asthma, TB, or chronic obstructive pulmonary disease in areas with

limited access to electricity.  This nebulizer generates a constant flow of air at sufficient

pressure to operate a nebulizer mouthpiece. Human power, generated either by

pedaling or hand cranking, causes air flow via pistons.  The output of the pistons is

connected via tubing and one-way valves with a flow regulator.  The flow regulator

constrains the air flow rate to be within a small range (WHO, 2012).  The benefits of this

device include:  no compliance or technique issues associated with inhalers; lower cost

and maintenance than existing nebulizers; electricity-free and portable.  In comparison,

the HPN was shown to be equivalent to an electric nebulizer in quality of induced

sputum for TB diagnosis.  Particle size distribution, pressure and flow, and volume of

liquid delivered between HPN and an electric nebulizer is equivalent (WHO, 2012).

Electrical nebulizers use electricity or battery power to convert liquid asthma

medicine into a fine mist that's inhaled into the lungs.  The three subtypes include the jet

nebulizer, the ultrasonic wave nebulizer, and the vibrating wave technology nebulizer.

The jet nebulizer is (also known as an atomizer) is connected by tubing to a

compressor, that causes compressed air or oxygen to flow at high velocity through a

liquid medicine to turn it into an aerosol, which is then inhaled by the patient.  These

devices are commonly used for patients in hospitals who have difficulty using inhalers,

such as in serious cases of respiratory disease, or severe asthma attacks (Hickey,

2004).  The ultrasonic wave nebulizer is a portable device that was invented in 1964.

The basis of its technology is that it contains an electronic oscillator that generates a

high frequency ultrasonic wave, which causes the mechanical vibration of a

piezoelectric element.  This vibrating element is in contact with a liquid reservoir and its

high frequency vibration is sufficient to produce a vapor mist (Lee, 2015).  These

nebulizers weigh approximately 6 ounces and have the advantage of being silent during

operation.  The last subtype known as the vibrating wave technology nebulizer was

introduced into the market in 2005.  This technology uses a mesh/membrane with 1000-

7000 laser drilled holes that vibrates at the top of the liquid reservoir, and thereby

pressures out a mist of very fine droplets through the holes. It is more efficient than

having a vibrating piezoelectric element at the bottom of the liquid reservoir, and

thereby shorter treatment times are also achieved.  The vibrating mesh technology is

advantageous over other technologies as it does not destroy the medication due to heat and high pressure (Olszewski, 2016).

3. <u>Sampling for Bioaerosols</u>

There are numerous airborne particles in the environment such as *Listeria* spp. that may affect living organisms directly and indirectly.  These particles are known as bioaerosols and they include viruses, bacteria, proteins, fungal spores, pollens, and plant/animal detritus.  Particle size for bioaerosols range typically from less than 1 um up to greater than 100 um.  Generally, these particles are part of components of larger organisms or contained within liquid droplets (Vincent, 2007).

Across the commercial landscape, there are a large number of bioaerosol samplers that have been developed for monitoring and sampling.  Basically, there are two principle means of monitoring the microbiological population of the air, passive monitoring and active sampling (Rapidmicrobiology, 2015).  Passive monitoring is usually done using "settle plates" or standard Petri dishes containing the appropriate media.  They are opened and exposed for a certain amount of time and then incubated to allow for colony growth.  This application is limited in that the plates are only capable of monitoring viable particles that sediment out of the air.  This method is mainly used for qualitative analysis and is very inexpensive.  Active monitoring requires the use of an air sampler to draw a known volume of air over or through a particle collection device.  The two subtypes of this application include impingers and impactors.  With impingers, they use a liquid medium for particle collection. Sampled air is drawn by a suction pump through a narrow inlet tube into a small flask containing the collection medium. This

accelerates the air towards the surface of the collection medium and the flow rate is determined by the diameter of the inlet tube. When the air hits the surface of the liquid, it changes direction abruptly and any suspended particles are impinged into the collection liquid. Once the sampling is complete the collection liquid can be cultured for growth of viable microorganisms.  Impactors, on the other hand, use a solid medium (agar) for particle collection and is commonly used in commercial applications.  Air is drawn into a sampling head by a pump or fan and accelerated, usually through a perforated plate (sieve samplers), or through a narrow slit (slit samplers). This produces laminar air flow onto the collection surface, often a standard agar plate. When the air hits the collection surface it makes a tangential change of direction and any suspended particles are thrown out by inertia, impacting onto the collection surface (Rapidmicrobiology, 2015).  Once the correct volume of air passage has been achieved, the plate can be removed and incubated without further treatment.

Other types of air samplers can be used as alternatives to impaction or impingement collection techniques.  The most commonly used alternative is filtration, where the air is drawn by a pump or vacuum line through a membrane filter (usually polycarbonate or cellulose acetate in material).  Also, instruments have been developed that are capable of detecting airborne microorganisms in real time. These employ laser technologies to induce fluorescence in any viable particles in air drawn through the instrument and provide immediate detection and enumeration of microbial contaminants.  Examples include the BioLaz® instrument from Particle Measuring Systems and TSI's BioTrak® Real-Time Viable Particle Counter instrument (Rapidmicrobiology, 2015).

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***Chapter in FOOD CONTROL format***

Title.

The Recovery and Transfer of Aerosolized *Listeria innocua* within a Bioaerosol Chamber and on Industrial Surfaces

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Highlights:
- The physical environment of a facility can affect the transmission of *Listeria.*
- Aerosolized *Listeria innocua* was recovered from HDPE coupons, steel coupons, Oxford agar, and air.
- Recovery from HDPE plastic was greatest (mean 1.21 cells/cm$^2$).
- Recovery from surfaces was significantly greater at a relative humidity of ~83% vs. ~65%.
- Recovery from air after 5 and 40 min, ranged from 2.5 to 0.1 CFU/L.

Keywords:

*Listeria innocua*, bioaerosol chamber, nebulizer, aerosolized, steel, HDPE

Abstract

Bioaerosol control in food production environments is important because of the potential for contamination to foods and food contact surfaces. The physical environment of a processing facility can affect the transmission and viability of *Listeria* (growth and death of cells) or other bacteria. The purpose of this work was to quantitate the ability of aerosolized *Listeria innocua* to contaminate surfaces over time.

*Listeria innocua* was nebulized into a customized bioaerosol chamber (~3 x $10^4$ CFU in 1 mL) adjusted to either a low (60-70%) or high (80-90%) humidity. Aerosolized bacteria were allowed to settle on to stainless steel coupons, high-density polyethylene coupons and Oxford agar plates, for either 5, 10, 20 or 40 min, placed at a specific distance from the nebulizer. Additionally, 100 L air samples (50 L/min for 2 min) were collected on gelatin filters either 5, 10, 20, or 40 min after the end of nebulization. All steel coupons, HDPE coupons and gelatin air filters were transferred to Oxford agar, incubated and enumerated.

The three surface samples yielded a greater mean recovery of 2.7 cells/cm$^2$ at ~83% humidity compared to 0.45 cells/cm$^2$ at ~65% humidity. Mean recovery of *L. innocua* from air samples was significantly higher 7 or 12 minutes at 83% humidity after nebulization (2.2 CFU/L) compared to 22 or 42 minutes after nebulization (0.4 CFU/L). Understanding the survival rate of aerosolized *Listeria* and the time interval that these aerosolized bacteria can still colonize a food contact surface will enhance our efforts to prevent transmission. If *Listeria monocytogenes* behaves in same manner as *Listeria innocua* under similar conditions, food industries could apply proper precautions when either organism is detected.

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1. Introduction

   In the public health spectrum, the *Listeria* species is a major concern for everyone who consumes processed and non-processed foods. *Listeria* is a genus of Gram-positive, rod-shaped bacteria, containing a number of species including *L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii and L. grayi.* Although the first four of these have all been implicated in human infection nearly all cases of *Listeria* infection are caused by *L. monocytogenes* (Lawley, 2013). *Listeria monocytogenes* has the potential to be present in all raw foods. Cooked or processed foods can also be contaminated, usually as the result of cross contamination. The latter more often than not is the way the bacteria is spread throughout food systems. The bacteria forms into a bioaerosol and is created from foot and wheeled vehicle traffic through standing water in which the microorganisms have grown. They can also be created by the application of high-pressure washers to contaminated surfaces, the use of compressed air lines that do not have appropriate point-of-use filters in which condensate has accumulated, and by various unhygienic designs of air handling units (Kornacki, 2014). Many consumers and food professionals often don't understand how significant the airborne transmission of *Listeria* can be when infecting food products. There are a very limited number of studies that quantify the airborne transmission and recovery of *Listeria*. This research provides new insight on how certain environmental factors can affect or limit the quantity of *Listeria* in the air and on industrial surfaces indirectly.

   The overall objective of this research was to determine if environmental factors affect the recovery and transmission of aerosolized *Listeria innocua* to a food contact

surface and in the air.  Additionally, this project explored the survival time for aerosolized bacteria within a bioaerosol chamber, the distance from the source that aerosolized bacteria can contaminate a surface in a bioaerosol chamber, and the role of relative humidity on bacteria survival and transmission.

Also in this study, *L. innocua* was used as a surrogate due to its similar growth properties, biological characteristics, guanine-plus-cytosine DNA content, 16S rRNA sequence, electroporation transformation rate, and morphology compared to *L. monocytogenes* (Zhang et al, 2007).  It is not hemolytic and not pathogenic, which makes it safe in experimentation.

Ultimately, understanding the survival rate of aerosolized *Listeria* and the time interval that these aerosolized bacteria can still colonize a food contact surface will enhance our efforts to prevent transmission. If *Listeria monocytogenes* behaves in same manner as *Listeria innocua* under similar conditions, correlations can be made from this small scale study to large platforms (processing plants, grocery stores, packaging facilities).  The food industry could apply proper precautions when either organism is detected.


2.  Materials and Methods

2.1.  Bioaerosol chamber

A polycarbonate box (154 L volume) was constructed to contain aerosolized bacteria.  The chamber inner dimensions were 73 cm long by 43 cm wide by 49 cm high with wall thicknesses of 1.1 cm.  The top side was a hinged lid for access to insert agar plates, surface coupons, and instruments.  Round ports on opposite sides were used to

insert an air sampling device or nebulizer.  The top edges of the chamber frame where

the lid meets were lined with neoprene cord to create a better seal when the lid was

closed for experimentation.  The chamber frame was held together with instant

cyanoacrylate adhesive, continuous steel hinges (sides), and socket cap screws.  The

nebulizer source opening for the chamber was 18.5 cm in height (middle of chamber

side) and 3.5 cm in diameter.  The air sampler source opening was 4.5 cm in height

(middle of chamber, opposite side) and 8 cm in diameter.  The chamber was used

inside a laminar flow hood within a Biological Safety Level 2 (BSL-2) laboratory

throughout the study.


2.2. *Listeria innocua* culture preparation

     *Listeria innocua* strain NCTC 11288 was purchased as a lyophilized pellet from

Microbiologics, Inc. (St. Cloud, MN).  The culture was hydrated and streaked to Tryptic

Soy Agar plus 0.6% yeast extract (Fisher Scientific, Suwanee, GA) and incubated at

35°C for 24 h. Colony isolates were transferred to Tryptic Soy Broth, vortexed, and then

incubated at 35°C for 24 h.  Culture purity was determined with phase contrast

microscope identification and the API *Listeria* biochemical test strip (bioMérieux, Inc.,

Durham, NC).


2.3. *L. innocua* aerosolization

     A portable Voyager™ Pro Vibrating Mesh Nebulizer (Just Health Shops, Fulton,

MD) was used to aerosolize approximately 1 mL of a *Listeria innocua* broth culture.  The

nebulizer was placed outside of the chamber and extended to its fitted chamber port for

bacteria dispersion.  The maximum capacity of the medicine cup used within the

nebulizer is 8 mL, but preliminary research showed that dispersing a 1 mL volume (over

6 minutes) was sufficient to disperse the culture without raising the humidity above 90%

in the chamber.

To aid in dispersion, an O2Cool Pocket Carabiner Fan, Model no. FP02001

(O2COOL, Chicago, IL) was running during and after nebulization (O2Cool, 2017).  This

fan was placed in the chamber corner (adjacent to the source of nebulization

(approximately 5 cm inside the back right corner of the chamber (viewed from the front)

and 14 cm from the source). The fan runs at one speed and had dimensions of 4 x 7 x

18 cm with two blades (7.5 cm length).  After each use, the fan was disinfected with

70% ethanol and short wave ultraviolet light (254 nm).


