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## **Invasion Properties of *Listeria monocytogenes* in Aged Cells**

Mina Lane Chasteen Burton  
*The University of Southern Mississippi*

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Invasion Properties of *Listeria monocytogenes* in Aged Cells

by

Mina Lane Chasteen Burton

A Thesis  
Submitted to the Honors College of  
The University of Southern Mississippi  
in Partial Fulfillment  
of Honors Requirements

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Approved by:

---

Janet Donaldson, Ph.D., Thesis Advisor,  
School of Biological, Environmental and Earth  
Sciences

---

Jake Schaefer, Ph.D., Director,  
School of Biological, Environmental and Earth  
Sciences

---

Sabine Heinhorst, Ph.D., Dean  
Honors College

## ABSTRACT

Listeriosis is a bacterial infection caused by the gram-positive pathogen *Listeria monocytogenes*. When compared to other foodborne illnesses, listeriosis has a higher death rate due to an increased incidence of complications such as meningitis, hydrocephalus, and sepsis in immunocompromised populations. Elderly individuals experience a condition known as immunosenescence, which is a gradual compromise of the immune system brought on by natural aging. Hutchinson-Gilford progeria syndrome (HGPS) is a rare genetic disorder causing premature aging, which allows HGPS cell lines to be used as models to further research in the field of biogerontology. This study utilizes the F2365 strain of *L. monocytogenes*, which was responsible for the 1985 listeriosis epidemic that killed around 50 people, to compare the pathogenesis of *L. monocytogenes* in aged and unaged cells. Through a series of time course invasions utilizing B cell lines donated from an individual with HGPS (AG03344) and their healthy sibling (AG03343), data were generated that concluded *L. monocytogenes* were able to cross the cell membrane quicker and proliferate longer within the B cell lines of aged cells. Oxygen consumption rates of AG03343 and AG03344 were also measured, which concluded HGPS cell lines demonstrate a reduction in oxygen consumption and utilization in cellular respiration. This may be responsible for the observed increase in pathogenesis. Further work is needed to determine the impact of reduced oxygen availability and pathogenesis of *L. monocytogenes*.

**Keywords:** *Progeria, Listeria monocytogenes, listeriosis, oxygen consumption, aging*

## **DEDICATION**

I dedicate my thesis to my family who has never ceased to recognize my potential and push me to succeed in areas of life I could not have dreamed possible. Through all the sleepless nights and endless study sessions their support was unwavering, and I cannot conceive of a world in which I could have achieved any of the successes I have today without them.

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## LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
CBC	Complete Blood Culture
FBS	Fetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
HGPS	Hutchinson Gilford Progeria Syndrome
LLO	Listeriolysin
PBSS	Phosphate-Buffered Saline Solution
RPMI	Roswell Park Memorial Institute Medium
ROS	Reactive Oxygen Species
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

## CHAPTER I: INTRODUCTION

*Listeria monocytogenes*, a facultative anaerobic bacterium, is a Gram-positive foodborne pathogen that is the etiology of listeriosis infections in humans. Colonies of *L. monocytogenes* have been observed in various environmental reservoirs, but listeriosis typically is caused by the ingestion of contaminated dairy or poultry products. This bacterium's ability to thrive in environments with low temperatures, including within the refrigerator *Listeria monocytogenes* contributes to its prevalence as a foodborne pathogen. Refrigeration is a method commonly used to preserve the safety of food before ingesting it, but the growth of *L. monocytogenes* is only slowed in this environment. As acidophiles, *L. monocytogenes* can also persist in environments with low pH. This allows the bacterium to evade the body's chemical barriers such as stomach acid to further colonize, invade, and infect the body's gastrointestinal tissue and evade the host immune defenses.