2.3.1  Humidity control and measurement

All experiments were conducted at ambient temperature (21-23°C) in a

laboratory and biological safety cabinet where the relative humidity was approximately

35-40%).  Humidity was continuously monitored with a Traceable™ Jumbo Thermo-

Humidity Meter (Fisher Scientific, Suwanee, GA).  The humidity level in the chamber

was adjusted to either a low level (60-70%) or high level (80-90%).  To obtain the lower

humidity level, a separate polyethylene tube air line from the biological safety cabinet

was inserted into a stoppered port on one side of the chamber.  Air was pumped into

the chamber, at 10 L/min, until the humidity dropped from ~40% to 25%.  The air supply

was closed and 1 mL of culture was dispersed over ~6 min which caused the humidity

to rise to approximately 70%.  When air samples were collected, the air supply would be

introduced again and replacement air would be pushed into the chamber on the opposite side until the end of the sample time.  After each air sampling, the humidity level would drop to a mean level of 65%.

To obtain the higher humidity level, an air supply was not introduced prior to nebulizing the culture.  After aerosolizing 1 ml over 6 min, the humidity would increase from ~40% to ~90%.  When air samples were collected, the air supply would be introduced and replacement air would be pushed into the chamber on the opposite side until the end of the sample time.  After each air sampling, the humidity level would drop to a mean level of 83%.

2.4.    Recovery of *L. innocua*

2.4.1.  Recovery from agar plates, steel coupons and plastic coupons

Oxford *Listeria* Agar (Neogen Corp., Lansing, MI) plates, stainless steel coupons, high density polyethylene coupons were placed in the chamber for *Listeria* collection. Oxford *Listeria* agar plates (~90 mm diameter, 59.4 cm$^2$ agar area) were prepared at least two days in advance to ensure a dry surface. After 24 hours of incubation, *L. monocytogenes* or *L. innocua* form olive-green colonies surrounded by a black halo.

Stainless steel coupons (Speedy Metals GB LLC; Little Chute, WI) were type 304, hot rolled, annealed and were pre-cut to approximately 2.5 cm x 2.5 cm squares (~6.25 cm$^2$ area).  This type of steel is commonly used in food processing and preparation equipment.  Test coupons were cleaned with acetone and sterilized with UV irradiation.  Each coupon was submerged in 400 mL of acetone for 20 min, and then blotted with paper towels and air dried for 10 min.  Next, the pieces were placed on a

sterilized plastic surface under a short wave UV lamp for 30 min on each side. After, disinfection, coupons were stored in sterile specimen cups prior to use.

High density polyethylene (HDPE) (Interstate Plastics, Sacramento, CA) coupons were pre-cut into approximately 2.5 cm x 2.5 cm squares (~6.25 cm$^2$ area). High density polyethylene (0.910-0.925 g/cm$^3$) is more rigid and less permeable than low density polyethylene (LDPE) (U.S. Plastic Corp., 2013). These coupons were initially rinsed in distilled water and sterilized under a long wave UV lamp for 30 min on each side. They were then stored in sterile specimen cups before use.

Prior to aerosolizing a *L. innocua* culture, six each of the plastic polyethylene coupons, stainless steel coupons, and Oxford agar plates (uncovered) were placed in the bioaerosol chamber in two rows. Each row included three zones with one steel coupon, one plastic coupon and one Oxford agar plate. The distance of the nebulizer to the center of the coupons or agar plates ranged from 55-62 cm in zones 1 & 4, 47-54 cm in zones 2 & 5, and 39-47 cm in zones 3 & 6. Specific locations of the plates and coupons are described in Appendix A.

Oxford *Listeria* Agar was used for the recovery and enumeration of aerosolized *Listeria innocua* from the steel and plastic coupons. After coupons were exposed to aerosolized *L. innocua*, they were aseptically removed from the chamber and the exposed side was placed onto an Oxford agar plate and rotated around the plate four times. Plates were incubated at 35° for 24 hours and then enumerated. The exposed agar plates from the chamber were similarly incubated and enumerated.

2.4.2. Air sampling and recovery

A Sartorius Airport MD8 Air Sampler (Sartorius Corp., Bohemia, NY) was used to sample the air after nebulization.  For each culture nebulized, the air sampler was placed at the end port constructed on the bioaerosol chamber and 100 L of air was sampled (50L per minute, for 2 min).  An air sample was collected, starting at either 5, 10, 20 or 40 minutes after nebulization ended.  This occurred simultaneously with coupons and agar plate samples in the chamber.

Aerosolized organisms were collected onto water-soluble gelatin filters (39 cm$^2$ area, 250 μm thickness) in the air sampler head.  After sample collection, filters were aseptically placed on to Oxford agar, incubated at 35° for 24 hours and then enumerated.  Each filter dissolved in to the agar within 15 minutes.  The hydrophilic gelatin filters are usually directly dissolved in fluids at 37°C for cell extraction (Chang et al., 2015).  Placing filters directly on the agar, rather than dissolving in broth, will enable a lower limit of detection of aerosolized organisms.


2.4.3.  Recovery from bioaerosol chamber surfaces

After removal of exposed coupons and plates, three interior chamber surfaces (inner lid – 73 x 43 cm, back wall – 73 x 49 cm, and one side wall – 43 x 49 cm) were swabbed entirely in a "z" motion in two passes with individual 3M™ Sponge-Sticks wetted with Buffered Peptone Water Broth.  The three sponge sticks were combined into 50 mL of peptone water within a stomacher bag.  This stomacher bag was agitated for 1 minute, then 0.1 mL was plated on Oxford agar in duplicate.  All plates were incubated at 37°C for 24 h and examined for growth.

2.5  Experimental design

Separate one mL portions of a *L. innocua* broth culture, adjusted to ~3 x $10^4$ CFU/mL were nebulized into a customized bioaerosol chamber.  The relative humidity in the chamber was adjusted to either a low (60-70%) or high (80-90%) level.  Aerosolized bacteria were allowed to settle on to six stainless steel coupons, six HDPE coupons six and Oxford agar plates for either 0-5 min, 0-10 min, 0-20 min, or 0-40 min.  The tested times were done in separate experiments.  All surfaces were exposed starting at 0 minutes. Additionally, a 100 L air sample (50 L/min for 2 min) was collected either 5, 10, 20, or 40 min after the end of nebulization.  All steel coupons, HDPE coupons and gelatin air filters were transferred to Oxford agar, incubated and enumerated.  Cell recoveries on all media were analyzed as concentrations per surface area or per liter of air and compared for statistically significant differences.  All test combinations of chamber humidity (2) and sample time (4) were repeated in triplicate for a total of 24 separate cultures of *L. innocua* aerosolized into the chamber.  Cleaning and sterilization occurred after each repetition (coupons and chamber surface).  Ethanol (70%) was used to clean the coupons, chamber surface, nebulizer, and air samplers, followed by UV irradiation to sterilize the inside and outside of the chamber surface.  The results of at least 100 other culture nebulizations conducted as preliminary research are summarized in Appendix A.  All *Listeria* populations were converted to $\log_{10}$ CFU/ml for analysis.  Analysis of statistical tests was performed using Minitab 17 (Version 17.1.3 Copyright 2013, Minitab Inc., USA).  Comparisons of log reduction values of specific surface types in conjunction with different factors (humidity level, contact time, and distance from the source) were determined using ANOVA with Tukey's HSD at $\alpha = 0.05$.

In cases where plates were too numerous to count (TNTC), a count of 250 cells was assigned.

3. Results

In this study, the objective was to determine if environmental factors (humidity, contact time, distance) affect the recovery and transmission of aerosolized *Listeria innocua* to a food contact surface and in the air within a bioaerosol chamber.  The mean cell recovery per area for each test surface, across time, are shown in Figures 1, 2 and 3.  Cell recovery was much lower for samples collected 20 or 40 minutes after nebulization, and was much higher under 83% humidity for all surfaces and times combined.  At the same time, these figures show that average cell recovery trended upward with 65% humidity for steel, plastic and agar surfaces exposed for 20 or 40 minutes after nebulization.

The mean cell recovery from air samples (gelatin filters), across time, is shown in Figure 4.  Recovery was significantly higher ($p<0.05$) from the air when samples were collected 5 or 10 minutes after nebulization at 83% humidity (mean 2.2 CFU/L) compared to collection after 20 or 40 minutes or compared to all times under 65% humidity (mean 0.4 CFU/L).

The highest air sample recovery was 2.5 mean cells recovered/L (or 376 cells in estimate recovery for the entire chamber), which occurred at 83% humidity and 5 minutes after nebulizing the bacteria.  The lowest air sample recovery was 0.1 mean cells recovered/L (or 12.5 cells in estimate recovery for the entire tank), which occurred at 65% humidity and 40 minutes after nebulizing the bacteria.  The mean number of *L.*

*innocua* recovered from air samples was generally lower than recovery from the combined surfaces at each sample time. Air samples were collected for 2 min, while surface samples were collected for either 5, 10, 20 or 40 min in length. For all combined air and surface samples tested in this study, mean cell recovery under high humidity conditions was 1.45% at each sample time, and 0.77% under low humidity conditions.

No statistically significant differences in recovery were found between plastic, steel and agar surfaces located at different distances from the nebulizer (Table 1). For each material the distance between the closest and farthest pieces, from the nebulizer, was only 16 cm. Recovery was generally higher for test surfaces located closes to the nebulizer (zones 3 & 6). At each distance from the bacteria source, CFU recovered per sample area was highest for HDPE coupons, followed by steel coupons, and then Oxford agar plates.

When looking at the three different types or surfaces evaluated, HDPE coupons showed the greatest mean cell recovery per square centimeter of surface area (Figure 5). Mean recovery of 1.21 CFU/cm$^2$ was 2.5 X recovery from Oxford agar (mean 0.49 CFU/cm$^2$), but the total cells recovered on Oxford agar was much higher than the number recovered on the coupons due to the larger surface area of the agar plates.

Maximum cell recovery per area (2.80 cells recovered/cm$^2$) occurred on plastic coupons at 83% humidity and when sampled 5 minutes after nebulization of the bacteria. By extrapolation to the entire base surface of the chamber, this would represent a total of 9,956 cells that could be recovered if the floor of the chamber was HDPE and could be sampled in the same manner as the HDPE coupons. This mean cell recovery represents approximately one-third of the cells nebulized. For samples

collected after 40 minute exposures on HDPE coupons (83% humidity), the recovery

per area (0.25 CFU/cm$^2$) was 11X less than for samples collected at 5 minutes.  The

minimum concentration of cells recovered, from all surfaces, was on stainless steel after

40 minutes (83 % humidity).  A mean concentration of 0.04 CFU/cm$^2$ can be

extrapolated to just 126 total cells recovered or ~0.4% of the cells that were aerosolized.

Even though Oxford agar plates yielded a lower overall number of *L. innocua*

cells per square centimeter of surface than the HDPE or steel surfaces, the mean

recovery after 20 or 40 minutes was higher than recovery from steel under the low and

high humidity conditions.  And, the mean recovery on Oxford agar after 40 minute

exposures was higher than recovery from HDPE after 40 min and under low humidity

(~65%).  Additionally, the recovery from Oxford agar decreased over time from 1.20

CFU/cm$^2$ to 0.16 CFU/cm$^2$ at higher humidity, but recovery increased over time at the

lower humidity from 0.1 CFU/cm$^2$ to 0.86 CFU/cm$^2$.

All tested surfaces combined (polyethylene coupons, stainless steel coupons,

and Oxford agar plates) yielded a significantly higher ($p < 0.05$) mean recovery under

83% humidity (2.7 cells/cm$^2$) than under 65% humidity (0.45 cells/cm$^2$).   And, for the

three surfaces combined, the mean total cells recovered for all sample times under high

humidity conditions (1,279 CFU) was nearly twice as high as the number of cells

recovered under lower humidity conditions (691 CFU).

4.  Discussion

In this first part of the research project, the main objective was to determine if

humidity, distance from the source, and time after nebulization for three different surface

types would affect the transmission of aerosolized *Listeria innocua* and its recovery from agar, food contact surface materials or air.  The surface type made a significant difference in bacteria recovery on average, as well as the humidity level.  Specifically, attachment and recovery was most likely dependent on surface finish/hardness, the amount of humidity present (moisture buildup), and hydrophobic/hydrophilic properties of the surface type. Additionally, air samples trended to yield lower recovery at longer times after nebulization due to the deterioration of viable cells over time (this followed the same for surfaces).  Lastly, the factor of distance from the source did not have a significant impact on recovery, probably because the chamber air was well-mixed due to the action of the fan inside it.

L. *innocua* recovery on a per area basis was highest on the HDPE plastic (1.21 cells/cm$^2$) while recovery from steel was only 0.86 cells//cm$^2$. This result is most likely due to the surface finish/hardness between the two industrial surfaces. In this experiment, the industrial coupons used were high density polyethylene plastic and 304 hot-rolled, annealed stainless steel.  The higher number of bacteria recovered on plastic may be due to its softer and more porous surface when compared to the stainless steel surface, allowing bacteria and moisture to attach and buildup easier and at a higher rate.  The microscopic crevices or pores within the plastic are more abundant/existent, which allows for more bacterial attachment and growth.  Since the steel coupons have a smoother finish with a denser makeup, bacteria have a more difficult time attaching and growing.  Stainless steel is generally considered to be a hydrophilic material, whereas plastics are considered hydrophobic materials. *Salmonella* and *L. monocytogenes* were

reported to attach in higher numbers to more hydrophobic materials such as rubber and plastic (Sinde and Carballo, 2000).

Numerous studies have cited that bacterial attachment is dictated by several factors, such as surface conditioning, surface charge, surface roughness, growth medium, and hydrophobicity of the contact surface to bacterial cells (Boulané-Petermann et al., 1997; Bayoudh et al., 2006; Palmer et al., 2007; Tresse et al., 2007; Goulter et al., 2009, Nguyen et al., 2011).

Several investigators have found that microorganisms attach more rapidly to hydrophobic, nonpolar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals (Dolan, 2002). These findings align with the result produced in this experiment, for recovery was significantly higher in the plastic coupons compared to the stainless steel coupons. Veluz et al., (2012) tested the adhesion of *L. monocytogenes* cells (Scott A, Brie 1, and ATCC 6744) to six different types of conveyor belts over 48 hours. Among the six materials tested, higher mean numbers of *Listeria* cells (4.0 to 4.3 cfu/cm$^2$) were found attached to the polyurethane with mono-polyester fabric conveyor belt compared with other polymer materials (acetal, polypropylene-meshtop, polypropylene) and two stainless steel belts (stainless steel-single loop (SSSL), and stainless steel-balance weave (SSBW)). Attachment of *Listeria* to the polyurethane belt was typically 1 to 2 cfu/cm$^2$ higher than the three other plastics or the steel belts. This previous study coincides with the results of this study, for it shows that softer/more porous and hydrophobic surfaces will be more liable to retain higher numbers of bacteria.

Oxford agar is specifically used for the recovery and enumeration of *Listeria innocua* in a laboratory setting.  In preliminary research, recovery of *L. innocua* on Oxford agar was similar to recovery on Tryptic Soy Agar (+ 0.6% yeast extract) and to recovery on Bio-Rad Rapid *Listeria* chromogenic agar.  Unexpectedly, out of the three surfaces compared, the Oxford Agar plates generated the least amount of bacterial recovery per surface area (average 0.49 cells/cm$^2$).  This relatively lower concentration of colonies per square centimeter could be due to the hydrophilic nature of the agar. Or, the higher surface concentrations found on the steel and plastic coupons could be due to the breakup of cell clusters that may have occurred when transferring these cells from coupons to agar.

Also, time after nebulization of the bacteria had an effect on the recovery of *Listeria innocua*.  Sampling 5 and 10 minutes after nebulization yielded significantly higher recovery across all surfaces (including air) compared to sampling 20 and 40 minutes after nebulization.  The average for 5 and 10 minutes after nebulization was 1.36 cells recovered/cm$^2$, while 20 and 40 minutes after was 0.35 cells recovered/cm$^2$. The lower recoveries over time (especially significant after 20 minutes) could simply be due to bacterial deterioration. Suspended cells will simply dry out, become injured, or non-viable over time.  Future research in this area should consider exposing sample surfaces for no more than 10 minutes, since samples that were exposed for 20 or 40 min in this study yielded much lower recoveries than surfaces exposed for 5 or 10 min.

Humidity played a significant role in cell recovery as well. When analyzing the results of all of the surfaces and air samples together, it was discovered that the tested humidity of 83% yielded a significantly higher recovery compared to that of tested 65%

humidity.  The 83% humidity level exhibited an average recovery of 1.24 cells/cm$^2$ across all sample types, while recovery was only 0.47 cells/cm$^2$ at 65% humidity.  A lowered or minimal humidity level in an industry setting would be desirable for reducing the presence of Listeria on food contact surfaces. In comparison with a surrogate, an increase in relative humidity (RH) was shown to prolong survival of *L. monocytogenes*, as well as encourage growth when inoculated on fresh produce, while a decrease in RH demonstrated a decreased survival of *L. monocytogenes* (Rodríguez et al., 2007).

In contrast to the other experimental factors, the factor of distance from the source statistically had no significant impact ($p > 0.05$) on the amount of bacterial recovery from the surfaces (Oxford agar, plastic, and steel).  It may be concluded from this and other previous experiments that distance from the source will not have a significant impact on bacterial survival and recovery within a close vicinity (0-100cm). For example, a previous study on *Pseudomonas syringae* and *Erwinia herbicola* determined that the slope of regression lines generated from bacterial survival in non-aerosolized control samplers and samplers located 1 m from the site of release were not significantly different from 0 m (Walter et al., 1990).  *P. syringae* aerosolized at 3 to 15 m from the site of release at a temperature of 12°C and a relative humidity of 80% survived 35 to 65-fold better that *P. syringae* released at 27°C and a relative humidity of 40% (Walter et al., 1990). The change in bacterial survival occurred mainly due to a change in relative humidity, which was the case in this performed research project.
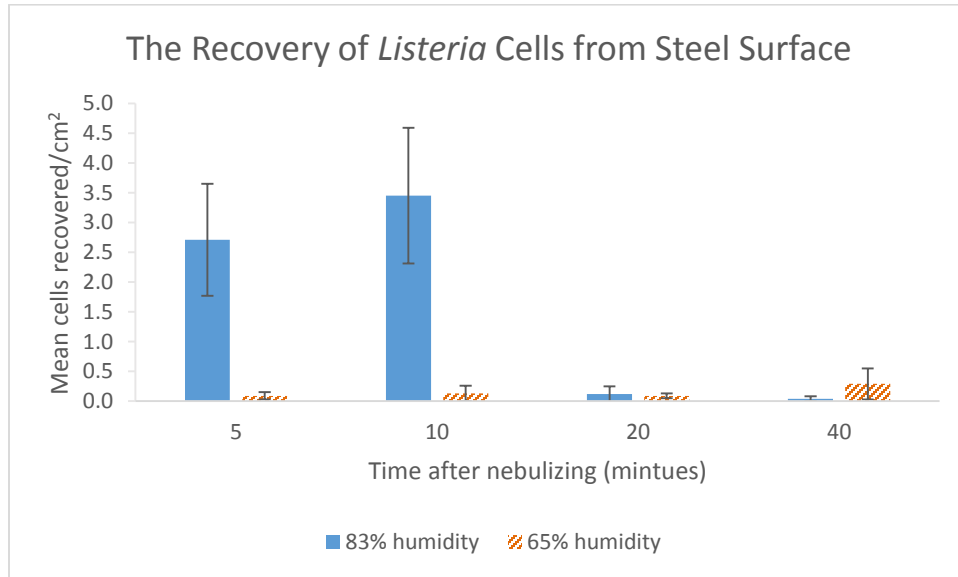
Acknowledgments

Figure 1.  The average cells recovered from plastic surface over time at 83% and 65% humidity (all distances combined). Bacteria was nebulized using the Voyager Pro Vibrating Mesh Nebulizer.



The Recovery of *Listeria* Cells from Plastic Surface
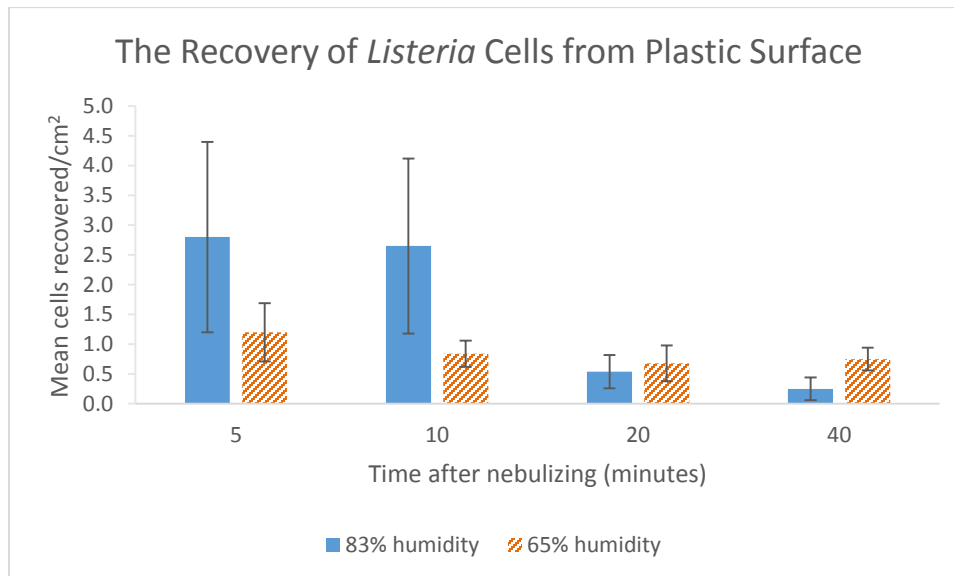
*n = 144 total plastic samples
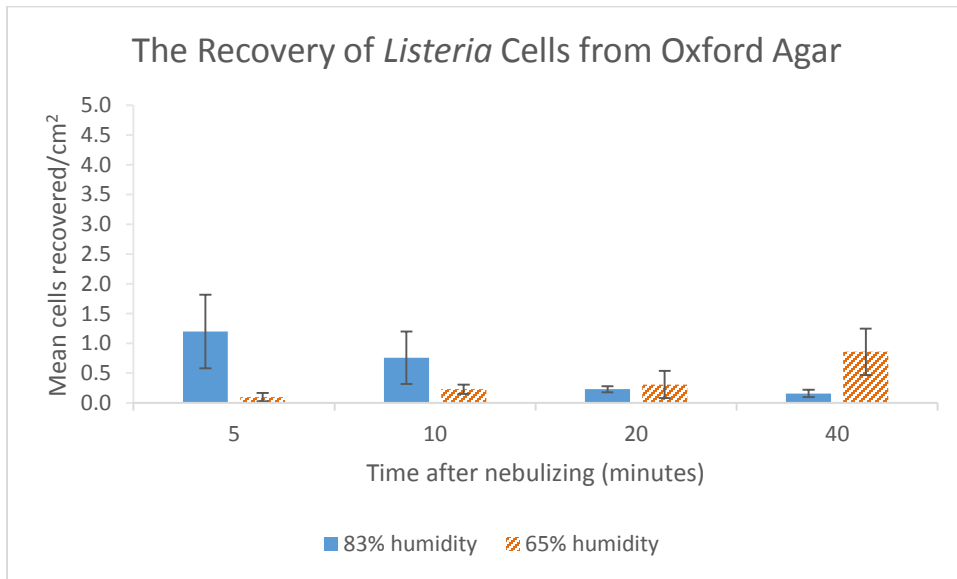*error bars represent standard deviation (SD)

Figure 2.  The average cells recovered from steel surface over time at 83% and 65%

humidity.  Bacteria was nebulized using the Voyager Pro Vibrating Mesh Nebulizer.



*n = 144 total steel samples
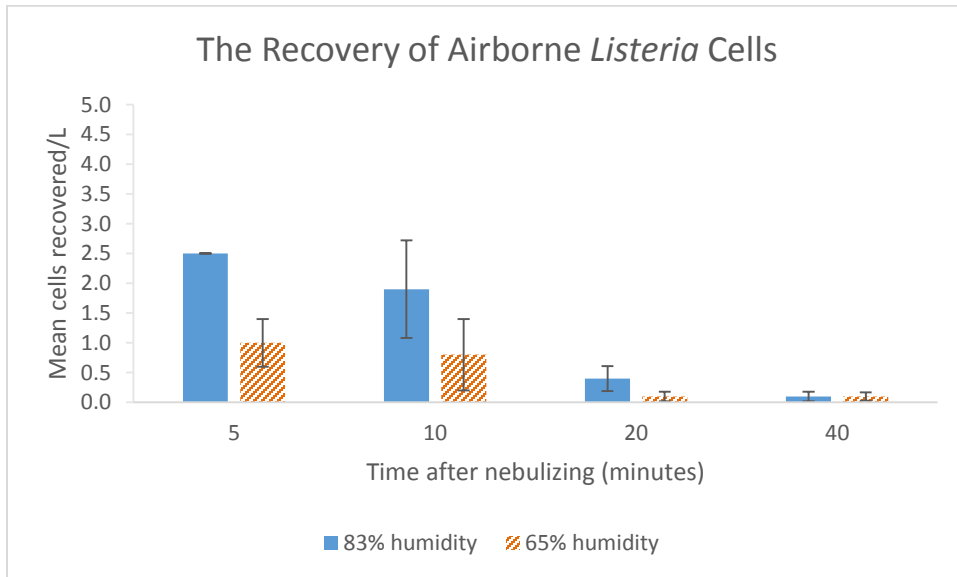*error bars represent standard deviation (SD)

Figure 3.  The average cells recovered from Oxford agar over time at 83% and 65%

humidity. Bacteria was nebulized using the Voyager Pro Vibrating Mesh Nebulizer.