Listeriosis infections are especially dangerous to immunocompromised individuals, pregnant women, and individuals over the age of 65. The Center for Disease Control (CDC) reports that elderly individuals account for over half of listeriosis infections in the United States every year. An invasive form of listeriosis is characterized by acute and widespread infections such as septicemia, meningitis, and encephalitis. In 2019, researchers analyzed U.S. Foodborne Diseases Active Surveillance Network data to determine the annual incidence rate of invasive listeriosis in individuals over the age of 65 per 100,000 was 1.33 cases. The annual incidence rate of invasive listeriosis in individuals among the general population per 100,000 is 0.28 (Pohl et al., 2019). Hutchinson-Gilford progeria syndrome is a genetic disorder caused by a frameshift

mutation on the LMNA gene that results in a deletion of amino acids and alteration of the protein product. This allows individuals with HGPS to be used to study the effects of aging on the body, as their bodies prematurely age using the same molecular mechanisms that occur in natural aging. Because *L. monocytogenes* are omnipresent in many environments and elderly individuals consistently suffer more severe forms of listeriosis, this study sought to observe differences in the pathogenesis and proliferation of *L. monocytogenes* in aged and unaged cytosolic environments.

## CHAPTER II: REVIEW OF LITERATURE

### *Listeria monocytogenes*

The *Listeria* genus consists of microbes that have been isolated from a diverse range of environments including feces, food, soil, and water although their natural habitat is decomposing plant matter (Vázquez-Boland et al., 2001). *Listeria* are resilient, tolerating acidic environments, and surviving food-processing techniques to proliferate at refrigeration temperatures. Listeriosis is a serious infection caused by the rod-shaped, gram-positive bacterium *L. monocytogenes*. In 1929 the first case of listeriosis was reported in Denmark, and there has been a steady rise in cases and outbreaks since (Denoon, 2011). Our research focuses on the F2365 strain, which was isolated from the listeriosis epidemic in California that was propagated by cantaloupes in 1985 (Nightingale et al., 2007). Epidemics typically arise because of contaminated meat and dairy products, but community-based outbreaks have occurred in hospital settings and environments where there is a considerable population of women birthing infected infants without proper sanitary precautions (Schlech & Acheson, 2000). From July 2017 to July 2018, South Africa experienced the largest Listeriosis outbreak in history with approximately one thousand laboratory-confirmed cases and two hundred and sixteen fatalities recorded. Polony, a processed meat made of pork and beef, was found to be the source of contamination in many of these cases (Kaptchouang Tchatchouang et al., 2020).

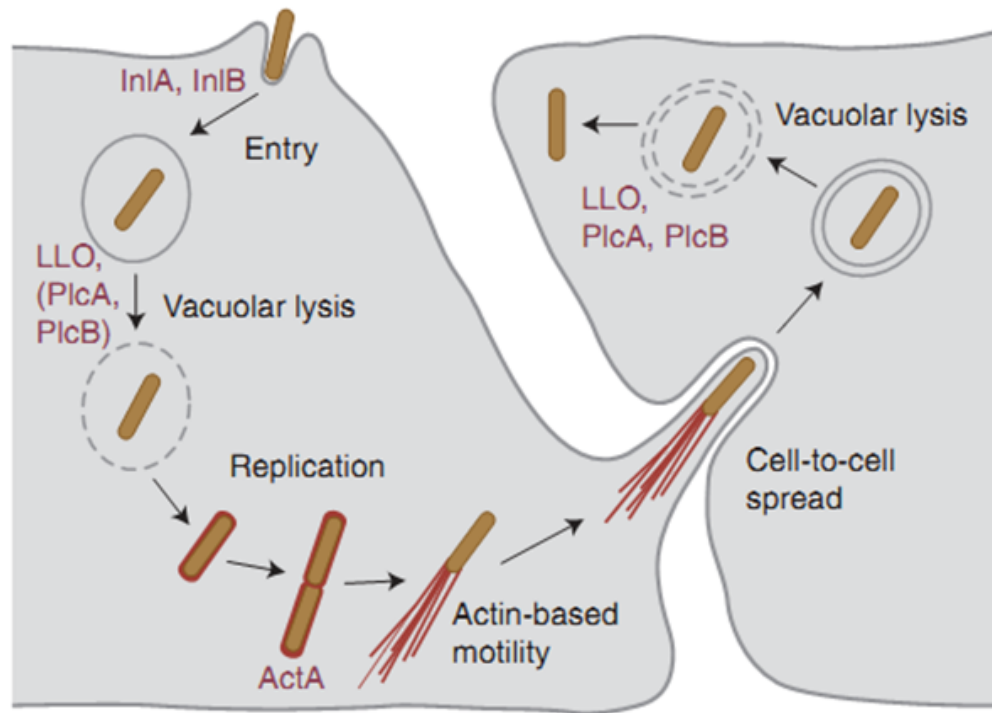
*Listeria monocytogenes* are unique as they have the potential to induce internalization in a diverse array of cell types including hepatocytes, fibroblasts, epithelial cells, and neurons. Typically entering the body using the oral pathway,

infection begins with the ingestion of contaminated food with symptoms appearing 24-48 hrs. post-consumption. Individuals with healthy immune systems often experience a mild form of gastroenteritis due to *L. monocytogenes* resulting in gastrointestinal and flu-like symptoms such as fever, vomiting, and diarrhea. Mild forms of listeriosis can be diagnosed using a stool or blood culture, and typically can be treated at home with supportive care. Immunocompromised individuals are more susceptible to invasive and severe forms of listeriosis such as sepsis, meningitis, and encephalitis. *Listeria rhombencephalitis*, a rare infection of the hindbrain, can be diagnosed by conducting an assay of a cerebrospinal fluid sample to determine if there is an elevation in protein level or mononuclear cell counts. This severe manifestation of listeriosis is characterized by signs and symptoms of double vision, headache, difficulty balancing, and confusion (Mansbridge et al., 2018).

When invading a host, *L. monocytogenes* first anchor themselves to a receptor on the host cell membrane and penetrate using a zipper mechanism to allow themselves to be engulfed within the host cell. The bacterium is transported into the cell via a phagocytotic vesicle and multiplies with a doubling time of an hour after reaching the cytosol. Actin filaments wrap around the bacterium to form an actin tail which is used to exit the cell membrane, penetrate nearby cells, and initiate further rounds of proliferation. Internalins are proteins produced by *L. monocytogenes* that code for InlA and InlB, which function as specialized tools required to adhere to the cell and enter. Hemolysins and phospholipases enhance mechanisms to facilitate the exit of *L. monocytogenes* and increase proliferation. Listeriolysin (LLO) is a form of hemolysin that functions synergistically with InlA and InlB to disrupt the membrane of phagosomes and induce



cell lysis. The movement of actin in the cytoplasm and invasion of bacteria into neighboring cells is facilitated by the surface protein ActA, which is coded for by the *actA* gene. As the membrane of phagosomes breaks down, they accrue waste which is normally cleared away in a process known as autophagy. *Listeria monocytogenes* secrete phospholipases PlcA and PlcB which aid the pathogen in leaving the phagosome to return to the cytosol and lysing the phagocytic vesicles that form as *L. monocytogenes* spread from cell to cell (Matereke & Okoh, 2020).



**Figure 2.1:** Intracellular replication of *Listeria monocytogenes*. This figure provides imagery detailing the intracellular replication cycle of *Listeria monocytogenes*, and the virulence factors it utilizes to enhance its ability to invade and proliferate within the tissue of the lymphatic system.

(From Pizarro-Cerda et al., 2012)

## **Immunosenescence**

Immunosenescence refers to the gradual decline in the immune system's ability to effectively recognize and respond to antigens resulting from natural aging. By reducing the body's ability to respond to pathogens, immunosenescence effectively reduces the ability to fight off infections and generate a comparable immune response to vaccinations. This juxtaposes with the increase in inflammation and immune responses targeting self-antigens combining to produce higher incidences of opportunistic infections and disease sequelae (Fuentes et al., 2017).