*n = 144 total Oxford agar samples
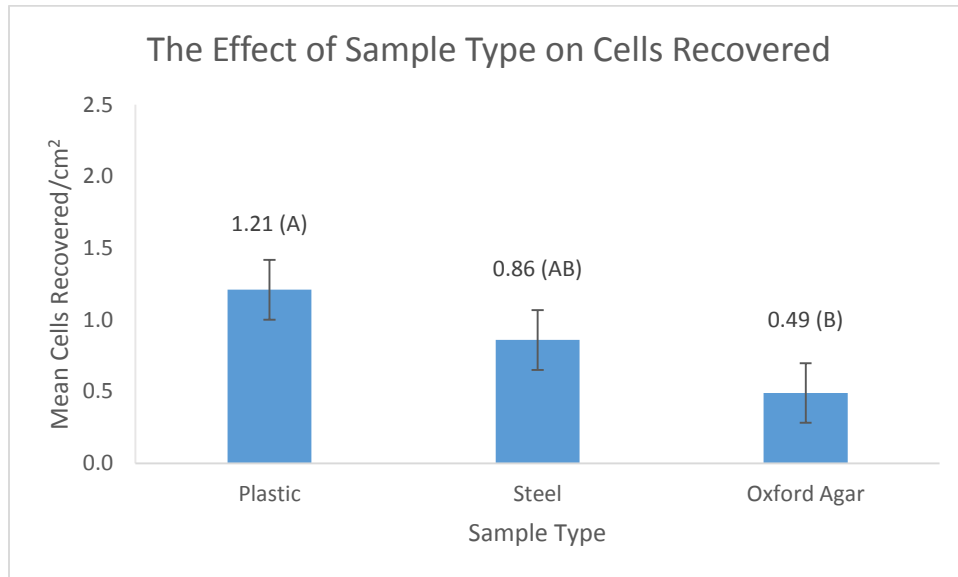*error bars represent standard deviation (SD)

Figure 4.  The average cells recovered from air sampling over time at 83% and 65%

humidity. Bacteria was nebulized using the Voyager Pro Vibrating Mesh Nebulizer.



*n = 24 total air samples (gelatin filters)
*error bars represent standard deviation (SD)

Figure 5. The effect of material type on the average cells nebulized. Bacteria was aerosolized using the Voyager Pro Vibrating Mesh Nebulizer.



The Effect of Sample Type on Cells Recovered

*n = 432 total surface samples
*error bars represent standard error (SE)

Tables

Table 1.  Mean cells recovered/cm$^2$ of *Listeria innocua* by sample type and distance from source.

| Location | HDPE | | Steel | | Oxford Agar | |
|---|---|---|---|---|---|---|
| | mean CFU/cm$^2$ | mean distance (cm) | mean CFU/cm$^2$ | mean distance (cm) | mean CFU/cm$^2$ | mean distance (cm) |
| *Zone 1/4* | 1.29 [Aa] | 55 | 0.55 [ABa] | 61 | 0.39 [Ba] | 62 |
| *Zone 2/5* | 1.15 [Aa] | 47 | 0.74 [ABa] | 53 | 0.43 [Ba] | 54 |
| *Zone 3/6* | 1.20 [Aa] | 39 | 1.31 [ABa] | 46 | 0.64 [Ba] | 47 |

n = 144 for total surface samples

Significant differences (p<0.05) in overall mean Tukey HSD statistic between rows are designated with a lower case superscript letter.

Significant differences (p<0.05) in overall mean Tukey HSD statistic between columns are designated with an upper case superscript letter.

Refer to Figure 4 in Appendix B for a diagram of zones.

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*Chapter in FOOD MICROBIOLOGY format*

Title.

Recovery of Aerosolized *Listeria innocua* within a Walk-in Refrigeration Unit

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Highlights: (3 – 5 bullet points on the core results, up to 85 characters each, submitted to journal as a separate file)

- Refrigeration units can affect the transmission and viability of *Listeria.*
- Explored the ability of *Listeria innocua* to contaminate a surface after aerosolization.
- More bacteria settled onto agar plates in the first 10 minutes, than over the next 50 minutes.
- After 20 min, *L. innocua* concentrations from air samples dropped from 6.8 to 3.2 CFU/L.

Keywords: *Listeria innocua*, nebulizer, aerosol transfer, Oxford agar, refrigeration

**Abstract**


The control of airborne *Listeria* is important because of the great health and economic impact it can present. Airborne pathogens can cause severe infection suspended in the air or by attaching to various food processing surfaces. These and other bioaerosols may be created from foot and wheeled vehicle traffic, high-pressure washers, air handling units, etc. The purpose of this work was to quantitate the ability of *Listeria innocua* (a surrogate for *L. monocytogenes*) to contaminate air or surfaces aerosolization into a walk-in refrigeration unit.

*L. innocua* was nebulized into an 11 $m^3$ walk-in cooler where RH ranged from ~29-37%. Aerosolized bacteria were collected on to Oxford agar plates for 10 min intervals and with 50 or 100 L air samples over 60 minutes. In the walk-in cooler, total estimated mean recovery from Oxford media at 10 min after nebulizing was 0.48%, but only 0.04% for samples collected after 60 minutes. The recovery of *L. innocua* from air samples after 5 min was 6.8 CFU/L, but 2.8 CFU/L after 60 min. No significant difference in recovery was found between plates at different distances (2 – 2.5 m) from the nebulizer in the walk-in cooler. Quantitative studies such as this give better insight on the survival rate of these airborne microorganisms. Understanding the survival of aerosolized *Listeria* and how it can colonize over time on a food contact surface will enhance our efforts to prevent transmission on a small and large scale.

1.  Introduction

Listeria monocytogenes is the pathogenic bacterial species in the genus Listeria that causes human listeriosis, a serious infection that can cause Encephalitis or meningoencephalitis in immuno-compromised adults.  Approximately 1,591 cases of foodborne listeriosis and 306 fatalities occur annually in the United States at an estimated cost of $2.8 billion, making listeriosis the third costliest foodborne illness after salmonellosis and Toxoplasma gondii (Food Safety News, 2015).

Many of these cases stem from factory environments containing strains of Listeria in and around the workplace.  Listeria monocytogenes is very widespread in the natural environment and is likely to be reintroduced into food production facilities (Tompkin, 2002).  Ready-to-eat food products (raw or cooked) can be contaminated via indirect and direct cross contamination.  It has been found that the introduction of Listeria to these products usually occurs after the products have been processed. This contamination may occur during additional handling steps such as peeling, slicing and repackaging. Also, in the retail and food service environment, contamination may be transferred between ready-to-eat products (Lianou and Sofos, 2007). In addition, the bacteria can travel through the air by forming into bioaerosols that are usually created from foot and wheeled vehicle traffic through standing water.  Its biological makeup also gives it an advantage over non-psychrotrophic microbes.  Extremely slow growth of L. monocytogenes has been recorded at temperatures as low as -1.5ºC and the maximum temperature for growth is generally accepted as 45ºC.  Its optimum temperature for growth is between 30 ºC and 37 ºC (Lado and Yousef, 2007).

In general, many consumers and food professionals often don't understand how significant airborne and cross transmission of *Listeria* can be when infecting food products.  Most studies that have been done do not quantitatively analyze the survival and recovery rates of *Listeria*, just qualitatively.  This research provides information on how distance and time can affect the quantity of *Listeria* in the air and on industrial surfaces.  It gives insight on the behavior of *Listeria* in a real world environment, which is limited application in food science research.

The overall objective of this research is to determine if contact time, distance from the source of nebulization, temperature, and humidity affect the survival and transmission of *Listeria innocua* to an industrial surface and airborne within a walk-in cooler.  This project can lead to comparisons being made between smaller scale studies and real world applications in the food industry, ultimately predicting the ability of aerosolized or airborne *Listeria* to contaminate a surface.

In experimentation, *L. innocua* was used as a surrogate for *L. monocytogenes* as mentioned previously.  A surrogate strain is usually used because *L. monocytogenes* is pathogenic in nature and is not feasible to test within commercial processing plants for thermal experiments.  *L. innocua* was used as a surrogate in this study due to its similar growth properties, biological characteristics, guanine-plus-cytosine DNA content, 16S rRNA sequence, electroporation transformation rate, and morphology compared to *L. monocytogenes* (Zhang et al, 2007).  It is not hemolytic and not pathogenic, which makes it safe in experimentation.

It is imperative to understand the survival and recovery rates of *Listeria* to determine what protocols and safety measures need to be amended.  Quantitative

studies such as this will give consumers and food professionals a more precise idea of how impactful *Listeria* can be under certain conditions.

## 2.  Materials and Methods

### 2.1  Aerosolization chamber

A commercial walk-in refrigerator (cooler) was used to contain aerosolized cultures of *Listeria innocua*.   Manufactured by Hillphoenix, Inc. (Conyers, Ga), the cooler exterior dimensions were 165.7 cm x 287.6 cm x 235.2 cm (w x d x h) with an interior volume of approximately 11,000 L (Appendix B, Figures 4, 5 and 6).  This interior volume was approximately 70X larger than the polycarbonate test chamber used in the previous chapter. This wood framed unit had a wall and ceiling finish of 26-gauge Stucco White internally and externally and the structure is reinforced with PVC Screed (Hillphoenix, 2016).  The flooring is concrete, which is the laboratory floor.  The door was hinged with a latch and accessories include one pole switch w/ pilot light and dial thermometer.  The refrigerator was used within a Biological Safety Level 2 (BSL-2) processing plant laboratory at the Virginia Tech Department of Food Science and Technology.

### 2.2.  *Listeria innocua* culture preparation

*Listeria innocua* strain NCTC 11288 was purchased as a lyophilized pellet from Microbiologics, Inc. (St. Cloud, MN).  The culture was hydrated and streaked to Tryptic Soy Agar plus 0.6% yeast extract (Fisher Scientific, Suwanee, GA) and incubated at

35°C for 24 h. Colony isolates were transferred to Tryptic Soy Broth, vortexed, and then

incubated at 35°C for 24 h.  Culture identity was confirmed with phase contrast

microscope identification and the API *Listeria* biochemical test strip (bioMérieux, Inc.,

Durham, NC).  Fresh cultures were prepared, and diluted to ~2 x $10^6$ CFU/mL, for each

of the four replicated experiments discussed below.

2.3.  *L. innocua* aerosolization

A portable Voyager™ Pro Vibrating Mesh Nebulizer (Just Health Shops, Fulton,

MD) was used to aerosolize approximately 1.5 mL of a *Listeria innocua* broth culture.

The maximum capacity of this nebulizer is 8 mL, and preliminary research determined

that 1.5 mL could be sprayed in ~9 minutes.  The nebulizer was placed in a sterile cup

and suspended 2 feet from the ceiling in the middle of the cooler (Appendix B, Figure 7).

The cup was suspended by cutting holes on each side of it and tying a loop using

bendable pipe wire, which securely hung around the cooler's light fixture.  The

maximum capacity of the medicine cup used within the nebulizer is 8 mL.

2.4  Humidity and temperature and measurement

All experiments were conducted with the cooler door closed and the refrigeration

unit turned off.  Temperature and relative humidity was continuously monitored with a

Traceable™ Jumbo Thermo-Humidity Meter (Fisher Scientific, Suwanee, GA).  The

mean temperature for each of the four trials (~60 min each) was 58 – 66°F (14 – 19°C).

The mean humidity during each trial was 29 to 37%.  Both humidity and temperature

would incrementally increase over the course of 60 minutes for each trial.

2.5.    Recovery of *L. innocua*

2.5.1. Recovery from agar plates

Oxford *Listeria* Agar (Neogen Corp., Lansing, MI) plates were used for the recovery and enumeration of aerosolized *Listeria innocua*.  Previous research indicated that recovery from Oxford agar was similar to recovery from Tryptic Soy Agar plus 0.6% yeast extract.  Oxford *Listeria* agar plates (~90 mm diameter, 59.4 cm$^2$ agar area) were prepared at least two days in advance to ensure a dry surface.  Plates were taped onto Readi-Board Foam Boards (20 x 30") using double sided adhesive tape. Five plates were taped to each board.  Four sets of boards were used for each trial and each set contained four boards (total of 80 agar plates).  Prior to aerosolizing a *L. innocua* culture, the Oxford agar plate lids were removed before the boards were stacked.  One board without plates was placed on top of each stack.  Stacks of boards (with agar plates) were placed in the cooler in four locations (Appendix B, figures 6, 8, 9).  All plates were located between 2.0 and 2.5 m from the nebulizer.

Nebulization of the culture lasted 9 min.  One minute afterwards, the top layer of agar plates was exposed to the air for 10 min (sample point "10 min"). Then, these plates were covered and the next layer of plates was exposed for 10 min (sample point "20 min").  Then, this set of plates was covered, and the third layer of plates was exposed for 10 min (sample point "30 min").  Then, this layer of plates was covered, and 20 minutes later the final layer of plates were exposed for 10 min (sample point "60 min").  After the final layer of plates are covered, all plates were removed from the cooler and incubated at 35°C for 24 hours and then enumerated.

### 2.5.2.  Recovery from air samples

Two Sartorius Airport MD8 Air Samplers (Sartorius Corp., Bohemia, NY) were used to sample the air after nebulization.  For each culture nebulized, the air samplers were suspended and mounted on opposite ends of the walk-in cooler as shown in Appendix B, Figure 8. The air samplers were suspended and secured against the middle short walls using adjustable shower curtain rods (117 cm from the floor), which fit into the handles of the air samplers.

Air was sampled at the rate of 50 L/min for 1 or 2 min.  One air sample was collected starting at 5, 10, 15, 20, 25, 30, 55, and 60 min after nebulization ended. Sampled air was drawn through a gelatin membrane filter.  The filters are water soluble with a pore size of 3 μm, approximate thickness of 250 μm and an area of ~39 sq. cm. After each air sample was collected, the gelatin filter was the removed from the air sampling device and placed into a sterile bag.  After all samples were collected, filters were aseptically placed on to Oxford Listeria Agar, incubated at 35°C for 24 hours and then enumerated.  Each filter dissolved in to the agar within 15 minutes.  Placing filters directly on the agar, rather than dissolving in broth, enabled a lower limit of detection of aerosolized organisms.  Air sample volumes were decreased when a previous trial yielded plate counts >250 CFU/plate (gelatin filter).

### 2.5.3.  Recovery from refrigeration unit surfaces

Interior wall surfaces were swabbed with individual 3M™ Sponge-Sticks wetted with Buffered Peptone Water Broth.  Four plates were used for sponges taken from the

cooler surface which was marked at 6 locations (2 plates each for 3 locations swabbed at 10 min after nebulization and 2 plates for 3 locations not previously sampled but in relative vicinity at 60 min after nebulization.  The rectangular swabbing areas were marked with masking tape and measured 78 x 46 cm each. One sponge was used per location per time and sponges were combined, for each time, into 50L of peptone water within a stomacher bag.  This stomacher bag was agitated for 1 minute, then 0.1 mL was plated on Oxford agar in duplicate.  All plates were incubated at 37°C for 24 h and examined for growth.  The cooler was sanitized after each session using Ster-Bac Quat by Ecolab Inc.  The floor was mopped with the solution and the walls were wiped down as well.

2.6  Experimental design

Separate 1.5 mL portions of a *L. innocua* broth culture, adjusted to ~2 x 10$^6$ CFU/mL were nebulized into a commercial walk-in cooler unit with the refrigeration apparatus turned off.  Aerosolized bacteria were allowed to settle on to 20 Oxford agar plates for (4 each) 10 min periods. Additionally, a 50 or 100 L air sample (50 L/min for 1 or 2 min) was collected 8 times after the end of nebulization.  All gelatin air filters were transferred to Oxford agar, incubated and enumerated.  All samples were collected within one hour after nebulization ended.  Sample collection was replicated four times (four separate cultures of *L. innocua* aerosolized into the chamber).  Cell recoveries on all media were analyzed as concentrations per surface area or per liter of air and compared for statistically significant differences.  All *Listeria* populations were converted to log$_{10}$ CFU/mL for analysis.  Analysis of statistical tests was performed using Minitab

17 (Version 17.1.3 Copyright 2013, Minitab Inc., USA).  Comparisons of log reduction values of specific surface types in conjunction with different factors (humidity level, temperature, contact time, and distance from the source) were determined using ANOVA with Tukey's HSD at $\alpha = 0.05$.  In cases where plate counts were recorded as TNTC, a count of 250 cells was assigned.

3.  Results

As a consensus, the Oxford agar plates yielded less recovery as time after nebulizing increased.  For each of the four trials, the total cells of *Listeria innocua* nebulized ranged between $3.75x10^6$ and $1.13x10^6$.  By extrapolating cell recoveries per $cm^2$ of agar to estimated cell recoveries for the entire base surface of the cooler, the mean recovery, 10 min after nebulizing the *Listeria innocua*, was 0.48% (average for all four experiments at this specific time).  The estimated recovery for the entire floor of the cooler for the 20 min sample (10 – 20 min after nebulization) was 0.18%. Estimated recovery of aerosolized bacteria after 30 min and 60 min was 0.08% and 0.04%, respectively (Figure 1).

The mean recovery from Oxford agar plates was highest for plates exposed for the first 10 min sampling period (0.24 $CFU/cm^2$).  Recovery on agar was lowest during the final sampling period, 60-70 min after nebulization (0.02 $CFU/cm^2$).  In each trial, the mean distance between the agar plates and the nebulizer was ~227 cm. The recovery on Oxford Agar on a per area basis was much lower for the current study than the recovery reported in the previous chapter.  In the smaller scale study within a benchtop chamber, the mean distance between the nebulizer source and the agar plates was ~61

cm. and recovery ranged from 1.20 CFU/cm$^2$ for the first 5 min sample period to a mean of 0.16 CFU/cm$^2$ after 40 minutes of plate exposures.  The five-fold lower recovery per agar surface area for this cooler study could be attributed to the greater distance of the plates from the bacteria source and also to the lower humidity (29-37%).

Distance from the source statistically had no significant impact on the amount of bacterial recovery from the surface (Oxford agar) in the walk-in cooler.  For example, run #2 yielded results for the different locations that were statically the same.  At 10 min after nebulization, 2256 mean cells were recovered/m$^2$ in combined locations RC and LC (239 cm from nebulizer) while RF and LF (214 cm from nebulizer) yielded 2458 mean cells recovered/m$^2$.  At 60 min after nebulization, 211 mean cells were recovered/m$^2$ in combined locations RC and LC while RF and LF yielded 185 mean cells /m$^2$.

The air samples taken for this study followed a similar trend as with the Oxford agar plate layers. As time after nebulizing increased in minutes, the mean recovered airborne organisms decreased (Figure 2).  The highest mean percentage recovered of *Listeria innocua* was 5.26% at 5 min after nebulizing.  At 20 min after nebulizing, the mean percentage recovered decreased by more than half to of 2.44%.  The lowest mean percentage was 1.35% at the final time of 60 min after nebulization.  Figure 3 displays a steady increase in the cumulative percentage rate at which airborne *Listeria* can be recovered of the course of 60 minutes.

Humidity was measured and monitored in conjunction with the temperature.  A constant holding humidity level was not feasible due to the nature of the cooler system.  Humidity levels ranged from 29% to 37% for these experiments.  There was no

significant trend in humidity level versus total percentage of recovery (Figure 4).  While the recovery (1.25%) was highest at the lowest humidity level (29%), the recovery was similar (0.55 to 0.65%) at the other mean humidity levels (32, 35, 37%).

*L. innocua* was aerosolized after the refrigeration unit was turned off, and when the temperature in the unit was ~60°F.  The mean temperature during each sampling period ranged from 58 to 66°F (14.4 to 18.9°C).  Estimated recovery for each trial ranged from 0.55% to 1.25% (Figure 5).  For run #1, the average temperature was 58 °F and yielded a recovery of 0.65%.  The highest recovery occurred when the mean temperature was 65°F, but the recovery was only half (0.64%) during the trial with a similar mean temperature (66°F).

Swabs of the cooler wall surfaces were taken at three specific locations.  The locations were swabbed at 10 minutes after nebulization and again at locations not previously sampled but in relative vicinity at 60 minutes after nebulization.  The cooler surface yielded zero bacterial counts at all three locations, at both sample times, and four all four runs performed.  During the 3rd and 4th run, the swabs were combined and diluted with *Listeria* enrichment broth, which was then incubated and plated on to Oxford agar.  Still, no bacterial counts were produced from the cooler surface.

## 4.  Discussion

In this part of the research study, the objective was to determine if contact time, distance, and temperature affect the survival and transmission of *Listeria innocua* to an industrial surface and in the air within a walk-in cooler while maintaining consistent

temperature and humidity. The surface type made a significant difference in bacteria recovery, for the swabs from the cooler surface yielded no bacterial counts while the Oxford agar layered plates did.  The result of no bacterial growth may be due to the orientation, roughness, and design of the cooler surface that was sampled.  The cooler walls were swabbed using sponge sticks, so the sampling process was vertical in orientation. The wall finish was 26 Ga. Stucco White internally and externally with a grained pattern (porous in nature).  The combination of surface orientation and roughness could have definitely contributed to the lack of bacterial growth from the cooler surface.  A recent study looked at the bacterial response to surface topography during biofilm formation using 5 µm tall line patterns of poly dimethylsiloxane (PDMS). Cell cluster formation per unit area on 5 µm wide line patterns was reduced by 14-fold compared to flat PDMS. Also, the cells attached on narrow patterns were longer and had higher transcriptional activities, suggesting that such unfavorable topography may present a stress to attached cells (Gu et al., 2016).   Another study on *Candida albicans* biofilms demonstrated similar results. It evaluated the influence of the substratum positioning.  The organism was added to poly methylmethacrylate (PMMA) disks, incubated, and the biofilm structure was analyzed. Vertically oriented disks showed less biofilm formation and lower metabolic activity than the horizontal groups (p< 0.05) (Cavalcanti et al., 2013).  The study pointed to gravity as a possible reason for the lower vertical counts.  Gravity may impair deposition in the vertical position but facilitate the deposition horizontally (Soll, 2002). These studies along with the result of this experiment shows that surface design (vertical and complex in pattern design) can help reduce microbial attachment.

Oxford agar plate layers of 10 min and 20 min after nebulization yielded significantly higher recovery compared to the layer of 60 minutes after nebulization. The average recovery for the entire room floor for all experiments at times 10 minutes and 20 minutes after nebulization was 0.41%, while the average recovery at time 30 minutes and 60 minutes after nebulization was 0.058%. The recovery was 7 times greater 20 minutes or less after nebulization compared to 30 minutes or more after nebulization. The lower recoveries over time are most likely could due to bacterial deterioration. Suspended cells will simply dry out, become injured, or non-viable over time.

When looking at the results for the Oxford agar plates and air samples together, it was revealed that the small changes in humidity during experimentation (29-37%) yielded no firm trend in cell recovery (Figure 4). For example, the first run with an average humidity of 29% yielded 1.25% total recovery and the third run with an average humidity of 35% yielded 0.65% total recovery. And surprisingly on the fourth run, total recovery was 0.64% but the average humidity was at 37%. There was no correlation to draw on, perhaps due to the small increment increase in humidity or the lack of repetitions of the same experimental runs. In most cases with bacteria, higher humidity levels can be expected to support more growth and survival, but the difference in humidity levels was small (mean 29-37%) between trials. One research study on *Listeria monocytogenes* looked at its survival on stainless steel at three temperatures (4, 10, and 21 °C) and three humidity levels (11, 50, and 85%). A portion of the results indicated that the medium humidity level enhanced survival (irrespective of temperature), while the lower humidity decreases recovery at all temperatures (Redfern, 2017). Another possible reason for the lack of high humidity equals growth trend is that

the bacteria may be selective for its optimal growth environment.  For example, Influenza (even though a virus) has distinct transmission patterns around the world. The virus peaks during the winter in temperate regions while in tropical regions it will peak during rainy seasons (Yang et al., 2012).  With that being said, *Listeria* may have a range of specific factors to yield optimal growth, with humidity being partially important.

Temperature did not have an effect on cell recovery either.  As temperature of the cooler increased the amount of cell recovery varied (Figure 5).  The difference in mean maximum and minimum temperatures (8C°) of the refrigeration unit may have been too small to appreciably effect the rate of settling of the bacteria, or the humidity or the agar surfaces.  Generally, it is known that as temperature increases, bacterial growth increases to a certain extent.  A study looked at a broader range of temperature changes and observed a similar concrete result.  It looked at the survival and growth of *Listeria monocytogenes* in fresh coleslaw.  In experimentation, a decline in viable numbers of *L. monocytogenes* in coleslaw at pH 6.0 occurred at 4 °C and at 15 °C, whereas at 25 °C the viable count of *L. monocytogenes* increased initially and remained high after incubation for 25 days. The organism grew rapidly in coleslaw at pH 7.0 at all three temperatures studied, followed by an equally rapid decline in viable count (George, 1990).  It can be said that the bacteria will increase in rate of growth as temperature increases.  However, a temperature greater than 170 °F (known critical control point) is the organism's limit and will be hot enough to kill the bacteria (Wagner, 2008).