In my research, B lymphocyte cell lines from an individual with Hutchinson-Gilford Progeria Syndrome (HGPS) were utilized to simulate the aged intracellular environment. B cells are generated within the bone marrow and produce immunoglobulins which recognize antigens and aid in clearing them from the body. Each B cell assembles a specific antibody, and collectively they respond to the wide array of antigens individuals are bound to incur in their lifetime (Oh et al., 2019). As humans grow older, they develop a limited heterogeneity in the B cell receptors produced, lessening their immune systems' ability to respond to a large repertoire of antigens. In 2016, researchers at the University of Miami-Miller School of Medicine conducted a retrospective analysis documenting the complete blood counts (CBC) in infants, children, adolescents, adults, and elderly study groups. Through the utilization of flow cytometry, the populations of T-Cells, B-Cells, and Natural Killer cells were quantified to determine that while T and NK cell populations grow with age, B cell populations begin to decline after childhood (Valiathan et al., 2016).

As the life expectancy of humans continues to lengthen it will be accompanied by a growing need for research in the field of gerontology, including research to further understand the mechanisms of cell signaling and physiological changes involved in immunosenescence, to protect a larger part of the world's population. Sepsis, a complication of systemic infection, cost the United States a whopping \$24 billion dollars in 2013, accounting for 13% of the money expended on U.S hospitals while only compiling 3.6% of hospital admissions. A retrospective study analysis was conducted using the discharge and billing records of approximately twenty percent of the United States hospital admissions. These records were analyzed to detect patterns in demographic or patient characteristics that equated to poor clinical and economic outcomes. Out of the 2,566,689 septicemia patients, 75-80% of septicemias were the result of an unknown etiology with mean age upon admission of 65 years (Paoli et al., 2018).

### **Oxygen Availability and Immune Suppression**

In 2021 an original article published by the European Journal of Allergy and Clinical Immunology disseminated data generated by researchers in Graz, Austria stating that hypoxic conditions caused decreased weight gain, and suppressed the acquired immune response. This suppression of the adaptive immune system is achieved by obstructing the ability of antigen presenting cells (APC), such as dendritic cells (DC), macrophages, and B-cells, to function by hindering their ability to communicate with T-cells (Hochgerner et al., 2021). Within the immune system, B cells are responsible for production of cytokines and presentation of antigens. In this study, B-lymphocyte cell lines were utilized to further our understanding of *L. monocytogenes* pathogenesis in aged

and unaged cells. Reactive oxygen species (ROS) are involved in controlling the maturation, activation, and apoptosis of B cells (Zhang et al., 2019). A previous study demonstrated that HGPS fibroblasts sustain preponderant levels of ROS, leading to an accretion of permanent double-stranded breaks in DNA (Richards et al., 2011).

### **Hutchinson-Gilford Progeria Syndrome**

Hutchinson-Gilford Progeria Syndrome (HGPS) belongs to a group of genetic disorders known as laminopathies that involve a mutation in the LMNA gene, which codes to produce a variety of lamin proteins, including lamin A and C. Individuals with HGPS possess a single-base substitution in the LMNA exon 11 which causes a deletion of nucleotides at exon 11 resulting in a frame deletion of amino acids to alter the protein produced. This LMNA mutation is sporadic, not inherited and occurs in approximately 1 in 4 million people, following no epidemiological or demographic patterns. Through alterations of the body's cellular integrity and signaling abilities, the LMNA mutation reduces the efficacy of DNA repair including the ability to carry out RNA polymerase II transcription (Gonzalo et al., 2017).

HGPS is known as a segmental aging disease because some tissues, such as tissues from the immune system have hallmark features of normal aging whereas tissues from the liver, lungs, kidneys, and gastrointestinal tract have fewer observable features of aging. This disease process begins in utero, with children developing signs and symptoms by 12 months of age. HGPS is characterized by normal cognitive abilities, skin alterations, alopecia, subcutaneous fat loss, bone and joint abnormalities, and atherosclerosis. The life expectancy of this disorder is 14.6 years of age with death often

occurring due to heart attack or stroke. There is currently no cure, but there have been many promising new discoveries in clinical trials (Ding & Shen, 2008).

## CHAPTER III: MATERIALS AND METHODS

### Cell Lines

Two B-lymphocyte cell lines (AG03344 and AG03343) were obtained from the Coriell Institute for Medical Research. Cell line AB03344 was collected from a patient that was clinically diagnosed with Hutchinson-Gilford Progeria Syndrome (HGPS); cell line AG03343 was obtained from their unafflicted sibling. These cell lines were stored at 37°C in a 5% CO<sub>2</sub> incubator in T175 mL flasks containing Roswell Park Memorial Institution (RPMI) medium and 20% Fetal Bovine Serum (FBS). To exchange the media, the initial media containing the cells was placed into 50mL conical tubes and centrifuged at 2500 x g for 5 minutes to form a cell pellet. The old media was then decanted, and the cell aggregate was resuspended in a fresh RPMI + 20% FBS solution.

### Bacterial Cultivation Conditions

This study utilized the F2365 strain of *L. monocytogenes*, which was responsible for the 1985 listeriosis epidemic that killed nearly 50 people (Nightingale et al., 2007). The F2365 strain was routinely stored -80°C. Approximately 48 hours before the study began a Tryptic Soy Agar (TSA) plate was inoculated with a progressive dilution of F2365 and incubated for 24 hours. After 24 hours this plate was taken from the incubator, and an overnight culture (~16 hrs.) was prepared in a 15 mL conical tube using a sample of the bacteria grown on the TSA plate and 5mL of Tryptic Soy Broth (TSB). An additional 5mL of TSB was placed in a separate 15 mL conical tube to detect any potential media contamination. To encourage more bacterial growth this culture was incubated in a shaking incubator at 37°C.

## Invasion

Following overnight incubation, 1 mL of bacterial cells was transferred into 1.5mL tubes (5 tubes total) and centrifuged for 5 minutes at  $7,500 \times g$ . The supernatant was subsequently decanted, and the remaining bacterial pellet was then resuspended in 0.9 mL PBS + 0.1 mL of FBS to opsonize the bacteria and encourage phagocytosis. This solution was then incubated at  $37^{\circ}\text{C}$  for 30 minutes for opsonization.

After the incubation period a spectrophotometer was used to measure the optical density of the solution at 600nm using  $2 \mu\text{L}$  of cells and PBS as a blank to determine the concentration of cell present. The Beer-Lambert law, seen below, demonstrates that the absorbance of a solution is proportional to the path length through the sample ( $l$ ) and the concentration of the absorbing species ( $c$ ).

$$A = \epsilon cl$$

The molar extinction coefficient ( $\epsilon$ ) is a measure of a substance's affinity for light at a particular time, with the molar extinction coefficient of *L. monocytogenes* strain F2365 known to be  $2.42 \times 10^{-10}$ . In this experiment the optical path length was 1 cm. By using the known values of the molar extinction coefficient and optical path length in this experiment, Beer's Law was used to determine the concentration of the cultures.

Approximately  $1 \times 10^9$  CFU in each flask of cells. Cells were incubated for 1 hour.