Air sampling also generated significant results in cell recovery during the large scale study.  As mentioned in the results, the mean number of cells recovered from the air decreased as the time after nebulizing increased.  At 25 minutes after nebulization, the mean percentage recovered decreased by more than half with a percentage of 2.32%.  This is because of the natural deterioration or decay of viable cells over time.  A similar qualitative study showed similar results over an extended period of time.  In the study, the number of *L. innocua*–positive plates decreased gradually from the 30 min to 3 h samplings for trials with either 105 or 103 CFU of *L. innocua* per liter of air.  Also, *L. innocua* positive plates decreased consistently to a low level of contamination (0 of 9 or 1 of *L. innocua* positive) at the 2.5-h and 3.0-h sampling times (Zhang et al., 2007). This further demonstrates the general decrease in viable cells over time qualitatively.  By analyzing and comparing studies over an hour time frame, it can be estimated that the mean recovered airborne proportion for the entire cooler over two or three hours will be less than 1.5% since the data shows 1.35% bacteria recovered airborne at 60 minutes. A quantitative study on the survival of *Listeria monocytogenes* Scott A on metal surfaces also supports this conclusion as well.  On copper, brass, aluminum bronze and silicon bronze, no viable bacteria could be detected after 60 min incubation during this study, indicating a 5 log reduction (the detection limit of the procedure was 100 bacteria) (Wilks et al., 2006).

Many studies and reports have found that infectious bacteria can be found a significant distance from the original source. Bacteria such as *Listeria* can easily enter a building through an HVAC air intake, spreading throughout via the air-handling system. Building materials, carpets, clothing, food, and pests are also known sources of

introduction of airborne particles into a commercial or industrial building (Fernstrom, 2013). Distances of 1 to 3 meters from the source seem to not affect the vitality of airborne *Listeria*.  For example, one study in a slaughterhouse showed where the highest counts of airborne positive air samples of *Listeria seeligeri* (3 positive air samples, n = 63) and the highest number of aerobic mesophilic bacteria (CFU/m$^3$) were determine on the location near the carcass evisceration at the sampling levels of 0.5, 1.0, and 2.0 m over the floor.  At this location the eviscerated entrails have to drop at least 0.5 m from the carcass and are splashed onto a trolley standing on the floor, which is lifted to the viscera conveyor into which the entrails are thrown (Dobeic et al., 2011). This study as well as our research demonstrate how *Listeria* can contaminate the air as well as various surfaces within 3 meters of a source).
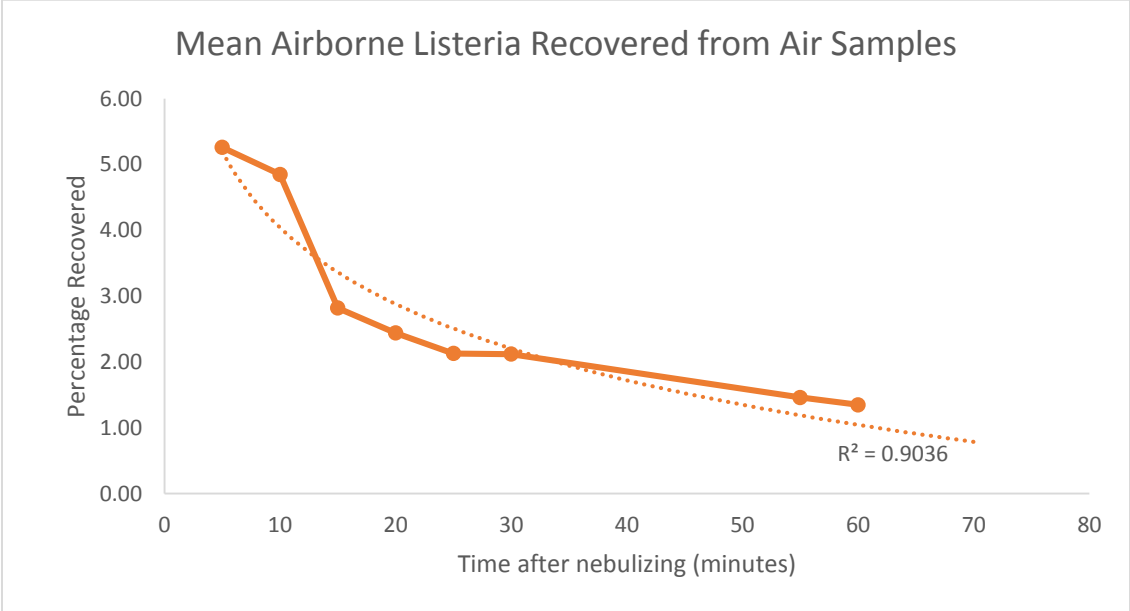
Figures

Figure 1.  The average cells recovered from the cooler floor.  Oxford agar plates represented the floor and extrapolation was calculated and applied. Bacteria was aerosolized using the Voyager Pro Vibrating Mesh Nebulizer.
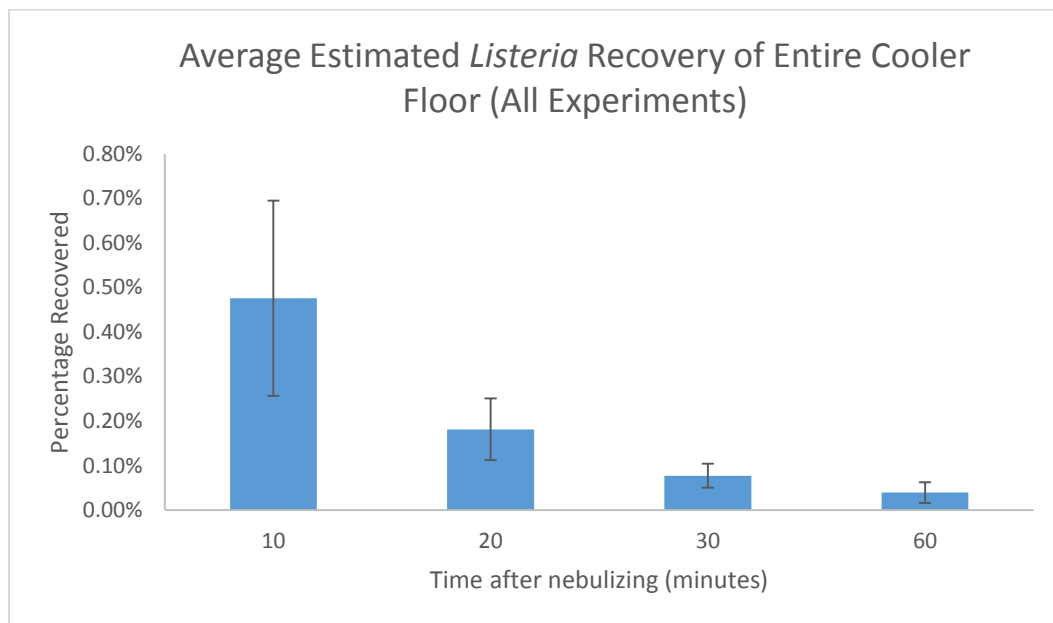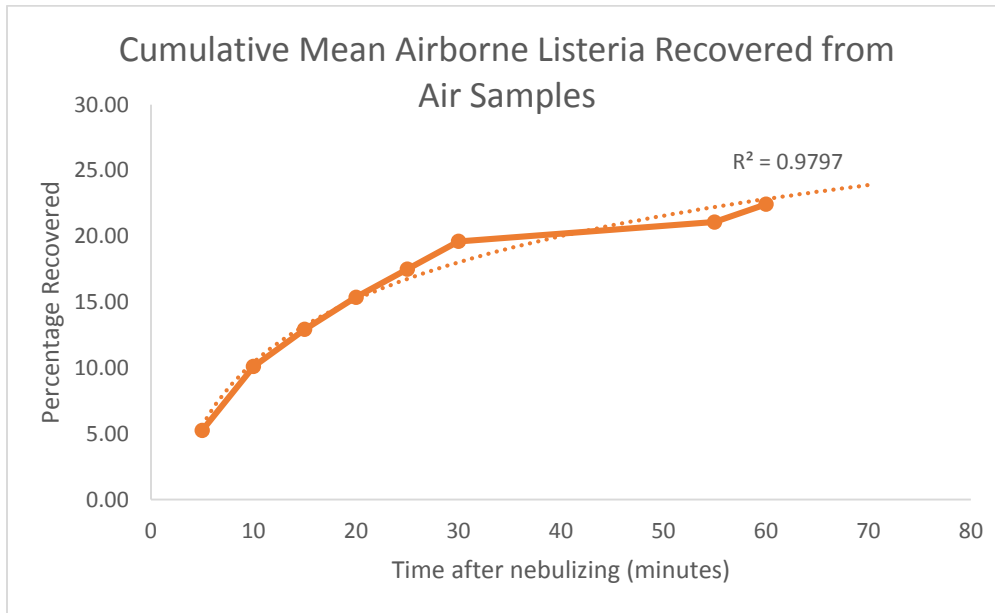


*n = 320 Oxford agar plates

*error bars represent standard deviation (SD)

Figure 2.  The average percentage of total cells recovered from the air.  Bacteria was
aerosolized using the Voyager Pro Vibrating Mesh Nebulizer.



**Mean Airborne Listeria Recovered from Air Samples**

$R^2 = 0.9036$

Percentage Recovered

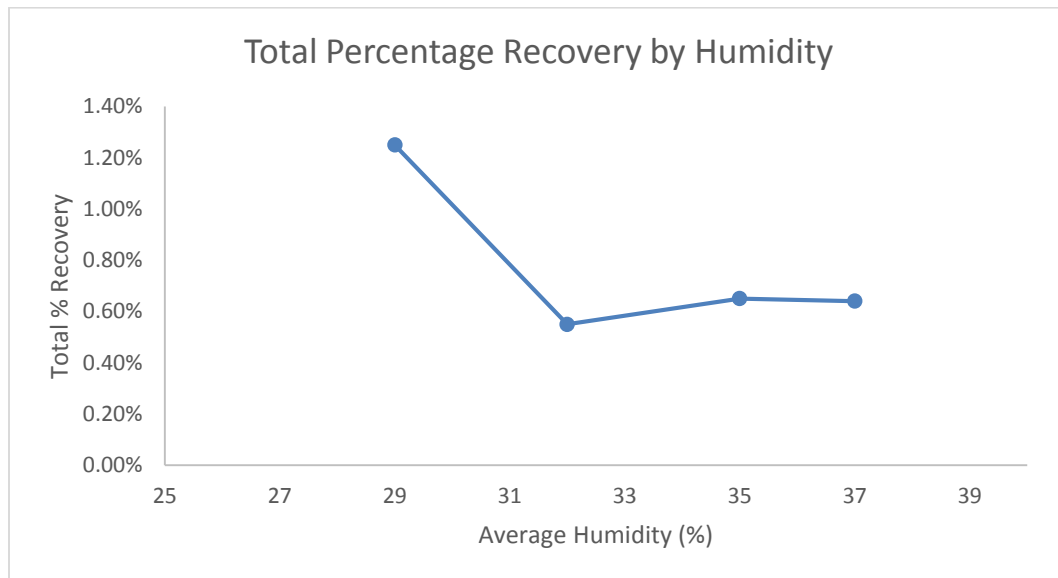Time after nebulizing (minutes)

*n = 32 air samples (gelatin filters)

Figure 3. The cumulative mean percentage of total cells recovered from the air.

Bacteria was aerosolized using the Voyager Pro Vibrating Mesh Nebulizer.



*n = 32 air samples (gelatin filters)

Figure 4.  The effect of humidity on the average percentage of total cells recovered from the cooler floor.  Oxford agar plates represented the floor and plate area was extrapolated to floor area. Bacteria was aerosolized using the Voyager Pro Vibrating Mesh Nebulizer.



*Total Percentage Recovery by Humidity* — chart plotting Total % Recovery (y-axis, 0.00% to 1.40%) against Average Humidity (%) (x-axis, 25 to 39).

*n = 320 Oxford agar plates

Figure 5.  The effect of temperature on the average percentage of total cells recovered from the cooler floor.  Oxford agar plates represented the floor and plate area was extrapolated to floor area. Bacteria was aerosolized using the Voyager Pro Vibrating Mesh Nebulizer.
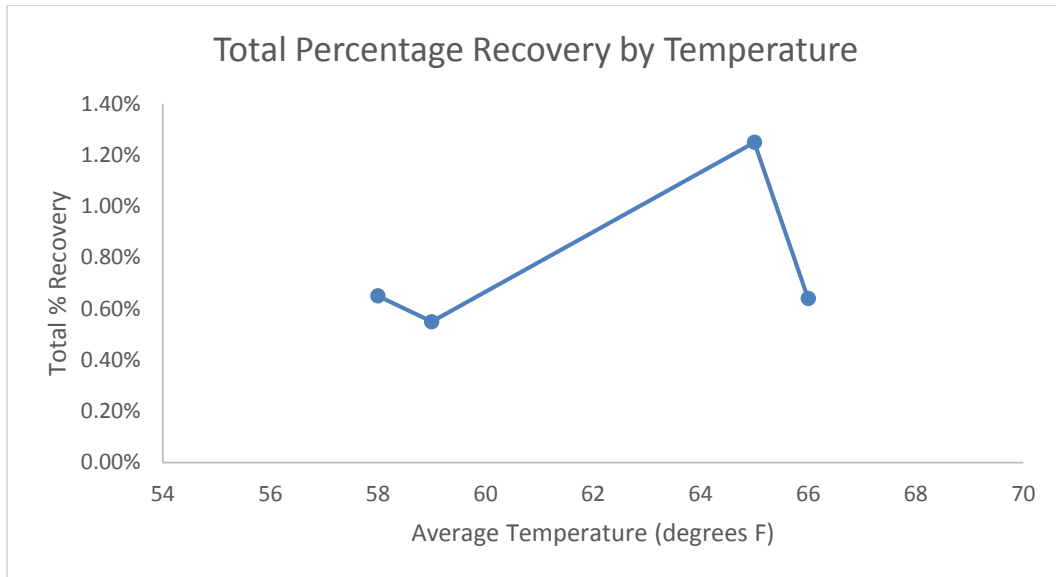


*n = 320 Oxford agar plates

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Conclusion

The aerosolized experiments within the bio chamber and the walk-in refrigerator unit correlated well with one another. Both experiments exhibited the potential for *Listeria* species to containment the air as well as industrial surfaces under various conditions.  The environmental factors of humidity, temperature, distance from the source, and contact time played a significant role in the recovery of the bacteria from the tested surfaces and air samples.  In general comparison, both studies yielded results that would in enable food professionals to predict the ability of aerosolized or airborne *Listeria* to contaminate a surface as well as its viability over time.

The tested samples that correspond for both experiments were the Oxford agar plates and the air samples.  When evaluating the results of the Oxford agar samples versus the air samples for both experiments, it was discovered that both experiments followed a similar trend in bacterial recovery.  In the first experiment with the chamber, the Oxford agar in high humidity (83%) on average decreased from 1.20 mean cells recovered per $cm^2$ at 5 minutes after nebulization to 0.23 mean cells recovered per $cm^2$ at 20 minutes after nebulization (a decrease of more than 5 times).  For the air samples in the chamber at the same humidity, the bacterial recovery decreased from 2.5 mean cells recovered per L at 5 minutes after nebulization to 0.4 mean cells recovered per L at 20 minutes after nebulization (a decrease of more than 6 times).  The trend was similar with the second experiment as well.  In the larger scale walk-in cooler, the Oxford agar on average decreased from 0.643% mean estimate recovery of the entire room floor at 10 minutes after nebulization to 0.181% at 20 minutes after nebulization (a

decrease of more than 3.5 times).  For the air samples in the walk-in cooler, the

bacterial recovery decreased from 5.26% mean recovered airborne at 5 minutes after

nebulization to 2.44% mean recovered airborne at 20 minutes after nebulization (a

decrease of more than 2 times).  These comparable results demonstrate significant

decreases in recovery and viability over time.  These results could be of importance in

applications such as cleaning or maintaining industrial systems.  For example, food

professionals will be able to make more precise recommendations on waiting times to

use a cleaned surface (when *Listeria* is considered nonviable or inactive).  Also, this

and similar studies could lead to the development of a universal system that models the

time of inactivation of *Listeria monocytogenes*.  For instance, a mathematical model was

developed to predict time to inactivation by high pressure processing of *L.*

*monocytogenes* in a broth system as a function of pressure, inoculum level, sodium

chloride, and sodium lactate from a 4° C initial temperature (Youart, 2010). Different

elements were used in this model but the goal remains the same, which is to help

manufacturers eliminate the threat of *Listeria* from processing environments.

In conjunction with these results, it was apparent that the plastic surface coupons

yielded the highest recovery compared to all other samples in the first experiment.  Its

average recovery was 1.213 cells recovered/$cm^2$ within the chamber (steel was 0.86

cells recovered/$cm^2$, 2nd in ranking).  With such a significant gap in recovery, it's

presumed that the recovered results would be similar if the plastic coupons were tested

in the large scale walk-in cooler of the second experiment.  Other studies have exhibited

similar results as well.  One study looked at the adhesion and viability of *Listeria*

*monocytogenes* on various surfaces qualitatively.  It was concluded that Polypropylene

surfaces (plastic) displayed the highest percentage of viable bacteria (nearly 100%), whereas marble and granite had a lower percentage of cultivable cells, 69.5 and 78.7%, respectively (Silva, 2007).

In the future, these two experiments could be expanded upon to gain more valuable information on the behavior of the *Listeria* species.  Time after nebulization could be extended from 60 minutes to 120 minutes or more.  A similar study with aerosolized *Listeria* actually showed that high populations of *L. monocytogenes* Scott A and *L. monocytogenes* V37CE survived in aerosol suspensions generated under these conditions for 210 minutes (Spurlock, 1991).  Also, the surface and air samples could be analyzed vertically instead of horizontally.  The orientation from which the bacteria is sampled could yield a different result and have specific benefits.  For example, many food plants have grill levels and conveyor lines that ascend vertically.  One particular study addressed this by evaluating the translocation of *Listeria* from floor drains to vertical food contact surfaces.  Results showed significantly higher translocation at 1 foot above the drain (up to 25%), followed by 3 feet (up to 11%) and 5 feet high (up to 2.7%) (Saini et al., 2012).

In closing, research on the recovery and transfer of *Listeria* is vital to the public spectrum as a whole.  Understanding the survival rate, time interval, and environmental conditions in which that aerosolized *Listeria* can colonize on a food contact surface will enhance our efforts to prevent transfer.

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Appendix A: Preliminary Research

The research described below was conducted over a nine-month period to optimize the methodology for quantitative recovery of aerosolized *Listeria innocua* from air and surface samples presented in Chapter II and III.

1.  Nebulization of *L. innocua* culture

    a.  Nebulizer selection

    Initially, two types of battery powered nebulizers were evaluated for dispersing a defined volume of a bacterial culture solution in less than 10 min.  The MiniBreeze Ultrasonic nebulizer and the Omron Micro-Air Electronic nebulizer were initially compared for their ability to dispense a consistent volume over time and proportion of viable cells that could be recovered from the sprayed solution.  Both are different in design, the MiniBreeze uses a piezoelectric crystal vibrating at high frequencies (1-3 MHz) to produce an aerosol, while the Omron Micro-Air Electronic uses multiple openings in a mesh plate or open plates to generate an aerosol (micro pump).  The MiniBreeze Ultrasonic nebulizer left a larger volume of solution behind after similar periods of nebulization.  Additionally, the MiniBreeze was determined to be less effective since a significantly lower proportion of viable cells could be recovered after nebulization.  The cause of the loss of cells is likely due to the heat generated by this ultrasonic nebulizer.  The Omron Micro-Air Electronic was considered to be preferable for future experiments, but the design of this particular nebulizer was not compatible

with the dimensions and openings of the custom designed bioaerosol chamber.  An

extension device would have been required to direct the spray from outside the

chamber through a port to the inside, but this could have compromised cell viability

during nebulization.  A similar style of nebulizer, the Voyager Pro Vibrating Mesh

nebulizer (Just Health Shops, Fulton, MD), was used for the work described in Chapters

II and III.  This nebulizer could be placed inside the chamber when operated.


b.  Rate of culture volume dispensed

Both the Omron Micro-Air Electronic Nebulizer System # NE-U22V1 and the

MiniBreeze Ultrasonic (vibrating mesh) Nebulizer were compared for their ability to

aerosolize 5 mL of a *L. innocua* culture. Three concentrations of the cultures (~$10^4$, $10^5$

or $10^6$ CFU/mL) were studied.  Cultures were nebulized into the bioaerosol chamber

which also contained a small electronic fan to help ensure that aerosolized *L. innocua*

was distributed throughout in the chamber.  The Ultrasonic Nebulizer was operated from

outside the chamber, while the Micro-Air Nebulizer was placed just inside the chamber

in a similar location.

For each nebulizer, at least 10 min was needed to disperse 5 mL of *Listeria*

*innocua* culture.  Temperature and humidity were recorded every 2 minutes for 10

minutes during nebulizing.  The chamber humidity increased to >90% after 5 mL

inoculum nebulized into chamber.  The large increase in humidity led to adjusting the

culture concentration so that only 1 mL, instead of 5 mL, needed to be aerosolized into

the chamber.  The Voyager Pro Vibrating Mesh nebulizer could nebulize 1 mL of culture

solution in approximately six minutes.

c. Determination of optimum culture concentration for nebulizing

Based on the trial experiments below that utilized different culture concentrations, nebulizing volumes, agar collection media, air sample volumes, sample collection times, optimal recovery of *L. innocua*, for 40 minutes after nebulization, could be achieved if the concentration of *L. innocua* solution for the nebulizer was ~$10^5$ CFU/mL.  Nebulizing 1 mL of this concentration would likely lead to colony growth on agar settle plates and agar plates from transfer of gelatin filters from the air sampler.  Since the variation in number of organisms recovered from the air was greater than the variation in organisms recovered on settle plates, the air sample volume was adjusted to 50 L, 100 L, or 200 L depending on the time (after nebulization) for sample collection.

2. Recovery from air samples

Initially, two trials for air sample collection were performed using the Sartorius Airport MD8 air sampler.  The air sampler was attached to the outside of the chamber, and a 100 L air sample was collected onto a gelatin filter.  Air samples (50 L/min for 2 min) were collected at 1 min and 3 min after the aerosolization was completed. After the collection, gelatin filters were transferred onto two Oxford *Listeria* Agar (Neogen Corp., Lansing, MI) plates or Tryptic Soy Agar plus 0.6% yeast extract (Fisher Scientific, Suwanee, GA) plates and incubated at 35°C for 24 hours.

Air samples (50 L/min for 2 min) collected at 3 min after aerosolization recovered 1.1 CFU/L air, while air samples (50 L/min for 2 min) collected at 5 min after

aerosolization recovered 0.7 CFU/L air. In addition, approximately 0.2 – 0.4% of the initial inoculum was recovered by a 2 min air sample (3 or 5 min after aerosolization)

Next, 100 L air samples were collected every 5 minutes for 30 minutes (after nebulization ended). Only one air sample could be collected for each culture nebulized, therefore organism recovery at each of six times (5, 10, 15, 20, 25, 30 min post-nebulization) and for each culture concentration ($\sim 10^4$, $10^5$ or $10^6$ CFU/mL), and for each nebulizer (2) required disinfection of the tank prior to introducing the culture.

The results of these studies revealed that the recovery of *L. innocua* from air (gelatin filters) was approximately 3.2 X higher when using the vibrating mesh nebulizer versus the ultrasonic nebulizer. Additionally, recovery from Oxford agar was within 25% of the number recovered from Bio-Rad Rapid *Listeria* chromogenic agar.


3.  Recovery from agar settle plates


In addition to air samples, sets of 6 agar plates without lids were placed in the chamber so that aerosolized organisms could be captured when they settled out of the air. Both Bio-Rad Rapid *Listeria* chromogenic agar and Oxford agar plates (3 of each) were used for each culture nebulized. For each nebulizer (2) and culture concentration (3) described above, agar plates were exposed for 5, 10, 20 or 30 min. In this experiment, the recovery of *L. innocua* from agar plates was ~3 X higher when using the vibrating mesh nebulizer versus the ultrasonic nebulizer. Additionally, recovery on Oxford agar was consistently higher (10 – 30%) than recovery from Bio-Rad Rapid *Listeria* chromogenic agar.

4.  Recovery of *L. innocua* from steel and HDPE coupons


In addition to the open agar plates, sterilized polyethylene and stainless steel coupons (2.5 cm x 2.5 cm) were placed in the chamber prior to nebulization of *L. innocua* cultures.  Several recovery methods were tried to maximize the number of viable cells that could be enumerated from the coupons after nebulization.  First the coupons were swabbed with poly tipped applicator swabs (6 for each type) and placed in 10 mL of peptone, then plated out (steel and plastic swabs were separate). No counts were produced.  Next coupons were combined in groups of 3 in a bag with 20 mL of peptone.  After vigorous shaking, the plated solutions yielded a small number of colonies.  Another method was tested in which the coupons were taken after nebulization and placed directly on the agar plates.  The side that was exposed was placed down on that side on the plate, and then rotated in a circular motion (four complete revolutions) to cover the entire plate.  Plate counts were generally more than 10 per coupon.  For future experiments, agar plates were thoroughly surface dried before use to ensure that isolated colonies could be enumerated.