Afterwards, we then transferred cells to 50ml conical tube and centrifuged them for 5 minutes at  $2,500 \times g$  to separate the contents using centrifugal force. 2mL of HBSS was then added to resuspend cells, and then they were centrifuged again for 5 minutes at  $2,500 \times g$ . This process was then repeated, and after the second HBSS resuspension we disposed of the liquid HBSS while the cell pellet remained in the conical tube. This was

done to remove all extracellular bacteria so only the intracellular bacteria were quantified. Then, 0.1% Triton X-100 in cold, distilled water was added to each tube to resuspend the cells. We used a sonicator to sonicate cells for 30 seconds using three 10-second pulses to release the intracellular bacteria. The concentration of the cells that invaded the B-lymphocytes was then quantitated through viable plate counts.

### **Intracellular Replication**

Cells were infected with  $1 \times 10^9$  CFU/flask as described for the invasion protocol above. After the 1 hr. incubation period, the cells were centrifuged for 5 minutes at  $2,500 \times g$ . The cells were then resuspended with 5 mL of RPMI and  $10 \mu\text{l}$  of 50mg/mL of the antibiotic gentamicin, to result in a final concentration of  $100 \mu\text{g/ml}$  gentamicin. Cells were incubated at  $37^\circ\text{C}$  at 5%  $\text{CO}_2$  for 6 hours. Four conical tubes were removed at 0, 2, 4, and 6 hours after the addition of gentamicin. The cells were centrifuged for 5 minutes at  $2,500 \times g$ . Then, 1 mL of 0.1% Triton X-100 in cold, distilled water was added to each tube to resuspend the cells. We used a sonicator to sonicate cells using three 10-second pulses to release the intracellular bacteria. After the cells were lysed, we serially diluted the extract in PBS and plated onto TSA plates. After the serial dilutions, plates were incubated at  $37^\circ\text{C}$  overnight and the colony forming units were counted the next day. A minimum of four independent replicates was analyzed.



## CHAPTER IV: RESULTS

### **Invasion of *L. monocytogenes* in B-lymphocytic cells differs between aged and non-aged cells.**

To determine if *L. monocytogenes* can adhere and invade B-lymphocytes, two cell lines of B-lymphocytes were analyzed. Cell line AG03344 was obtained from an individual with Hutchinson-Gilford Progeria Syndrome (HGPS), a genetic disorder characterized by premature aging, while cell line AG03343 was donated by an unafflicted sibling. To measure invasion potential of *L. monocytogenes*, cells were allowed to adhere and invade these two cell lines for 1 hr, after which intracellular concentrations of cells were measured through viable plate counts. Both cell lines were infected with  $1 \times 10^9$  CFU per flask (~MOI of 100). Table 4.1 demonstrates the invasion differences between the two cell lines tested. AG03344 had more bacteria invade than the AG03343 cell line. However, neither cell line achieved the desired rate of 100 MOI per cell.

<b>Differences in the Invasion of B-lymphocytic Cell Lines with <i>L. monocytogenes</i></b>	
<b>Cell line</b>	<b>Invasion</b>
AG03344	$2.35 \times 10^4$ CFU/ 5mL flask
AG03343	$1.5 \times 10^4$ CFU/ 5 mL flask

**Table 4.1. Differences in the Invasion of B-lymphocytic Cell Lines with *L. monocytogenes***

### ***Listeria monocytogenes* replicates within AG03344 and AG03343 cell lines.**

In this study, a growth curve was constructed using data generated in a series of time course invasions where cell lines AG03344 and AG03343 were inoculated with *L. monocytogenes*. When quantifying the number of intracellular bacteria present after

various incubation periods, there was an increase in the intracellular concentrations of *L. monocytogenes* in both the AG03343 and the AG03344 cells (Figure 4.2). These data indicate that *L. monocytogenes* can invade and replicate intracellularly within B-lymphocyte cells.

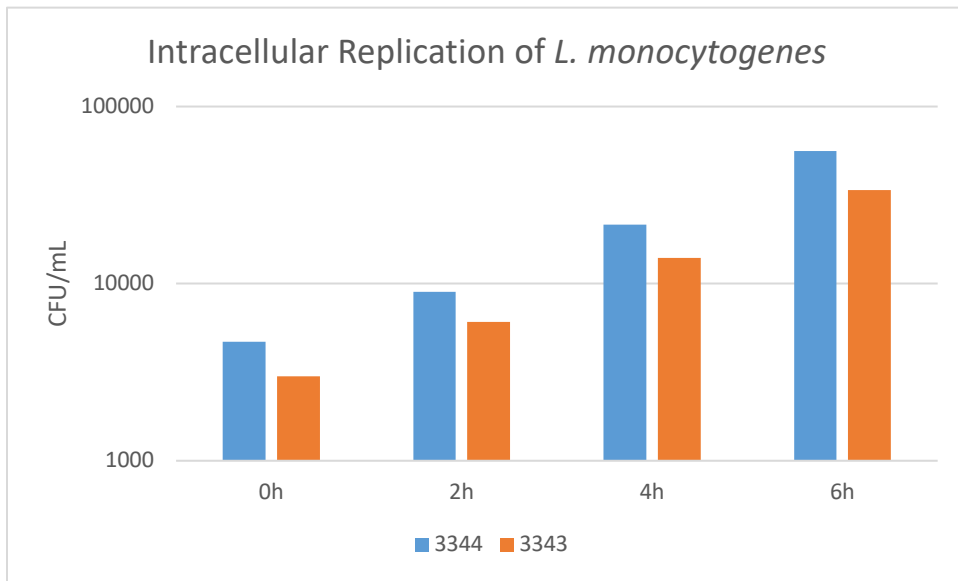
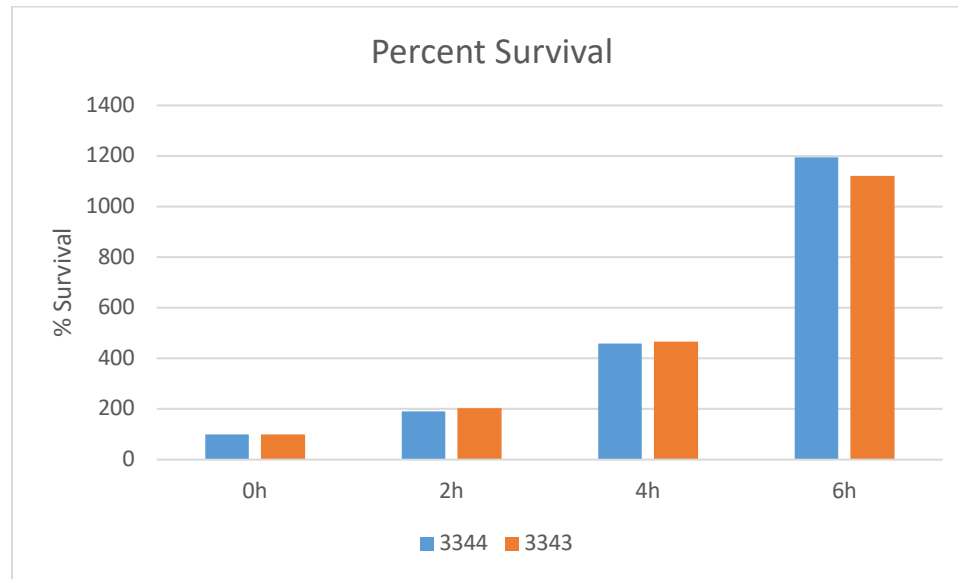


Figure 4.1. Intracellular *L. monocytogenes* populations post-incubation periods. *L. monocytogenes* was grown within AG03344 (blue) or AG03343 (orange) cell lines for 6 hrs. and intracellular concentrations were quantified by viable plate counts. Values represent the average of three independent replicates.