5.  Location of steel and plastic coupons and agar plates in the bioaerosol chamber


Steel coupons, plastic coupons and agar plates were placed in the bioaerosol chamber simultaneously to compare the relative recovery of bacteria that settled on to their surfaces.  For the work described in Chapter II, six each of the plastic polyethylene

coupons, stainless steel coupons, and Oxford agar plates (uncovered) were placed in

the bioaerosol chamber in two rows, prior to aerosolizing a *L. innocua* culture.  Each

row included three zones with one steel coupon, one plastic coupon and one Oxford

agar plate.  The zones were equivalently spaced across the chamber to examine the

effect of distance on bacteria viability and transfer (zones 1 and 4 being the furthest

from the source, while zones 3 and 6 being the closest).  Below are the zone

measurements for each specific surface sample (all measurements are from the

distance from the source):

- Plate zone 1 = 54 cm horizontal and 9.5 cm +vertical,
- Plate zone 2 = 46 cm horizontal and 9.5 cm +vertical,
- Plate zone 3 = 38 cm horizontal and 9.5 cm +vertical,
- Plate zone 4 = 54 cm horizontal and 9.5 cm –vertical,
- Plate zone 5 = 46 cm horizontal and 9.5 cm –vertical,
- Plate zone 6 = 38 cm horizontal and 9.5 cm –vertical
  (Plates horizontal rows = 8 cm apart and vertical columns = 20 cm)

- Steel zone 1 = 61 cm horizontal and 7 cm +vertical,
- Steel zone 2 = 53 cm horizontal and 7 cm +vertical,
- Steel zone 3 = 45 cm horizontal and 7 cm +vertical,
- Steel zone 4 = 61 cm horizontal and 7 cm –vertical,
- Steel zone 5 = 53 cm horizontal and 7 cm –vertical,
- Steel zone 6 = 45 cm horizontal and 7 cm –vertical.

- Plastic zone 1 = 61 cm horizontal and 12 cm +vertical,
- Plastic zone 2 = 53 cm horizontal and 12 cm +vertical,
- Plastic zone 3 = 45 cm horizontal and 12 cm +vertical,
- Plastic zone 4 = 61 cm horizontal and 12 cm –vertical,
- Plastic zone 5 = 53 cm horizontal and 12 cm –vertical,
- Plastic zone 6 = 45 cm horizontal and 12 cm –vertical.

6.  Recovery from bioaerosol chamber surfaces


Pre-wetted sponges (3M™ Sponge-Sticks with Buffered Peptone Water Broth)

were used to sample the interior surfaces of the chamber after nebulization and

collection of any air samples or settle plates.  The sponges were combined then placed

in a stomacher bag of 20 mL of peptone buffer which was then agitated, plated,

incubated, and counted.  However, no viable colonies could be recovered from these

sponges when solutions were plated on TSA or Oxford agar.  Therefore, the research

presented in Chapter II did not include sampling of the inner chamber surfaces after

each nebulization.  The lack of bacteria recovered from the chamber surfaces may be

due to possible antimicrobial properties of the chamber.  Poly resins may be present on

the surface as well as the finish of the chamber surface may discourage bacterial

attachment and survival.


7.  Sanitation of bioaerosol chamber


Lastly, the polycarbonate chamber was cleaned with a 70% ethanol solution and

exposed to UV light both inside and out prior to each nebulization experiment. To

confirm the absence of *Listeria* bacteria, a sponge-stick moistened with buffered

peptone water broth was used to scrub the inside of the box, and the sponge would be

cultured in 50mL of *Listeria* enrichment broth for 24 hours and plated to Oxford agar.

No *Listeria* were ever recovered from these analyses after the chamber walls were

cleaned, dried and exposed to UV light.

Appendix B

Figure 1.   Bioaerosol chamber (used in Chapter II experimentation).

Figure 2.  Bioaerosol chamber (used in Chapter II experimentation).

Figure 3.  Bioaerosol chamber with surface samples, environmental monitor, and portable fan inside.  Surfaces to be sampled include HDPE coupons, Stainless Steel coupons, and Oxford agar plates (used in Chapter II experimentation).
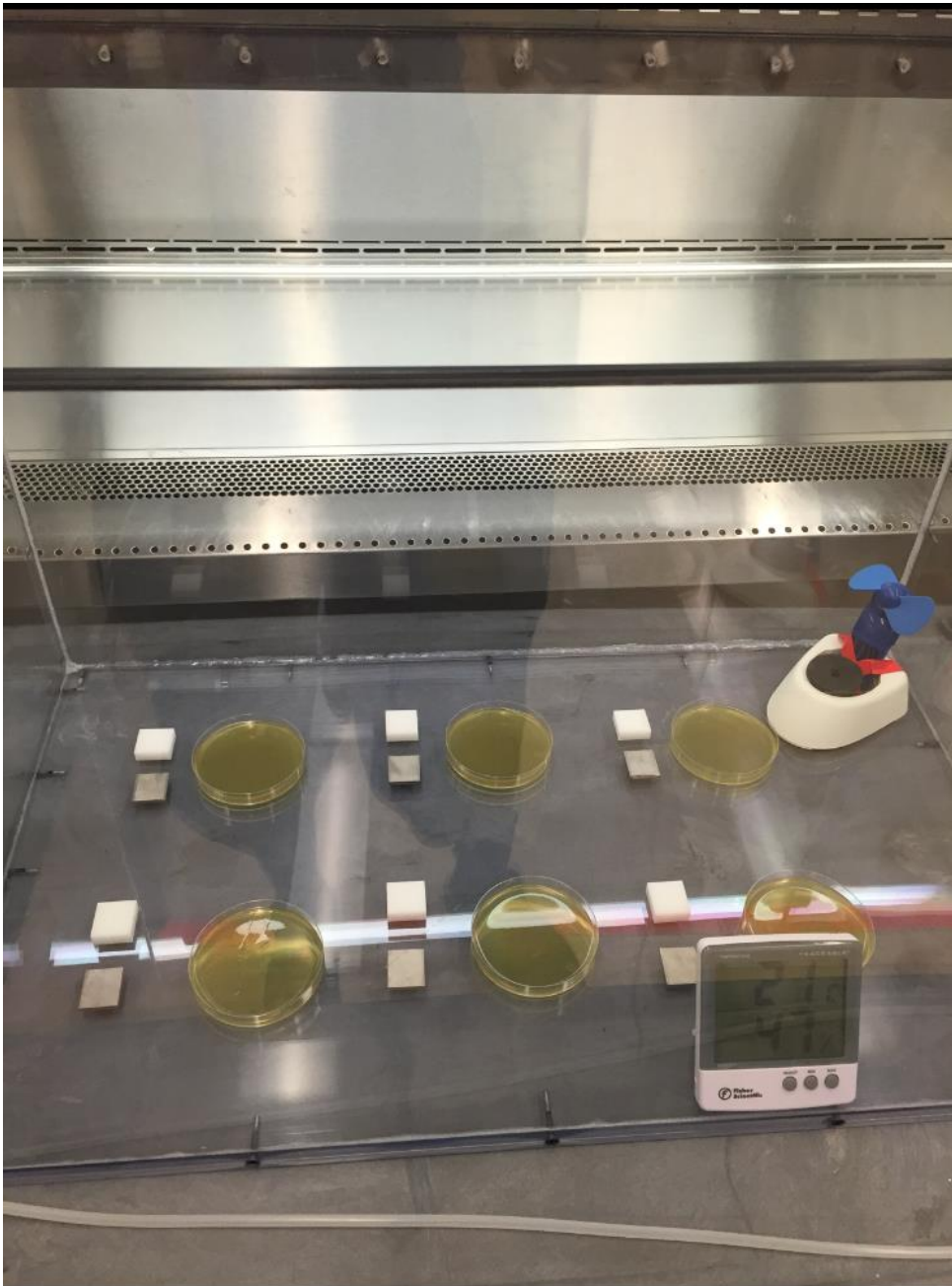
Figure 4.  Diagram of zones (1-6) in bioaerosol chamber which included HDPE coupons, Stainless Steel coupons, and Oxford agar plates (used in Chapter II experimentation).
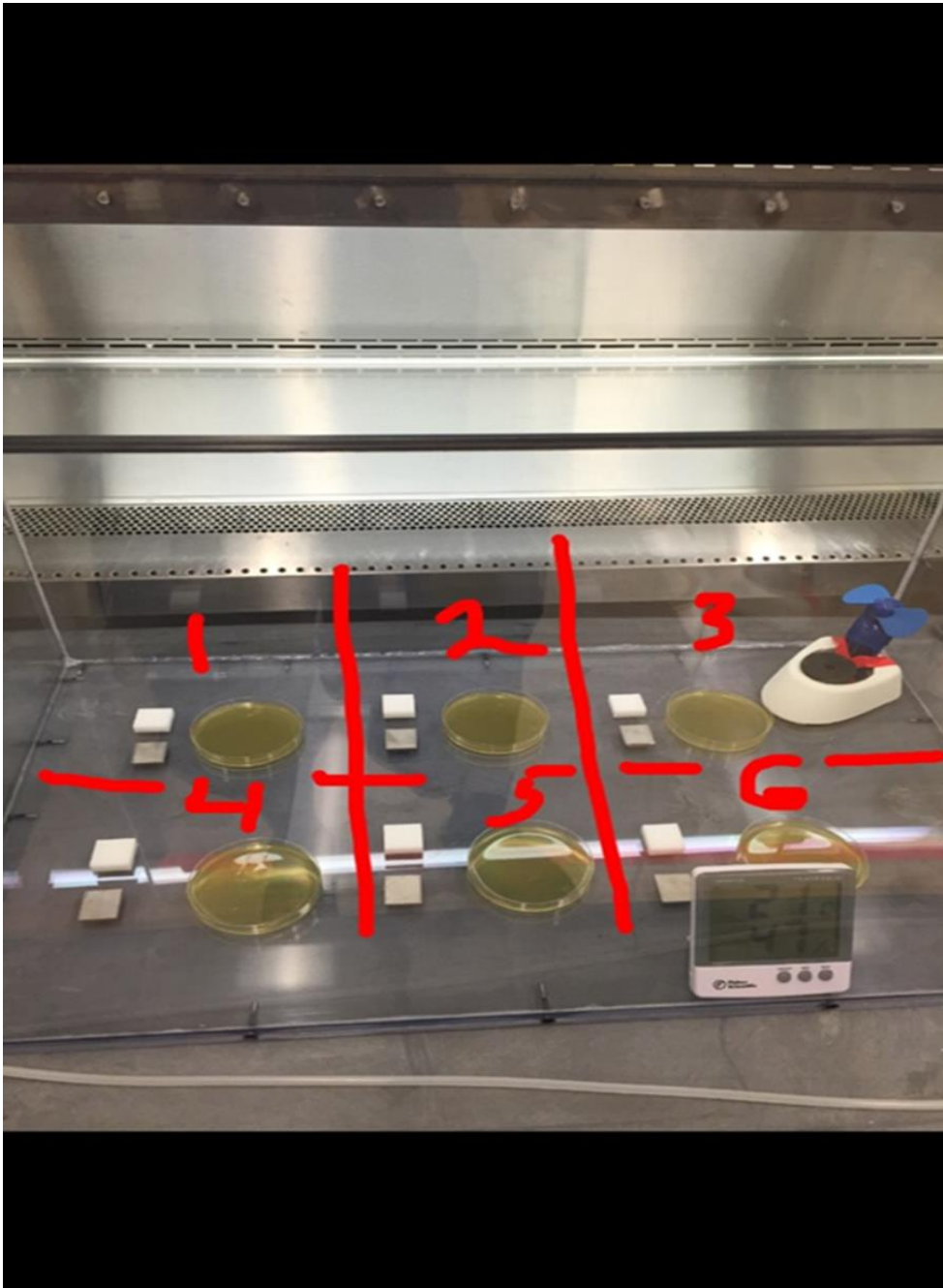
Figure 5.  Outside, front of Walk-in Cooler (used in Chapter III experimentation).

Figure 6.  Opened, front of Walk-in Cooler (used in Chapter III experimentation).

Figure 7.  Voyager™ Pro Vibrating Mesh Nebulizer suspended by sterile cup (used in Chapter III experimentation).

Figure 8.  Marked of areas of swab sampling for Cooler surface, Sartorius Airport MD8

Air Sampler, and layered Oxford agar plates (used in Chapter III experimentation).

Figure 9.  Layered Oxford agar plates. Each of the 4 sets had 4 layers, and 5 Oxford agar plates were attached to each layer. An additional 2 TSA plates were attached to each layer in the last trial (used in Chapter III experimentation).