### **Intracellular survival does not significantly differ between AG03344 and AG03343 B-lymphocytic cell lines.**

There was a slight increase in intracellular growth within the AG03344 in comparison to the AG03343 cell line, which may be indicative of immunosenescence (Figure 4.2). However, analyzing this as a percent of survival based on how many

bacteria were initially invaded indicated that there was not a significant difference in the survival rate of *L. monocytogenes* within these two B-lymphocytic cell lines. There was a slight trend in increasing survival for AG03344 (Figure 4.3). However, extended observations are needed to determine if this trend persists.



*Figure 4.2: Percent survival of *L. monocytogenes* within HGPS and B-lymphocytic cell lines. There are two cell lines, 3344 and 3343, with 3344 being taken from an individual with HGPS. Using two-hour incubation periods, a survival percent range of approximately 150%-1200%. Values represent an average survival from three independent replicates.*

## CHAPTER V: CONCLUSION

The ubiquitous nature of the *Listeria* family combined with their resistance to environmental sanitation efforts, evasion of immune system defense mechanisms, and increased mortality rates in elderly individuals merits more research to qualify the pathogenic nature of this microbe in aged hosts. Several virulence factors such as hemolysin, phospholipases, internalin, and the *actA* gene enhance *L. monocytogenes*' ability to colonize, evade and suppress the host's immune system, and enter and exit into host cells to cause listeriosis (Matereke & Okoh, 2020). Immunocompromised individuals' contract listeriosis at a higher incidence and are more likely to suffer severe consequences such as systemic infections, and brain abscesses and infections. One of the most common sources of immunodeficiency is immunosenescence, which is the progressive decline in the immune system's ability to protect itself from harmful pathogens. In this state, the lymphatic system is hypersensitized to the body's own tissues causing an increase in inflammation and a likelihood of developing autoimmune disorders. As the body grows older, its ability to produce a diverse array of antibodies to respond to pathogens is inhibited as the number of B-lymphocytes circulating the body is lessened and the quality of the remaining ones is reduced. These changes leave the body more susceptible to pathogens such as *L. monocytogenes* (Valiathan et al., 2016). This study utilized the B-lymphocyte cell lines from a donor with Hutchinson-Gilford Progeria Syndrome (HGPS) to study differences the differences pathogenesis of *L. monocytogenes* in aged and unaged environments.

Hutchinson-Gilford Progeria Syndrome is characterized by an aggressive form of premature aging that eventually results in fatal cardiovascular episodes. This condition is a result of a rare and sporadic mutation of the LMNA exon. Individuals with HGPS utilize the same molecular aging mechanisms that are observed in natural aging, so their cell lines can be used to simulate the environment within a naturally aged cell (Ding & Shen, 2008). A time course measuring intracellular survival of *L. monocytogenes* was performed to determine whether *L. monocytogenes* could survive the intracellular environment of the B-lymphocytes from individuals with HGPS better than normal B-lymphocytes.

Our results indicate that there is slight improvement of intracellular survival within HGPS cells as compared to control B-lymphocytic cells. Initial invasion was similar between the two cell lines, but continued intracellular growth increased with HGPS in comparison to controls. However, this difference was not significant ( $p > 0.05$ ). To determine if the differences observed between the two cell lines were due to variations in the oxygen consumption rate, the OCR was measured using a colorimetric assay. Previous results from our laboratory indicated that the OCR was reduced in the AG03344 cell line in comparison to the AG03343 cell line. Previous studies in mice demonstrated that reduced access to oxygen can suppress the acquired immune response by inhibiting the ability of B-lymphocytes, and other antigen presenting cells, to communicate with T-lymphocytes (Hochgerner et al., 2021). These studies suggest that immunosenescence includes physiological changes that reduce an individual's mitochondrion from functioning efficiently, resulting in a semi hypoxic cytosolic environment responsible for the increased incidence of communicable diseases in individuals over the age of 65 (ref).

Further research is needed to determine what causes the reduction in the body's ability to efficiently use oxygen, and what mechanisms induce the decline in the ability to continue genetic recombination to maintain a diverse population of antigen receptors.

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